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Ligation of TLR7 on CD19^{+}CD1d^{hi} B cells suppresses allergic lung inflammation via regulatory T cells

Adnan R. Khan[1], Sylvie Amu[2], Sean P. Saunders[1], Emily Hams[1], Gordon Blackshields[3], Martin O. Leonard[4], Casey T. Weaver[5], Tim Sparwasser[6], Orla Sheils[3] and Padraic G. Fallon[1,2,7]

[1] Trinity Biomedical Sciences Institute, School of Medicine, Trinity College Dublin, Dublin, Ireland
[2] Institute of Molecular Medicine, School of Medicine, St James's Hospital, Trinity College Dublin, Dublin, Ireland
[3] Department of Histopathology, Trinity College Dublin, Sir Patrick Duns Research Laboratory, St. James's Hospital, Dublin, Ireland
[4] School of Medicine and Medical Sciences, The Conway Institute, University College Dublin, Belfield, Dublin, Ireland
[5] Department of Pathology, University of Alabama, Birmingham, AL, USA
[6] Institute of Infection Immunology, TWINCORE, Centre for Experimental and Clinical Infection Research, a Joint Venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Hanover, Germany
[7] National Children's Research Centre, Our Lady's Children's Hospital, Crumlin, Dublin, Ireland

B cells have been described as having the capacity to regulate cellular immune responses and suppress inflammatory processes. One such regulatory B-cell population is defined as IL-10-producing CD19^{+}CD1d^{hi} cells. Previous work has identified an expansion of these cells in mice infected with the helminth, *Schistosoma mansoni*. Here, microarray analysis of CD19^{+}CD1d^{hi} B cells from mice infected with *S. mansoni* demonstrated significantly increased *Tlr7* expression, while CD19^{+}CD1d^{hi} B cells from uninfected mice also demonstrated elevated *Tlr7* expression. Using IL-10 reporter, *Il10*$^{-/-}$ and *Tlr7*$^{-/-}$ mice, we formally demonstrate that TLR7 ligation of CD19^{+}CD1d^{hi} B cells increases their capacity to produce IL-10. In a mouse model of allergic lung inflammation, the adoptive transfer of TLR7-elicited CD19^{+}CD1d^{hi} B cells reduced airway inflammation and associated airway hyperresponsiveness. Using DEREG mice to deplete FoxP3^{+} T regulatory cells in allergen-sensitized mice, we show that that TLR7-elicited CD19^{+}CD1d^{hi} B cells suppress airway hyperresponsiveness via a T regulatory cell dependent mechanism. These studies identify that TLR7 stimulation leads to the expansion of IL-10-producing CD19^{+}CD1d^{hi} B cells, which can suppress allergic lung inflammation via T regulatory cells.

Keywords: AHR · Helminth · IL-10 · Regulatory B cells · TLR7 Treg cells

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Correspondence: Prof. Padraic G. Fallon
e-mail: pfallon@tcd.ie

Introduction

A subset of B cells that has garnered intense interest are regulatory B (Breg) cells [1–4]. These include B cells that produce the anti-inflammatory cytokine IL-10, termed B10 cells [5–7], including murine $CD19^+CD1d^{hi}CD5^+$ B cells [8, 9]. However, there is still no universally recognized phenotype for these cells. In naïve mice, Breg cells that secrete IL-10 exist as no more than 1–2% of all $B220^+$ cells [8, 10] with such IL-10-secreting B cells potently suppressing inflammation in several murine disease models [10–14] and have been shown to have effects in man [14].

Specific factors and mechanisms that induce or regulate Breg cells in vivo have recently been explored. We have previously demonstrated that helminths can expand a Breg-cell population that can suppress in allergic inflammatory conditions in mice in an IL-10-dependent manner [10, 11]. Given that helminths can generate IL-10-producing Breg cells in both mouse and man [10, 11, 15], it is important to understand and define noninfective mechanisms by which to generate such cells both in vitro and in vivo [9]. Paradigm shifts in the role of B cells have described their effects in both innate and adaptive arms of the immune response [2, 16–18]. In particular, engagement of Toll-like receptors (TLR) on B cells can produce a plethora of cytokine responses [18].

In order to gain further insight into the function of IL-10-secreting B cells in mice, we undertook RNA microarray analysis of Breg cells from helminth-infected mice and compared its gene expression profile with that of B cells from both infected and uninfected animals. On analysis, we discovered that Breg cells from *Schistosoma mansoni* infected mice contained upregulated expression of genes associated with pattern recognition of bacteria and viruses. Of particular significance was the elevated expression of the endosomal-resident TLR, *Tlr7*.

Previously, we have shown that helminth-derived, IL-10-producing B cells can mediate airway hyperresponsiveness (AHR) through the induction of $CD4^+FoxP3^+$ regulatory T (Treg) cells [10]. Here, we now demonstrate that TLR7 is expressed on $CD19^+CD1d^{hi}$ B cells and that agonism of this receptor can induce IL-10 production, both in vitro and in vivo. Furthermore, using adoptive transfer strategies we describe how these TLR7-elicited $CD19^+CD1d^{hi}$ B cells facilitate the suppression of AHR in mice through the induction of lung-resident Treg cells. We also indicate that these ligands can generate IL-10-secreting B cells in man, thus supplementing the current evidence base that TLR7 has the potential to be a therapeutic target in respiratory disease and providing novel insight into IL-10-producing Breg cell function.

Results

Preferential upregulation of Tlr7 on $CD19^+CD1d^{hi}$ B cells determined using helminth-infected mice

Splenic $CD19^+CD1d^{hi}$ B cells are expanded in mice infected with *S. mansoni* [19]. These helminth-generated B cells have similar characteristics to other reported regulatory B cells [9, 20, 21]. We have previously demonstrated that these helminth regulatory B cells can prevent and reverse AHR when transferred to OVA-sensitized mice via an IL-10-depenent mechanism [10]. Thus, we sought to investigate the gene expression profile of $CD19^+CD1d^{hi}$ B cells from helminth-infected mice in order to identify genes of interest pertaining to regulatory B cell function. To do so, $CD19^+CD1d^{hi}$ B cells from *S. mansoni* infected mice were isolated (see Supporting Information Fig. 2 for sorting strategy) and compared via microarray to $CD19^+$ B cells from healthy, uninfected animals (ArrayExpress no. E-MTAB-2100). Analysis of array data generated 416 genes that were either significantly (more than twofold change in expression over uninfected $CD19^+$ B cells; $p < 0.01$) up- or downregulated (See Supporting Information Fig. 1 for comparative heatmap analysis and Supporting Information Tables 1 and 2 for a full list of significantly regulated genes).

As helminths are known to modulate immune cells via multiple interactions with the TLR pathway [22, 23], an area of interest was the gene expression profile of $CD19^+CD1d^{hi}$ B cells in relation to innate activation by pathogens. Comparative analysis unexpectedly indicated that the endosomal TLR, *Tlr7*, was significantly upregulated on $CD19^+CD1d^{hi}$ B cells from *S. mansoni* infected mice (Fig. 1A). To validate the findings of the array, splenic $CD19^+$ B cells, $CD19^+CD1d^{lo}$B cells, and $CD19^+CD1d^{hi}$ B cells from both uninfected and *S. mansoni* infected mice were isolated via flow cytometric cell sorting. Quantitative gene expression analysis for *Tlr7* and *Il10* demonstrated that *S. mansoni* infection increased the expression of both *Tlr7* and *Il10* in B cells, with significant ($p < 0.05$) increases observed in $CD19^+CD1d^{hi}$ B cells (Fig. 1B). Gene expression data were subsequently validated via Western blot and densitometry analysis (Fig. 1C), which indicated elevated protein expression of TLR7 on $CD19^+$ B cells as well as $CD19^+CD1d^{hi}$ B cells from *S. mansoni* infected mice. This suggests that infection drives a preferential increase in the expression of *Tlr7* in $CD19^+CD1d^{hi}$ B cells. In uninfected mice (Fig. 1D), the $CD19^+CD1d^{hi}$ B cell subpopulation also had significantly higher ($p < 0.05$) *Tlr7* expression relative to $CD19^+$ B cells. We sought to investigate whether this preferential upregulation of *Tlr7* on $CD19^+CD1d^{hi}$ B cells conferred any regulatory activity when ligated.

Activation of TLR7 induces IL-10 production from B cells

To address the role of TLR7 in the generation of IL-10-producing B cells, IL-10 reporter (10BiT) mice were treated in vivo with TLR7 ligands (Fig. 2A). Following the treatment of 10BiT mice with the TLR7 ligands imiquimod (IMQ) and R848 (100 μg injection for 24 h), there was a significant ($p < 0.05$) increase in the proportion of spleen IL-10-producing B cells ($Thy1.1^+$(IL-10)$CD19^+$) compared to control mice (Fig. 2A and B). The relative difference in potency of IMQ and R848 in expanding IL-10-producing B cells ($p < 0.05$) is consistent with the respective activity of each ligand on TLR7 [24]. Work by Govindaraj and colleagues has demonstrated that despite the reported TLR7/TLR8 activity of R848 in man [24, 25], there is a lack of TLR8 ligation in mouse [26]. To address this, we generated 10BiT/$Tlr7^{-/-}$ mice and confirmed

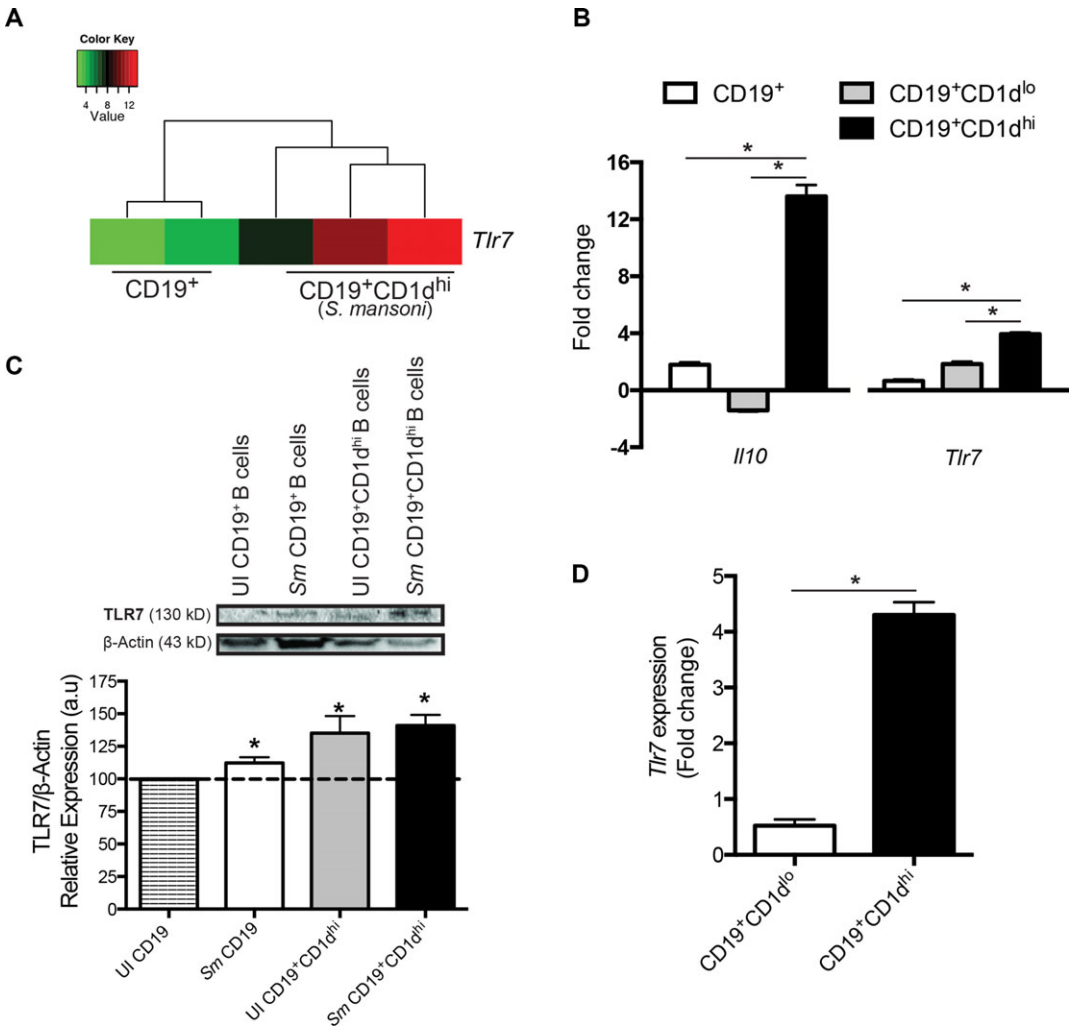

Figure 1. *Tlr7* is upregulated in CD19$^+$CD1d^{hi} B cells from both helminth-infected and uninfected mice. (A) mRNA was isolated from CD19$^+$ B cells ($n = 2$) from uninfected mice and CD19$^+$CD1d^{hi} B cells ($n = 3$) from *S. mansoni* infected mice to evaluate gene expression via microarray. Relative high or low expression of *Tlr7* is shown compared with that of all other *Tlr* genes. The data are presented as a single chromographic bar with columns that represent each sample type. The colors red or green in cells reflect relative high or low expression levels, respectively, which is indicated in the scale bar (log_2-transformed scale; $p < 0.01$). (B) Quantitative gene expression of *Il10 and Tlr7* on whole CD19$^+$ B cells, CD19$^+$CD1d^{lo}, and CD19$^+$CD1d^{hi} B cells from *S. mansoni* infected mice. Changes in expression are denoted as fold change compared with their uninfected counterparts. GAPDH was used as an endogenous expression control. Significance was determined by two-way ANOVA. (C) Western blot and densitometry analysis of TLR7 on sorted B cells from both uninfected (UI) and infected (*Sm*) mice. β-Actin is used as a loading control. Representative blot is shown. Ratio of TLR7:β-Actin was calculated with all values normalized against UI CD19$^+$ (Hatched bar; 100 a.u.). Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison test. (D) Quantitative gene expression of *Tlr7* in sorted CD19$^+$CD1d^{lo} and CD19$^+$CD1d^{hi} B cells from uninfected mice. Changes in expression are denoted as fold change compared to whole CD19$^+$ B cells from uninfected mice. GAPDH was used as an endogenous control. Significance was determined by two-way ANOVA. All data are shown as mean + SEM ($n = 3$, unless stated) pooled from three independent experiments (*$p < 0.05$).

that R848 expands IL-10-producing B cells in a TLR7-dependent manner (Fig. 2A and B). Furthermore, serum levels of IL-10, 24-h posttreatment, confirmed that R848 significantly ($p < 0.05$) elevated IL-10 levels via a TLR7-mediated pathway (Fig. 2C).

TLR7-elicited IL-10-producing B cells were characterized by flow cytometry. IL-10$^+$ B cells demonstrated elevated expression of CD1d, CD21, CD23, CD25, IgD, and IgM but did not express CD24 (Fig. 2D). Interestingly, the increase in CD1d on TLR7-elicited IL-10$^+$ B cells suggested that ligation of this receptor expanded this B cell population, which is synonymous with regulatory activity [6] and belonged to the marginal zone compartment as previously described [27, 28]. To validate the IL-10-inducing effect of TLR7 ligation on B cells, CD1d^{hi} and CD1d^{lo} B cells were sorted and cultured in the presence of various TLR agonists (Pam3CSK4 (TLR 1/2), Poly I:C (TLR3), LPS (TLR4), Flagellin (TLR5), R848 (TLR7/8), or ODN 1826 CpG (TLR9); Fig. 2E). TLR7 treatment of CD19$^+$CD1d^{hi} B cells demonstrated significant IL-10 production, comparable to cells treated with CpG. Crucially, TLR7 treatment of CD19$^+$CD1d^{lo} B cells yielded significantly lower IL-10 concentrations, suggesting a predisposition for CD1d^{hi}-expressing

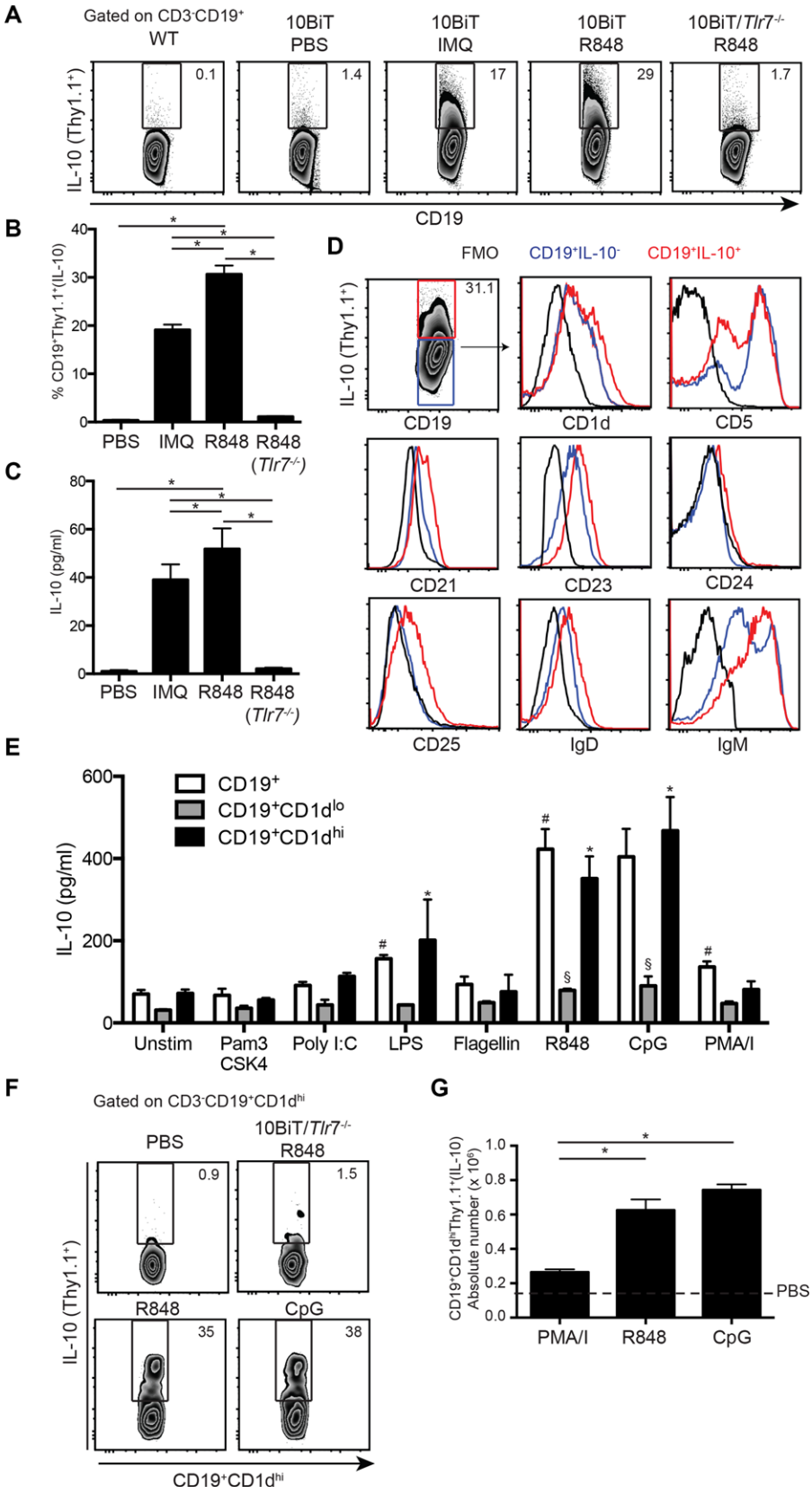

Figure 2. TLR7 ligation drives IL-10-producing B cells with expansion within the CD19$^+$CD1d^{hi} compartment. (A) Flow cytometry plots of IL-10-producing B cells from the spleens of 10BiT mice following treatment with the known TLR7 ligands IMQ and R848. IL-10-producing cells are denoted by their expression of Thy1.1. (B) Analysis of IL-10-producing B cells from spleens of 10BiT mice following the treatment with TLR7 ligands. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison test. (C) Serum IL-10 analysis following TLR7 ligand treatment via ELISA. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison test. (D) R848-elicited CD19$^+$ B cells were characterized using flow cytometry for CD1d, CD5, CD21, CD23, CD24, CD25, IgD, and IgM expression. Cells were compared on the basis of IL-10$^+$ (Thy1.1$^+$; red) or IL-10$^-$ (Thy1.1; blue) expression. (E) Concentration of IL-10 from culture supernatant of sorted CD19$^+$ (clear), CD19$^+$CD1d^{lo} (gray), and CD19$^+$CD1d^{hi} (black) B cells after culture with TLR agonists (Pam3CSK4, Poly I:C, LPS, Flagellin, R848 or ODN 1826 CpG) as measured by ELISA. #significance of CD19$^+$ cells, §significance of CD19$^+$CD1d^{lo}, and *significance of CD19$^+$CD1d^{hi} cells compared to respective unstimulated control populations. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison test. (F) Flow cytometry plots of gated CD19$^+$CD1d^{hi} B cells from 10BiT and 10BiT/*Tlr7*$^{-/-}$ mice stimulated in vitro with either R848 or ODN 1826 CpG. (G) Cell counts of IL-10$^+$ (Thy1.1$^+$) CD19$^+$CD1d^{hi} B cells as determined via flow cytometry when stimulated with either R848 or CpG. Dashed line represents PBS control. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison test (p <0.05). Flow cytometry are from single experiments representative of three experiments performed. (B, C, E, and G) Data are shown as mean + SEM (n = 3 mice/group) pooled from three independent experiments.

B cells to respond to TLR7 ligation. Using IL-10 reporter mice, it was demonstrated that the expansion of IL-10-producing B cells via TLR7 ligation in vitro compared favorably with CpG treatment. TLR7 ligation is a known inducer of IL-10-producing B cells [29] (Fig. 2F and G). Crucially, TLR7 ligation demonstrated an expansion in both the proportion (Fig. 2F) and the number of $CD19^+CD1d^{hi}$ B cells that produced IL-10 (Fig. 2G), suggesting a targeted expansion of this subset.

TLR7-ligated $CD19^+CD1d^{hi}$ B cells ameliorate OVA-induced AHR and drive an increase in $CD4^+FoxP3^+$ T cells

As we have shown previously that $CD19^+CD1d^{hi}$ B cells from helminth-infected mice can suppress AHR in an IL-10-mediated manner [10], and that in vivo TLR7 activation leads to an expansion in $CD19^+CD1d^{hi}$ B cells that produce IL-10, we assessed if TLR7-elicited IL-10-producing B cells were functional in suppression of AHR. 10BiT mice were sensitized to OVA and treated with 100 μg R848 i.p injection, following a standard OVA sensitization regimen (Fig. 3A). Flow cytometry analysis demonstrated R848 treatment increased the frequency of IL-10-producing B cells in the spleen ($p < 0.05$) but not in the lung or lung draining mediastinal lymph nodes (MLNs) (data not shown). On examination of lung function, R848-treated OVA-sensitized mice had significantly ($p < 0.05$) lower airway resistance compared to the untreated OVA-sensitized group (Fig. 3C), as reported previously [30, 31].

Having demonstrated that R848 treatment of OVA-sensitized mice suppressed AHR with the concordant in vivo expansion of spleen IL-10-producing B cells (Fig. 3B and C), we sought to determine whether R848-generated IL-10-producing B cells could ameliorate lung inflammation and associated AHR on adoptive transfer to OVA-induced recipient mice. B cells isolated from the spleens of WT and *Tlr7*$^{-/-}$ mice were treated in vitro with R848 for 24 h before being transferred into OVA-sensitized mice (Supporting Information Fig. 3A). As the $CD19^+CD1d^{hi}$ compartment demonstrated the highest proportion of IL-10-producing B cells, this phenotype was used for isolation via FACS. Although CD5 is coexpressed on some IL-10-producing $CD19^+CD1d^{hi}$ B cells [8, 9], TLR7 treatment expanded a $CD1d^{hi}CD5^{lo/neg}$ population that also produced IL-10 (Fig. 2D and data not shown) and as such CD5 was not used for cell selection.

OVA-sensitized mice recipients of TLR7-elicited $CD19^+CD1d^{hi}$ B cells had significantly ($p < 0.05$) lower airway resistance compared to the untreated OVA-sensitized group (Supporting Information Fig. 3B). As a control B-cell population, R848-treated $CD19^+CD1d^{hi}$ B cells from *Tlr7*$^{-/-}$ mice, which demonstrated no IL-10 production (Fig. 2B), were also transferred into OVA-sensitized mice. Transfer of these control B cells demonstrated no effect on airway resistance with comparable responses as the OVA control group ($p > 0.05$; Supporting Information Fig. 3B). Collectively, these data demonstrate that TLR7-elicited $CD19^+CD1d^{hi}$ B cells can suppress AHR when passively transferred into allergen-sensitized mice.

AHR suppression is mediated by $CD19^+CD1d^{hi}$ B cells through $CD4^+FoxP3^+$ induction

Having established that TLR7-elicited $CD19^+CD1d^{hi}$ B cells suppress AHR, we investigated the mechanism by which protection is conferred. As we have previously shown that IL-10-producing B cells from helminth-infected mice suppress AHR via IL-10 mediated induction of regulatory $CD4^+FoxP3^+$ T cells [10], we sought to determine whether TLR7-elicited $CD19^+CD1d^{hi}$ B cells acted in a similar manner. R848-treated $CD19^+CD1d^{hi}$ B cells were transferred into OVA-sensitized FoxP3eGFP mice. Identification of $CD4^+FoxP3^+$ T cells accounted for both natural and adaptive/induced Treg cells as described previously [32]. Flow cytometry analysis of $CD4^+FoxP3^+$ T cells indicated a significant ($p < 0.05$) increase in the percentage and number of $CD4^+FoxP3^+$ T cells in the spleen and lung of recipients of R848-treated $CD19^+CD1d^{hi}$ B cells (Fig. 3D). While transfer of isolated R848-treated $CD19^+CD1d^{hi}$ B cells from *Il10*$^{-/-}$ animals into OVA-sensitized FoxP3eGFP mice increased $CD4^+FoxP3^+$ T cells in the lungs and spleens, this was at a significantly reduced level ($p < 0.05$) relative to mice that received *Il10*$^{+/+}$ $CD19^+CD1d^{hi}$ B cells. Therefore, the capacity of TLR7-elicited $CD19^+CD1d^{hi}$ B cells to expand $CD4^+FoxP3^+$ T cells in vivo is in part IL-10 mediated. Transfer of R848-treated $CD19^+CD1d^{hi}$ B cells from *Tlr7*$^{-/-}$ mice demonstrated no significant increase in $CD4^+FoxP3^+$ T cells (Supporting Information Fig. 3C and D), thus demonstrating that TLR7 ligation drives activation of $CD19^+CD1d^{hi}$ B cells for downstream induction of $CD4^+FoxP3^+$ T cells.

To further define whether TLR7-elicited $CD19^+CD1d^{hi}$ B cells suppress AHR through $CD4^+FoxP3^+$ T cells, R848-treated $CD19^+CD1d^{hi}$ B cells were transferred into OVA-sensitized "depletion of Treg cell" DEREG mice. The efficiency of depletion of $CD4^+FoxP3^+$ T cells via diphtheria toxin was confirmed by flow cytometry (Fig. 4A). Untreated DEREG mice receiving R848-treated $CD19^+CD1d^{hi}$ B cells had reduced AHR compared to PBS-treated mice (Fig. 4B), as seen above (Fig. 3C). In contrast, DT-treated DEREG, with depletion of $CD4^+FoxP3^+$ T cells (Fig. 4A), that received TLR7-elicited $CD19^+CD1d^{hi}$ B cells developed marked AHR, with significantly ($p < 0.05$) elevated lung resistance compared to DEREG receiving treated $CD19^+CD1d^{hi}$ B cells (Fig. 4B). Transfer of R848-treated $CD19^+CD1d^{hi}$ B cells to DT-treated DEREG mice did not 'rescue' the depletion of $CD4^+FoxP3^+$ T cells in either the lung or spleen (Fig. 4C).

Furthermore, lung histology confirmed the suppression of lung inflammation by TLR7-elicited $CD19^+CD1d^{hi}$ B cell transfer. While OVA-sensitized DEREG recipients of TLR7-elicited $CD19^+CD1d^{hi}$ B cells had limited peribronchial airway inflammation, consistent with lung resistance analysis (Fig. 4B), when Treg cells were depleted, frank lung inflammation developed (Fig. 4D). Cell counts of bronchoalveolar lavage fluid (Fig. 4E) correlated with histological and lung function analyses, with significant cell infiltration and eosinophilia ($p < 0.05$) in $CD19^+CD1d^{hi}$ B cell transfers with $CD4^+FoxP3^+$ T cells depleted. Together, these data demonstrate that TLR7-elicited $CD19^+CD1d^{hi}$ B cells suppress OVA-induced AHR in mice via $CD4^+FoxP3^+$ T cells.

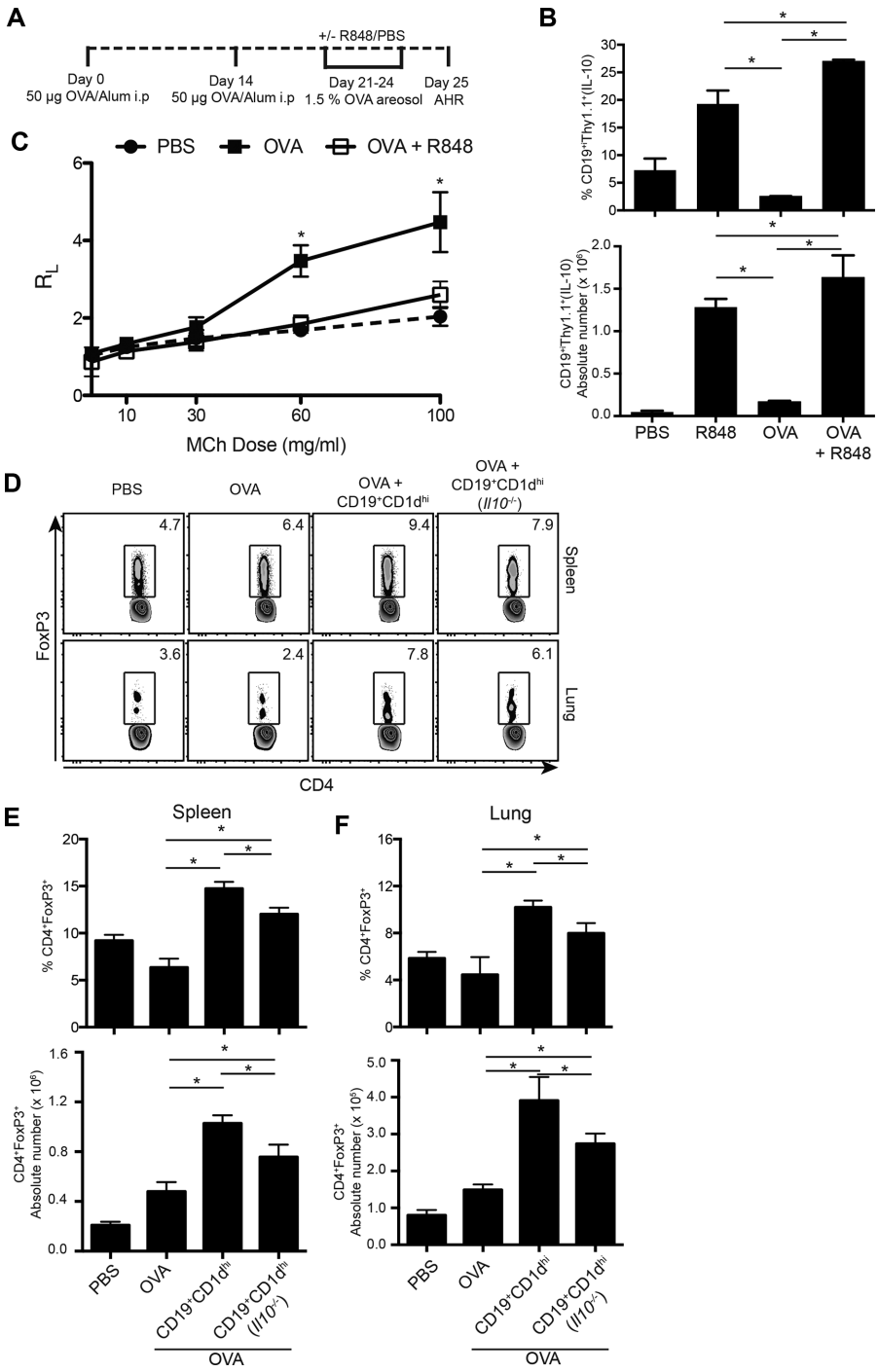

Figure 3. TLR7 ligation of $CD19^+CD1d^{hi}$ B cells can confer suppression of allergic lung inflammation. (A) OVA sensitization model: mice were sensitized with 50 µg OVA in alum i.p. on days 0 and 14. Airway challenge with 1.5% OVA was performed on days 21–24, concurrent with i.p injection of 100 µg R848. Spleen, MLN, and lung analysis was performed on day 25. (B) The proportion (top) and number (bottom) of IL-10-producing ($Thy1.1^+$) B cells in the spleen of R848-treated OVA-sensitized 10BiT mice were determined by flow cytometry. Significance determined by a one-way ANOVA with a Tukey's multiple comparison test ($p < 0.05$). (C) Methacholine-induced lung resistance (R_L) in R848-treated, OVA-sensitized mice. Significant responses were measured against a baseline of PBS administration. Significance determined via Students *t* test ($p < 0.05$). (D) Flow cytometry plots of splenic and lung $CD4^+FoxP3^+$ cells following adoptive transfer of R848-elicited $CD19^+CD1d^{hi}$ B cells from wild-type or $Il10^{-/-}$ mice into OVA-sensitized FoxP3eGFP mice. (E, F) The proportion (top) and number (bottom) of $CD4^+FoxP3^+$ in the (E) spleen and (F) lung were measured by flow cytometry. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison test ($^*p < 0.05$). Flow cytometry plots shown are representative of three independent experiments. Data are shown as mean ± SEM ($n = 3$–6 mice/group) pooled from three independent experiments.

TLR7 ligands can drive IL-10 production in human B cells

To address the relevance of these findings in man, we conducted studies into the induction of IL-10-producing B cells via TLR7 ligation of human cells. $CD19^+CD27^-$ B cells were isolated from peripheral blood mononuclear cells (PBMCs) and treated in vitro with a variety of TLR stimuli (LPS, IMQ, R848, or CpG) before restimulation with PMA/ionomycin (Fig. 5A). As negative and positive IL-10 inducing controls, LPS (10 µg/mL) and the human TLR9 ligand CpG-2006 (5 µg/mL) were used, respectively. IL-10 secretion was assessed via flow cytometry (Fig. 5B) and cytokine ELISA (Fig. 5C). Stimulus of $CD19^+CD27^-$ B cells with the TLR7 ligands IMQ (10 µg/mL) or R848 (10 µg/mL) both drove significant ($p < 0.05$) IL-10 production compared to unstimulated cells (Fig. 5A–C).

Phenotypic analysis of TLR7-elicited IL-10 positive B cells was conducted using flow cytometry. As there is no definitive phenotype for $IL\text{-}10^+$ B cells in man [14, 15, 29, 33], cells were screened for expression of relative markers including CD1d, CD5,

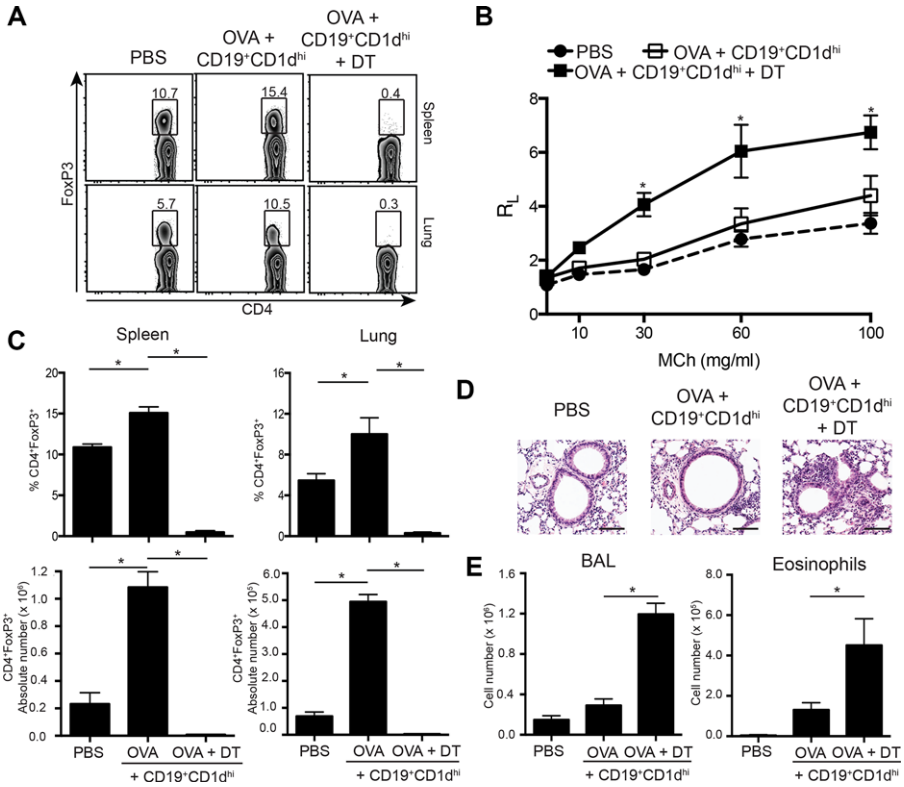

Figure 4. R848-treated CD19$^+$CD1d^{hi} B cells require CD4$^+$FoxP3$^+$ cells to reduce allergic lung inflammation. (A) Total CD4$^+$FoxP3$^+$ depletion via diphtheria toxin (DT) was confirmed via flow cytometry. (B) Methacholine-induced lung resistance (R_L) in DT-treated, OVA-sensitized DEREG mice after transfer of CD19$^+$CD1d^{hi} B cells. Significant responses were measured against a baseline of PBS administration. Significance determined via Student's *t*-test ($p < 0.05$). (C) The proportion (top) and number (bottom) of CD4$^+$FoxP3$^+$ cells in the spleen and lung of DT-treated, OVA-sensitized DEREG mice after transfer of CD19$^+$CD1d^{hi} B cells was determined by flow cytometry. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison test. (D) Representative hematoxylin and eosin stained lung sections of (left to right) nonsensitized, OVA-sensitized mice, which received R848-treated CD19$^+$CD1d^{hi} B cells, and DT-treated, OVA-sensitized mice, which received R848-CD19$^+$CD1d^{hi} B cells. Images acquired at 20× magnification (scale bar = 100 μm). (E) Analysis of bronchoalveolar lavage (BAL) fluid for pulmonary cell infiltration in DT-treated, OVA-sensitized mice was evaluated by cytocentrifugation of BAL, followed by staining with Kwik-Diff cytological staining kit and counted using morphologic criteria. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison test (*$p < 0.05$). Flow cytometry plots shown are representative of three independent experiments. (B, C, D, and E) Data are shown as mean ± SEM (n = 3–5 mice/group) pooled from three independent experiments.

CD24, CD25, and CD38. Figure 5D illustrates that TLR7-elicited human IL-10-producing B cells are CD19$^+$CD27$^-$IL-10$^+$, with elevated expression of CD1d$^+$, CD24$^+$, and CD25$^+$. However, they do not express significantly more CD5 or CD38 when compared to their IL-10$^-$ counterparts.

Discussion

In this study, we have been able to formally demonstrate that CD19$^+$CD1d^{hi} B cells express elevated TLR7, which can be exploited to drive a potent B-cell population that produces copious amounts of IL-10 and can suppress allergic lung inflammation. Identification of this upregulation in *Tlr7* expression stems from evidence of mice infected with the helminth *S. mansoni*, expanding a CD19$^+$CD1d^{hi} B cell population with regulatory activity [10, 11]. Microarray analysis of these cells from helminth-infected mice demonstrated increased expression of *Tlr7*. Through validation of the array data it was noted that CD19$^+$CD1d^{hi} B cells from uninfected mice also expressed significantly higher levels of *Tlr7* compared to CD19$^+$CD1d^{lo} or whole CD19$^+$ B cells.

Alterations in TLR function and expression on several immune cell subsets has previously been reported in helminth-infected mice [22, 34]. It is hypothesized that secretory/excretory molecules from pathogens are responsible for the suppression of TLR-associated effector proteins, leading to TLR tolerance [35–37]. This could be indicative of a scenario seen during a chronic, long-term infection, as observed with helminths. Pathway analysis of the microarray data did not infer the role of signaling molecules downstream of *Tlr7*, suggesting that upregulation of this receptor is due to another, yet undefined, mechanism. Further work is required to understand the functional relevance of *Tlr7* upregulation in the context of both helminth infection and expression in CD19$^+$CD1d^{hi} B cells.

It has previously been shown that OVA-sensitized mice treated with the TLR7 ligand R848 developed significantly lower AHR, with separate studies implicating roles for invariant NKT cells [31] and Treg cells [30]. Using IL-10 reporter mice, we were

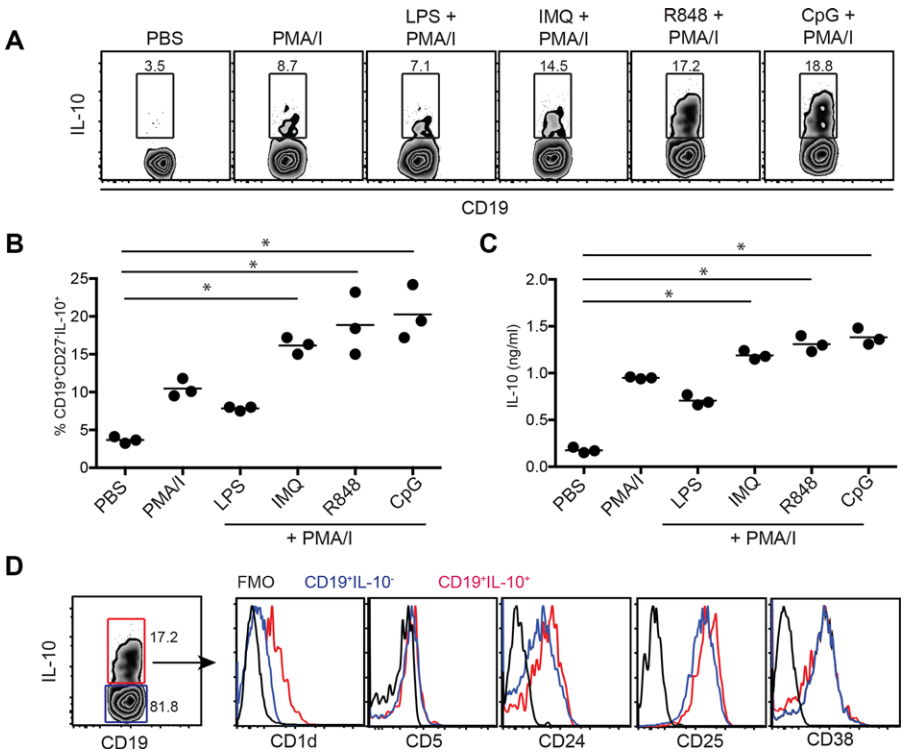

Figure 5. TLR7 ligands drive IL-10 production in human naïve CD19$^+$CD27$^-$ B cells. (A) Human CD19$^+$CD27$^-$ B cells were treated with LPS, IMQ, R848, or ODN 2006 CpG. Cells were restimulated with PMA/Ionomycin (PMA/I). CpG and LPS were used as positive and negative controls, respectively. CD19 and IL-10 expression were measured by flow cytometry. (B) Percentage of human CD19$^+$CD27$^-$ B cells stimulated with TLR ligands, which then went on to produce IL-10 was measured by flow cytometry. (C) Culture supernatants of human CD19$^+$CD27$^-$ B cells stimulated with TLR ligands were analyzed for IL-10 via ELISA. (B, C) Each symbol represents an individual donor and bars represent the mean of samples run in triplicate. (D) IL-10$^+$ human B cells were phenotyped for relevant markers CD1d, CD5, CD24, CD25, and CD38. Representative flow cytometry plots are shown. Data shown are from a single experiment representative of three experiments performed. Statistical significance was assessed by ANOVA ($p < 0.05$).

able to identify the existence of an expanded IL-10-competent B-cell population that was CD19$^+$CD1d^{hi}. To address the significance of these TLR7-elicited CD19$^+$CD1d^{hi} B cells, we generated this population *in vitro* and transferred to OVA-sensitized mice. Transfer of R848-elicited CD19$^+$CD1d^{hi} B cells conferred protection against AHR with concurrent increases in CD4$^+$FoxP3$^+$ T cells in the spleen and lung in a TLR7-mediated manner. Although a formulation of the TLR7 ligand IMQ (Aldara) has been demonstrated to function in a non-TLR7 dependent manner [38], Walter et al. were able to delineate that an excipient within the formulation (isostearic acid) had considerable biological activity. Given that the vehicle for IMQ and R848 in this study was endotoxin-free water, this was not considered a factor.

The fact that TLR7-elicited CD19$^+$CD1d^{hi} B cells expand CD4$^+$FoxP3$^+$ T cells suggests that these cells are similar to other reported Breg cells [10, 12, 33, 39]. As transfer of TLR7-elicited CD19$^+$CD1d^{hi} B cells increased CD4$^+$FoxP3$^+$ T cells in the lungs, it was suggested that their site of action was localized, at the site of allergen challenge. B-cell tracking studies, using R848-elicited CD19$^+$CD1d^{hi} B cells from CD45.1$^+$ mice, indicated that the cells traversed to the spleen and were not present in the lungs or the draining MLN of OVA-challenged mice (data not shown). This suggests transferred CD19$^+$CD1d^{hi} B cells return to the spleen and there prime CD4$^+$FoxP3$^+$ T cells. As the suppression of AHR by TLR7-elicited CD19$^+$CD1d^{hi} B cells is CD4$^+$FoxP3$^+$ T cell dependent, the site and mechanisms of B-T regulatory cell interplay requires further investigation. Synergistic treatment of human B-T cell cultures generates greater IL-10 production, suggesting that cell maturation is key for suppressive cell generation [40].

As demonstrated through the use of IL-10 reporter mice, TLR7-elicited CD19$^+$CD1d^{hi} B cells were significant producers of IL-10. *Il10*$^{-/-}$ mice indicated that TLR7-elicited CD19$^+$CD1d^{hi} B cells only partially use IL-10 to drive an increase in CD4$^+$FoxP3$^+$ T cells. This supports the notion that B reg cells may function through a number of suppressive mechanisms [41, 42], including a role for TGF-β [43–46] and more recently IL-35 [47, 48]. We have also recently described how B cells can suppress T-cell responses, via elevated PD-L1 expression, independent of IL-10 [49]. Indeed, there is evidence of the requirement for TGF-β in R848-mediated AHR suppression [30].

Studies of IL-10-producing B cells in man have indicated their dichotomy in function in different disease states [39]. Dysfunction of IL-10-producing B cells in patients with systemic lupus erythematous has been reported [14], while their presence in chronic hepatitis C infection is thought to contribute to poor prognosis [50]. Our data demonstrates that TLR7 ligation can drive an IL-10-producing B cell population in man. Elegant work by Lin et al. have identified subtle changes in a small proportion (~ 0.7%) of genes that are differentially expressed between IL-10$^-$ and IL-10$^+$ human B cells following stimulation with CpG [51]. Given that TLR7 shares a similar signaling pathway to TLR9 [52], it is plausible to suggest that some of the changes in gene expression described by Lin et al. would be conserved through TLR7 treatment. Crucially, the indication that TLR9 ligation can push human B-cell differentiation toward a germinal center (GC) fate is supported by murine data, which demonstrated that intrinsic TLR7 signaling is required for spontaneous GC development [53]. This raises the importance of further investigating B cell-TLR7 signaling within disease.

TLR7 is being investigated as a potential target for the treatment of asthma [30, 54, 55]. Association studies have indicated that mutations in *TLR7* and *TLR8* may confer susceptibility to asthma [56], while Roponen et al. report that in adolescents with asthma, TLR7 function is impaired that may also contribute to an increased susceptibility to respiratory viral infections [57]. Therefore, efforts have been made to target TLR7 in airway disease. Recent work by Matsui et al. has demonstrated that a TLR7 prodrug has inhibited features of the allergic airway inflammatory response in rat and murine allergic airway models [58]. They suggest that Type I IFN plays a role in the suppression of Th2 cytokines produced from murine splenocytes with a reduction in Th2-driven eosinophilia [59]. Although they ascribe no role for B cells, studies with the novel TLR7 agonist SA-2 demonstrated the production of IL-10 by B cells [54]. These studies indicate a potential for B cells with regulatory activity playing a key role in allergic airway disease.

In summary, we are able to formally demonstrate that *Tlr7* is upregulated on murine $CD19^+CD1d^{hi}$ B cells that contain suppressive B cells. TLR7 activation of $CD19^+CD1d^{hi}$ B cells drives significant expansion of IL-10-producing cells that can potently suppress AHR when adoptively transferred to allergen-primed recipients. Crucially, these cells require $CD4^+FoxP3^+$ T cells to mediate suppression, indicating a key B cell–T cell interaction, which is not solely dependent on IL-10. The formal identification TLR7 activation as a means to generate IL-10-producing B cells opens new avenues to define the mechanisms of regulatory B-cell activity.

Materials and methods

Mice

C57BL/6J mice, C57BL/6J Foxp3eGFP, $Il10^{-/-}$, and $Tlr7^{-/-}$ mice were originally from the Jackson Laboratories (Bar Harbor, ME, USA) and bred in-house. C57BL/6J IL-10 reporter (10BiT) mice [60] and C57BL/6J DEREG mice [61, 62] were also bred in-house. $Tlr7^{-/-}$ and 10BiT mice were crossed to generate 10BiT/$Tlr7^{-/-}$ mice. Mice were housed in a pathogen-free facility in individually ventilated and filtered cages under positive pressure (Techniplast, Northants, UK). The experiments were carried out in compliance with Irish Medicines Board regulations and approved by the Trinity College Dublin BioResources Ethical Review Committee.

Flow cytometry—murine cells

Single-cell suspensions from spleen, MLNs, and lung homogenates were prepared and surface marker expression on cells was assessed by flow cytometry or used for cell sorting as described [10, 63]. Cells were washed in flow cytometry staining buffer (PBS with 2% fetal calf serum (FCS) and 0.02% sodium azide) followed by blocking with anti-mouse CD16/32 (clone 93) (eBioscience). The following mAbs from BD Biosciences: CD1d-PE (IB1), CD3-FITC (17A2), CD4-V450 (RM4–5), CD19-allophycocyanin (1D3), CD21-FITC (7G6), CD25-biotin (7D4), IgD-PE (11–26c.2a), and CD90.1-allophycocyanin (HIS51); and eBiosciences: CD5-PE/Cy7 (53–7.3), CD23-PerCP-eFluor®710 (B3B4), CD24-PerCP-Cy5.5 (M1/69), and IgM-allophycocyanin-eFluor®780 (II/41) were used at optimally titrated concentrations. Streptavidin-PE-CF594 (BD Biosciences) was used as a secondary antibody for biotin labeling. For intracellular staining, cells were fixed and permeabilized with a commercial Foxp3 staining kit (eBiosciences) according to manufacturer's instructions, and the cells were stained with AlexaFluor647-conjugated rat anti-mouse Foxp3 mAb (MF23) rat anti-mouse mAb (BD Biosciences).

Viable cells were distinguished using LIVE DEAD Aqua (Invitrogen). Populations of interest were gated according to appropriate "fluorescence minus one" controls [64]. Representative gating strategies for murine B and T cells are illustrated in Supporting Information Figure 2. Samples were acquired on a CyAn flow cytometer (Beckman Coulter) and were analyzed with FlowJo software (Tree Star).

Flow cytometry—human cells

Single-cell suspensions from human PBMCs, prepared as below, were washed in flow cytometry staining buffer (PBS with 2% FCS and 0.02% sodium azide) followed by blocking with 10% FCS. The following mAbs from BD Biosciences were used at optimally titrated concentrations: CD1d-allophycocyanin (51.1), CD5-V450 (UCHT2), CD19-FITC (HIB19), CD24-PerCP-Cy5.5 (ML5), CD25-PE-CF594 (M-A251), CD27-V500 (M-T271), CD38-AF700 (HIT2). Viable cells were distinguished using Fixable Viability Dye eFluor® 780 (eBioscience).

Populations of interest were gated as above. Representative gating strategy is illustrated in Supporting Information Figure 2. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and were analyzed with FlowJo software (Tree Star).

Cell sorting

Spleen cells from mice were stained with CD1d-PE, CD3-FITC, and CD19-APC for cell sorting on a MoFlo cell sorter (Beckman Coulter, California, USA) as described [10]. Whole B cells were gated as $CD3^-CD19^+$ and sorted for $CD3^-CD19^+CD1d^{hi}$ B cells (Supporting Information Fig. 2B). Live cells were distinguished and gated via propidium iodide (Sigma) staining. Routinely the purity of sorted cell populations was > 95%. A representative gating strategy for cell sorting is provided in Supporting Information Figure 2. Sorted cells were used for adoptive transfer, microarray analysis, or Western blotting.

Preparation of RNA for RT-qPCR and microarray analysis

Sorted cells were washed and RNA prepared using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to

manufacturer's protocol. The concentration and purity of the prepared RNA was measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). Affymetrix Mouse Genome 430 2.0 arrays were performed on triplicate samples of each B cell subset (Source BioScience, Nottingham, UK).

RT-qPCR

Isolated RNA was reverse transcribed by using the QuantiTect® Reverse Transcription kit (Qiagen) and assayed by qPCR using a StepOnePlus real-time PCR system and TaqMan gene expression assays according to manufacturer's instructions (all Applied Biosystems). TaqMan gene expression assays for murine *Tlr7 and Il10* were used. All gene expression was normalized to GAPDH content.

Bioinformatics analysis

Full details of bioinformatic analysis of array data can be found in the Supporting Information.

Western blotting

Isolated cells were pelleted and then lysed for 30 min at 4°C with a buffer containing 150 mmol NaCl, 10 mmol Tris-HCL (pH 7.9), 0.5% v/v Triton X-100, and 0.6% v/v NP-40. One complete, Mini tablet (Roche) was added per 10 mL of lysis buffer to provide protease inhibition. The lysate was then centrifuged at 10 000 rpm for 10 min and the supernatant containing the soluble protein fraction removed. Seventy-five micrograms of lysate was then run on a 10% SDS-PAGE gel under reducing conditions before transfer to a nitrocellulose membrane. Membrane was blocked using 5% nonfat dry milk powder in PBS/Tween before incubation for 18 h with rat anti-mouse TLR7 mAb (clone #726606; R&D Systems) at 4°C. Blots were washed repeatedly in PBS/Tween before incubation with anti-rat IgG-HRP (R&D Systems) for 60 min at ambient temperature. Blots were developed using ECL Western Blotting Substrate (Thermo Scientific Pierce) and visualized via the ChemiDoc MP system (Bio-Rad). Blots were then stripped using Re-Blot-Mild buffer (Millipore) and reprobed for β-actin (clone C4; SantaCruz Biotech) before development as described above.

In vivo/in vitro TLR7 treatment

For in vivo TLR7 treatment, mice were injected i.p. with 100 μg IMQ or R848 (Invivogen), as described previously [30, 31], and spleens isolated 24 h later for analysis using flow cytometry. Mice were bled via cardiac puncture for serum cytokine analysis. For in vitro TLR7-treatment, spleens were isolated from mice of interest as described below. Cells were cultured at 2×10^6/mL in the presence of 10 μg/mL R848 (Invivogen) for 24 h before surface staining in order to be analyzed using flow cytometry or sorted for adoptive transfer experiments.

In vitro stimulation for analysis of IL-10-expressing murine B cells

Spleens from 10BiT mice were collected and single-cell suspensions created after erythrocyte lysis using BD PharmLyse® (BD Biosciences). Splenocytes or enriched B cells were cultured at a concentration of 2×10^6 cells/mL in the presence of RPMI-1640 (Invitrogen) supplemented with 10% heat-inactivated FCS (Sigma Aldrich), 2 mmol L-glutamine (Invitrogen), and 50 IU/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). For B cell enriched cultures, splenocytes were labeled with B220 microbeads (Miltenyi Biotec) before magnetic separation via AutoMACS™ Pro. Purity was determined by flow cytometry at > 90% (Supporting Information Fig. 2C). Cultures were stimulated with either 5 ng/mL PMA + 50 ng/mL ionomycin (Sigma Aldrich), 250 ng/mL Pam3CSK4 (Invivogen), 10 μg/mL Poly I:C (Sigma Aldrich), 1 μg/mL Flagellin (Invivogen), 10 μg/mL LPS (Sigma Aldrich), 1 μg/mL IMQ (Invivogen), 1 μg/mL R848 (Invivogen), or 5 μg/mL ODN 1826 CpG (Invivogen). Cultures were collected after 24 h with identification of IL-10-expressing cells determined by flow cytometry, as described above.

In vitro stimulation for analysis of IL-10-expressing human B cells

Buffy coats were provided by the Irish Blood Transfusion Service, under approval of the Trinity College Dublin Ethical Review Committee. Briefly, PBMCs were isolated via density centrifugation over Histopaque-1077 (Sigma Aldrich). Naïve B cells possessing the phenotype $CD19^+CD27^-$ were isolated using the EasySep™ Human Naive B Cell Enrichment kit (StemCell Technologies). Purity was assessed by flow cytometry and determined to be > 95% (Supporting Information Fig. 2F). Cells were cultured at a concentration of 2×10^6/mL in the presence of RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS (Sigma Aldrich), 2 mmol L-glutamine (InvitroGen), and 50 IU/mL penicillin, and 50 μg/mL streptomycin (InvitroGen). Cultures were stimulated with either 5 ng/mL PMA + 50 ng/mL ionomycin, 10 μg/mL LPS, 10 μg/mL IMQ (Invivogen), 10 μg/mL R848 (Invivogen), or 5 μg/mL ODN 2006 CpG (Invivogen) for 20 h followed by restimulation with 50 ng/mL PMA (Sigma Aldrich) + 1 μg/mL Ionomycin (Sigma Aldrich) for another 4 h. IL-10-secreting cells were then detected using the IL-10 Secretion assay (Miltenyi Biotec) as per manufacturer's instructions. Briefly, cells were labeled with a "catch" reagent on ice before incubation for 45 min at 37°C. Cells were then labeled with anti-IL-10-PE for analysis via flow cytometry. For the cytokine analyses, supernatants from B-cell cultures, stimulated as stated above, were collected for analysis via ELISA.

Experimental allergic airway inflammation

Mice were sensitized with OVA to induce allergic airway inflammation, as described previously [10, 63]. In the model, mice were administrated 50 μg OVA in alum (Pierce) i.p. on days 0 and 14, and subjected to airway exposure with 1.5% OVA in Dulbecco's phosphate buffered saline for 20 min on days 21–24. On days 21–24, an hour before airway exposure, mice were treated with either 100 μg R848 or 2×10^5 adoptively transferred R848-elicited $CD19^+CD1d^{hi}$ B cells from WT, *Tlr7*$^{-/-}$, or *Il10*$^{-/-}$ mice.

Treg cells were depleted from DEREG mice on days 21–24 using 1 μg diphtheria toxin i.p 18 h before adoptive transfer of R848-elicited $CD19^+CD1d^{hi}$ B cells. Twenty-four hours after the last OVA aerosol challenge, lung function was analyzed by an invasive method in which mice were tracheostomized and ventilated using a whole-body plethysmography with a pneumothechograph linked to a transducer (EMMS, Hants, United Kingdom) to determine changes in lung resistance in response to increasing doses of inhaled methacholine (10, 30, 60, and 100 mg/mL; Sigma-Aldrich).

Lung histology

Processing of lung tissue and immunologic analysis was as described previously [10, 63]. In brief, the following method was carried out:

–Bronchoalveolar lavage fluids were collected by cannulating the trachea and lavaging the lungs twice with 0.8-mL ice-cold Dulbecco's phosphate buffered saline. Bronchoalveolar lavage cells were pelleted, washed, and counted before cytospinning. For cytospins, the numbers of eosinophils was determined by performing a differential count, with at least 400 cells per slide, followed by staining with Kwik-Diff cytological staining kit (Thermo Scientific) and counted using morphologic criteria.
–Lung homogenates and digests: Whole lungs were removed from mice and placed in 10% formaldehyde saline for histology, or digested with collagenase-D (Roche) for flow cytometry analysis.
–Lung histology: Lungs were fixed in 10% formaldehyde saline for histology.

Paraffin-embedded tissue sections were stained with hematoxylin and eosin, and eosinophil infiltration was counted on Giemsa-stained sections. Airway mucus occlusion was analyzed on periodic acid Schiff stained lung sections. Images were acquired using the Aperio scanscope.

IL-10 ELISA

ELISA on the collected supernatants from human cell cultures and murine serum samples was performed to quantify levels of IL-10 using DuoSet ELISA kits (for murine and human samples, respectively) (R&D systems, Minneapolis, MN, USA) according to manufacturer's protocol.

Statistics

Statistical analysis was performed by GraphPad Prism (GraphPad Software Inc, La Jolla, USA). Data are shown as means ± SEM. Specific statistical tests are described in the figure legends. In all cases, differences were considered significant where $p < 0.05$.

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Abbreviations: **AHR**: airway hyperresponsiveness · **IMQ**: imiquimod

Full correspondence: Prof. Padraic G. Fallon, Trinity Biomedical Sciences Institute, School of Medicine, Trinity College Dublin, Dublin, Ireland
Fax: +353-1-8964040
e-mail: pfallon@tcd.ie

Current address: Martin O. Leonard, Centre for Radiation, Chemical and Environmental Hazards, Public Health England, Chilton, Didcot, Oxon OX11 0RQ, UK

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