IgG_4 production is confined to human IL-10–producing regulatory B cells that suppress antigen-specific immune responses

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Background: IL-10–producing regulatory B cells suppress immune responses, and lack of these cells leads to exacerbated symptoms in mouse models of chronic inflammation, transplantation, and chronic infection. IgG_4 is a blocking antibody isotype with anti-inflammatory potential that is induced in human high-dose antigen tolerance models.

Objective: We sought to characterize human inducible IL-10–secreting B regulatory 1 (B_R1) cells and to investigate their immunoregulatory capacity through suppression of cellular immune responses and production of anti-inflammatory immunoglobulins.

Methods: Highly purified IL-10–secreting B cells were phenotypically and functionally characterized by means of whole-genome expression analysis, flow cytometry, suppression assay, and antibody production. B cells specific for the major bee venom allergen phospholipase A_2 (PLA) were isolated from beekeepers who displayed tolerance to bee venom antigens and allergic patients before and after specific immunotherapy.

Results: Human IL-10^+ B_R1 cells expressed high surface CD25 and CD71 and low CD73 levels. Sorting of $CD73^-CD25^+CD71^+$ B cells allowed enrichment of human B_R1 cells, which produced high levels of IL-10 and potently suppressed antigen-specific $CD4^+$ T-cell proliferation. IgG_4 was selectively confined to human B_R1 cells. B cells specific for the major bee venom allergen PLA isolated from nonallergic beekeepers show increased expression of IL-10 and IgG_4. Furthermore, the frequency of IL-10^+ PLA-specific B cells increased in allergic patients receiving allergen-specific immunotherapy.

Conclusion: Our data show the characterization of IL-10^+ B_R1 cells and *in vivo* evidence for 2 essential features of allergen tolerance: the suppressive B cells and IgG_4-expressing B cells that are confined to IL-10^+ B_R1 cells in human subjects.

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Peripheral tolerance to allergens uses multiple mechanisms to suppress allergic inflammation, including suppression of dendritic cells that support the generation of effector T cells; induction of dendritic cells that support the generation of regulatory T (Treg) cells; suppression of effector T cells; suppression of mast cells, basophils, and eosinophils; interaction with resident tissue cells and remodeling; suppression of allergen-specific IgE; and induction of allergen-specific IgG_4.[1] Peripheral tolerance as a result of high-dose allergen exposure, as occurs in beekeepers, cat owners, or helminth-infected subjects, is accompanied by increased levels of specific IgG_4 antibodies.[2-4] Because of its unique structural features in the hinge region, IgG_4 has several noninflammatory properties, including low affinity for the classical Fcγ receptors and the capacity to form bispecific, functionally monovalent antibodies through Fab arm exchange.[5] IgG_4 antibodies lack the ability to cross-link antigen and form immune complexes. In addition, IgG_4 is unable to activate the complement cascade.[6,7]

Studies on the mechanisms of immune responses to allergens have demonstrated that inducible T regulatory 1 (T_R1) cells are dominant in healthy subjects.[8,9] T_R1 cells not only suppress T_H1 or T_H2 cell responses but also have a direct influence on B cells. T_R1 cells, as well as natural $CD4^+CD25^+$ Treg cells, suppress IgE and induce IgG_4. Both subsets of Treg cells (inducible and natural) reduce the IgE-secreting plasma cell frequency and simultaneously augment the IgG_4-secreting plasma cell frequency.[10] A useful tool to study the *in vivo* regulation of immune tolerance in response to high-dose antigen exposure in human subjects is the beekeeper model.[9] Continuous exposure to high doses of bee venom antigens in nonallergic beekeepers diminishes T cell–mediated cutaneous late-phase swelling reactions, suppresses allergen-specific T-cell proliferation, and induces an *in vivo* switch from allergen-specific T_H1 and T_H2 cells toward IL-10–producing T_R1 cells.[9] Although they produce IgE antibodies against venom antigens, beekeepers do not show anaphylaxis. Furthermore, beekeepers have high levels of circulating IgG_4 antibodies specific for the major bee venom allergen

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Abbreviations used

B_R1:	B regulatory 1
Breg:	Regulatory B
IL-10R:	IL-10 receptor
PLA:	Phospholipase A_2
PPD:	Purified protein derivative
SIT:	Specific immunotherapy
TLR:	Toll-like receptor
TLR9-L:	TLR9 ligand
T_R1:	T regulatory 1
Treg:	Regulatory T

phospholipase A_2 (PLA).[11,12] On the other hand, patients with bee venom allergy have IgE antibodies mainly to PLA but show increased production of specific IgG_4 in response to bee venom specific immunotherapy (SIT).[13]

B cells contribute to immune responses essentially through antigen presentation to T cells, secretion of cytokines, and production of antibodies after differentiation to plasma cells.[14] When they receive the right survival signals, plasma cells can reside for many years in dedicated niches in the bone marrow and continuously produce antibodies independent of antigen exposure.[15] On activation, IgM^+IgD^+ naive B cells can undergo class-switch recombination, leading to the expression of IgA, IgG, or IgE antibodies. Human B cells express several Toll-like receptors (TLRs), including TLR1, TLR6, TLR7, TLR8, TLR9, and TLR10. TLR7 (activated by single-stranded RNA) and TLR9 (activated by hypomethylated CpG DNA) are the most highly expressed TLRs on B cells.[16]

IL-10 is a key regulator of inflammatory responses and protects the host from tissue damage as a result of excessive inflammation.[17] It suppresses antigen presentation and the production of proinflammatory chemokines and cytokines. On the other hand, IL-10 enhances survival, proliferation, differentiation, and isotype switching of human B cells.[17] IL-10 augments IgG_4 production, whereas it inhibits IL-4–induced IgE class-switch recombination.[18,19] IL-10–mediated immunosuppressive functions of B cells have been described in murine models of autoimmunity,[20-23] infection,[24,25] cancer,[26] and allergic airway inflammation.[27,28] The relevance of immunoregulatory functions of human B cells was illustrated in patients with rheumatoid arthritis treated with the B cell–depleting antibody rituximab who showed exacerbation of ulcerative colitis and development of psoriasis.[29,30]

We hypothesize that if a B cell plays an anti-inflammatory role, the antibody isotype produced by the plasma cell originating from this B cell should also be anti-inflammatory. In the present study we characterized human inducible IL-10–producing B cells, which we designate B regulatory 1 (B_R1) cells, and their immunoglobulin production. Whole-genome microarray expression analysis was performed to characterize human circulating B_R1 cells. Functional experiments demonstrated that B_R1 cells could directly suppress antigen-specific $CD4^+$ T-cell responses and significantly upregulate IgG_4, but not IgG_1, IgA, or IgE, on differentiation to plasma cells. High-dose bee venom–exposed beekeepers and patients with bee venom allergy before and after bee venom SIT were used to demonstrate these findings *in vivo*.

METHODS

Heparinized peripheral blood samples from healthy donors and beekeepers, as well as allergic patients, before and after ultrarush bee venom SIT were used in the study. For staining of IL-10–secreting PLA^- and PLA^+ B cells, 4 allergic patients aged 18 to 65 years with a history of moderate-to-severe systemic allergic reactions to honeybee stings grade II to IV, positive intracutaneous skin test results to bee venom of less than or equal to 1024 g/L, and bee venom–specific serum IgE levels of 0.7 kU/L or greater in the ImmunoCAP FEIA were included in the study. Details on the immunotherapy protocol have been described elsewhere.[31] Blood samples were taken before the start of therapy and on day 110. The study was approved by the Ethical Commission of the Canton of Graubünden, Switzerland, and by the Ethical Commission of the Canton of Bern, Switzerland.

B-cell subsets were sorted by means of immunomagnetic separation and flow cytometric cell sorting. Whole-genome expression profiling was performed with a gene array, and select candidate genes were confirmed by using real-time PCR and flow cytometry. Suppression of antigen-specific proliferation was measured by using tritiated thymidine and carboxyfluorescein succinimidyl ester (CSFE) dilution experiments. Production of IL-10, IgG_1, IgG_4, IgA, and IgE mRNA and protein were measured with real-time PCR and ELISA or a bead suspension array, respectively. Allergen-specific cells were labeled with fluorescently labeled allergens. A detailed description of the methods used in this article can be found in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Purification and characterization of human IL-10–secreting B_R1 cells

Stimulation with the TLR9 ligand (TLR9-L) CpG2006 was found to optimally induce IL-10 in purified B cells. Stimulated B cells were labeled with anti–IL-10/CD45 antibody-antibody conjugates and subsequently incubated to allow secretion and capture of IL-10 to the cell surface for the purification of alive IL-10–secreting ($IL-10^+$) B cells. Both resting and proliferating B cells expressed IL-10 in response to TLR9 stimulation (see Fig E1, *A-C*, in this article's Online Repository at www.jacionline.org).

To characterize B_R1 cells, $IL-10^+$ B cells and $IL-10^-$ B cells were sorted, and IL-10 mRNA expression was strongly confined to $IL-10^+$ cells (Fig 1, *A*). Purified $IL-10^+$ and $IL-10^-$ B cells were subjected to whole-genome microarray expression analysis. Several genes involved in IL-2 signaling were upregulated in $IL-10^+$ cells, including IL-2 receptor α *(IL2RA)*, IL-2 receptor β *(IL2RB)*, Janus kinase 1 *(JAK1)*, signal transducer and activator of transcription 1 *(STAT1)*, *STAT3*, *STAT5A*, and *PTPN11* (see Fig E1, *D*, and Table E1 in this article's Online Repository at www.jacionline.org). *JAK1* and *STAT3* are also key factors in IL-10 signaling. Several genes encoding surface markers were differentially expressed between $IL-10^-$ and $IL-10^+$ B cells. A heat map of the 5 genes showing the highest median differential expression between $IL-10^-$ versus $IL-10^+$ genes is shown in Fig 1, *B*. Genes encoding surface markers that showed higher expression in $IL-10^+$ cells included *CD80* (4.5-fold), *IL2RA* (4.4-fold), *TFRC* (4.1-fold), *CD274* (4.1-fold), and *IL2RB* (2.8-fold). Genes encoding surface markers that showed lower expression in $IL-10^+$ cells included *PTPRJ* (2.5-fold), *NT5E* (2.3-fold), *CD79B* (2.2-fold), *CD37* (2.0-fold), and *FCGR2B* (1.9-fold; Fig 1, *B*, and see Table E2 in this article's Online Repository at www.jacionline.org). Differential expression of IL2RA (CD25), TFRC (CD71), CD274 (PD-L1), and NT5E (CD73) was

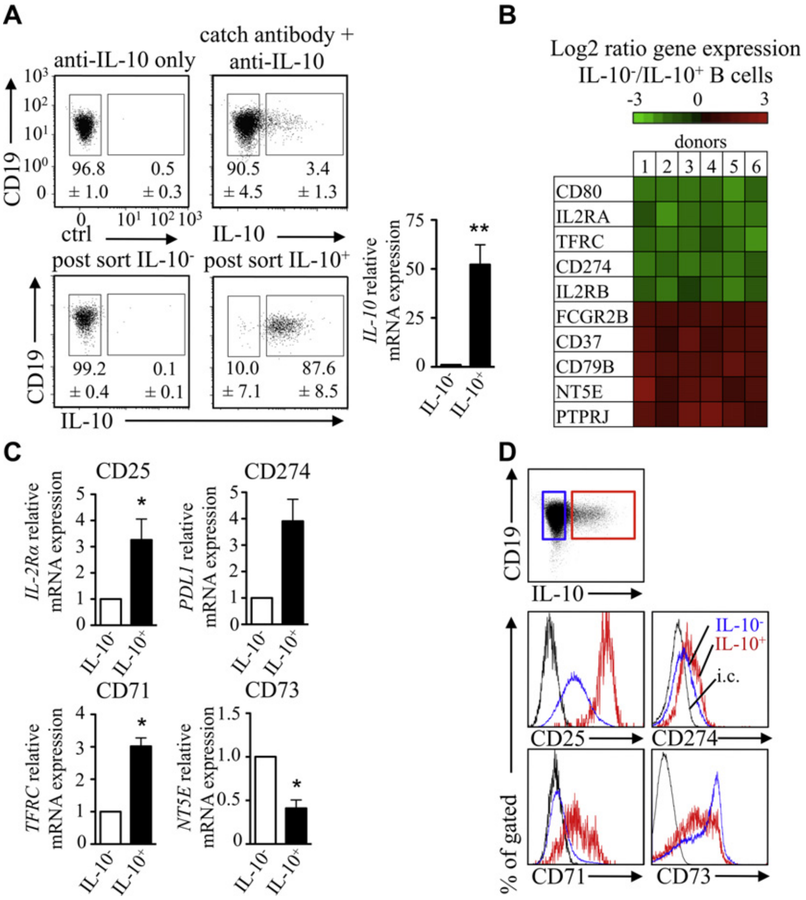

FIG 1. Characterization of IL-10$^-$ and IL-10$^+$ B cells. **A,** Sorting of IL-10$^-$ and IL-10$^+$ B cells. IL-10 expression was confirmed by using real-time PCR. **B,** Gene array result showing upregulated or downregulated surface marker genes in IL-10$^-$ versus IL-10$^+$ B cells. **C,** mRNA expression (relative to IL-10$^-$) of selected surface markers (n = 6). **D,** CD25, CD71, CD274, and CD73 surface staining (representative example of 3). *$P < .05$ and **$P < .01$.

confirmed by means of real-time PCR (Fig 1, *C*) and flow cytometry (Fig 1, *D*). Thus IL-10$^+$ B cells showed increased expression of CD25, CD71, and PD-L1, whereas CD73 expression was reduced. We then investigated the expression of other B-cell markers and surface markers that have been linked to regulatory B (Breg) cells in previous reports. Surface expression of CD38, CD24, CD5, CD27, IgM, and IgD was also measured to further characterize IL-10$^+$ B cells. TLR9-L–induced IL-10$^+$ cells had similar frequencies among CD24^{int}CD38^{int}, CD24^{hi}CD38$^-$, and CD24^{hi}CD38^{hi} B cells (see Fig E1, *E*). The frequency of CD27$^+$ B cells was similar between IL-10$^-$ and IL-10$^+$ B cells, whereas the level of CD27 expression was lower among CD27$^+$ B cells in the IL-10$^+$ population. The frequencies of CD5$^+$ and IgM$^+$IgD$^-$ cells were 2-fold higher in IL-10$^+$ cells (see Fig E1, *F*). Again, purified CD27$^-$ and CD27$^+$ B cells produced similar levels of IL-10 in response to TLR9-L (see Fig E1, *G*). These data suggest that B cells that produce IL-10 in response to TLR9-L stimulation are not restricted to a single subset of B cells.

IL-10–producing B cells are enriched in CD73$^-$CD25$^+$CD71$^+$ B cells

To isolate IL-10–producing B cells, we stained CD25 and CD71 as high expression markers and CD73 as a low/negative expression marker on circulating B cells without any stimulation. PD-L1 surface expression showed only a mild upward shift in IL-10$^+$ B cells and therefore was not suitable as a distinguishing marker. Among CD73$^-$ B cells, CD25$^+$ and CD71$^+$ cells formed a separate population (see Fig E2 in this article's Online Repository at www.jacionline.org), and CD25$^+$CD71$^+$ B cells had a 4-fold higher frequency in CD73$^-$ cells than in CD73$^+$ cells (Fig 2, *A*). CD73$^-$CD25$^+$CD71$^+$, CD73$^-$CD25$^-$CD71$^-$, CD73$^+$CD25$^+$CD71$^+$, and CD73$^+$CD25$^-$CD71$^-$ B cells were sorted (Fig 2, *B*) and subsequently stimulated with TLR9-L. We found that CD73$^-$CD25$^+$CD71$^+$ B cells secreted the highest amounts of IL-10. In contrast, CD73$^+$CD25$^+$CD71$^+$ cells secreted 3-fold less IL-10, and CD73$^-$CD25$^-$CD71$^-$ and CD73$^+$CD25$^-$CD71$^-$ cells showed the lowest IL-10 production (Fig 2, *C*).

IL-10 plays an essential role in T-cell suppression by human B$_R$1 cells

Next, we wanted to determine whether B$_R$1 cells have suppressive capacity on antigen-specific proliferative responses. IL-10$^+$ and IL-10$^-$ B cells were purified by using the IL-10 secretion assay and cocultured at different ratios with autologous PBMCs that were stimulated with purified protein derivative (PPD). With a

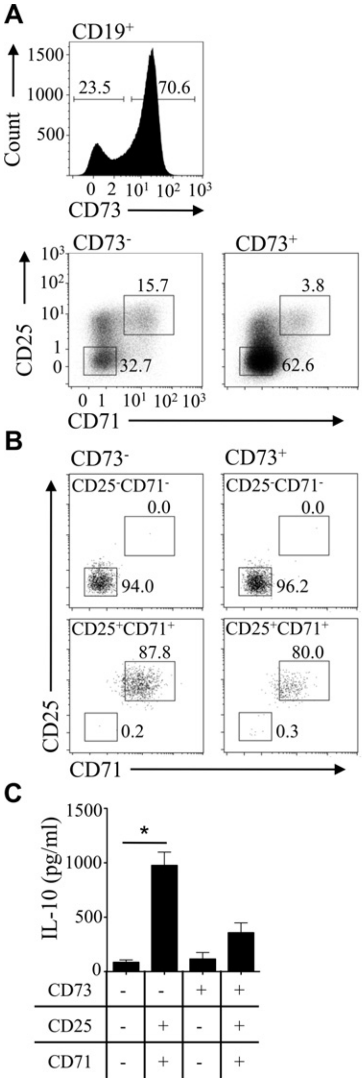

FIG 2. Sorting of cell populations from resting B cells based on expression of CD73, CD25, and CD71. **A,** Staining of CD25 and CD71 on $CD73^-$ and $CD73^+$ B cells (n = 3; 1 representative example is shown). **B,** Sorted B-cell populations were reanalyzed to assess purity. **C,** Sorted B-cell populations (n = 3, 5 × 10^4 cells in 100 μL) were stimulated for 48 hours, and IL-10 levels were measured in supernatants. *$P < .05$.

ratio of 1 B cell to 25 PBMCs, we observed up to 50% suppression by IL-10^+ B cells on PPD-induced proliferative responses in PBMCs, whereas other B cells did not show suppressive capacity. This effect was reversed on blocking the IL-10 receptor (IL-10R). Carboxyfluorescein succinimidyl ester dye analysis confirmed these findings and furthermore demonstrated that proliferation of $CD4^+$ T cells in response to PPD stimulation was strongly suppressed by IL-10^+ B cells. PPD-induced $CD4^+$ T-cell proliferation was suppressed approximately 50% with a ratio of 1 IL-10^+ B cell to 25 responder cells (Fig 3, *A*, and see Fig E3 in this article's Online Repository at www.jacionline.org). IL-10^- B cells could also partially suppress $CD4^+$ T-cell proliferation. In the presence of a blocking anti–IL-10R antibody, the percentage of proliferating $CD4^+$ T cells was generally higher in cultures with added B cells (both IL-10^- and IL-10^+ cells) than in PBMCs (Fig 3, *A*), possibly because of increased antigen presentation by B cells and neutralization of endogenous IL-10.

$CD73^-CD25^+CD71^+$, $CD73^-CD25^-CD71^-$, $CD73^+CD25^+CD71^+$, and $CD73^+CD25^-CD71^-$ B cells were sorted and cocultured at different ratios with autologous PPD-stimulated PBMCs to identify the suppressive capacity of B cells that differentially express CD25, CD71, and CD73. Only $CD73^-CD25^+CD71^+$ B cells could suppress $CD4^+$ T-cell proliferation, whereas the other 3 B-cell subsets did not show any suppressive effect (Fig 3, *B*). However, this suppressive effect was not fully reversed through addition of blocking anti–IL-10R antibody. Because of cell number limitations, higher B cell/PBMC ratios could not be used.

Immunoglobulin production of B_R1 cells

We then investigated the isotype of the produced immunoglobulin by circulating human B_R1 cells on their differentiation to plasma cells. TLR9-L stimulation of purified B cells induced expression of IgG_1, IgG_4, and IgA, but not IgE, at the mRNA level, whereas protein secretion of all 4 subclasses was induced. TLR9-L plus IL-10 stimulation showed a synergistic effect on IgG_4 production, whereas IgG_1 and IgE induction were not affected. IgA production showed a tendency to be suppressed by IL-10 (Fig 4, *A*). The small amount of IgG_4 induced by TLR9-L was mainly derived from $CD27^+$ B cells and was dependent on endogenous IL-10 production because blocking of IL-10R largely suppressed IgG4 production by $CD27^+$ cells. Dual stimulation with TLR9-L and IL-10 particularly increased IgG_4 production in $CD27^-$ B cells (see Fig E4 in this article's Online Repository at www.jacionline.org).

In the next step, IL-10^- and IL-10^+ B cells were sorted to investigate their immunoglobulin profiles on activation with TLR9-L. IL-10^- and IL-10^+ B cells were cultured for 5 days, and the expression of immunoglobulin mRNAs was analyzed. IgG_4 transcripts were expressed, on average, 10-fold higher in the IL-10^+ population than in the IL-10^- population (Fig 4, *B*). IgG_1, IgA, and IgE were not differentially expressed between the 2 populations.

IL-10^+ and IL-10^- cells were isolated from $CD27^-$ and $CD27^+$ B cells that were sorted before stimulation with TLR9-L and immunoglobulin production was measured to further delineate this distinct upregulation of IgG_4. IL-10^- and IL-10^+ B cells derived from $CD27^+$ B cells showed no significant differences in the expression of IgG_1, IgG4, or IgA. On the other hand, IL-10^+ B cells derived from $CD27^-$ B cells produced 15-fold higher IgG_4 levels than IL-10^- cells, whereas IgG_1 and IgA production was not significantly increased (Fig 4, *C*).

Allergen-specific B_R1 cells are confined to IgG_4 production in high-dose bee venom tolerance

PLA-specific B cells from highly bee venom–exposed tolerant beekeepers were enriched by using labeled PLA. Between 0.1% and 0.9% of beekeeper-derived $CD19^+$ peripheral B cells were specific for PLA (Fig 5, *A*). Subsequently, PLA-specific (PLA^+) and non–PLA-specific (PLA^-) B cells were sorted (Fig 5, *A*). Immunoglobulin isotypes and IL-10 mRNAs were measured immediately after sorting of the cells without any stimulation. IgG_4 expression was significantly higher (median increase, 14.1-fold) in PLA^+ B cells than in PLA^- B cells. IgA expression showed a modest decrease (median decrease, 2-fold) in PLA^+ B cells, whereas expression of IgE and IgG_1 transcripts showed no significant difference between PLA^+ and PLA^- B cells. IL-10

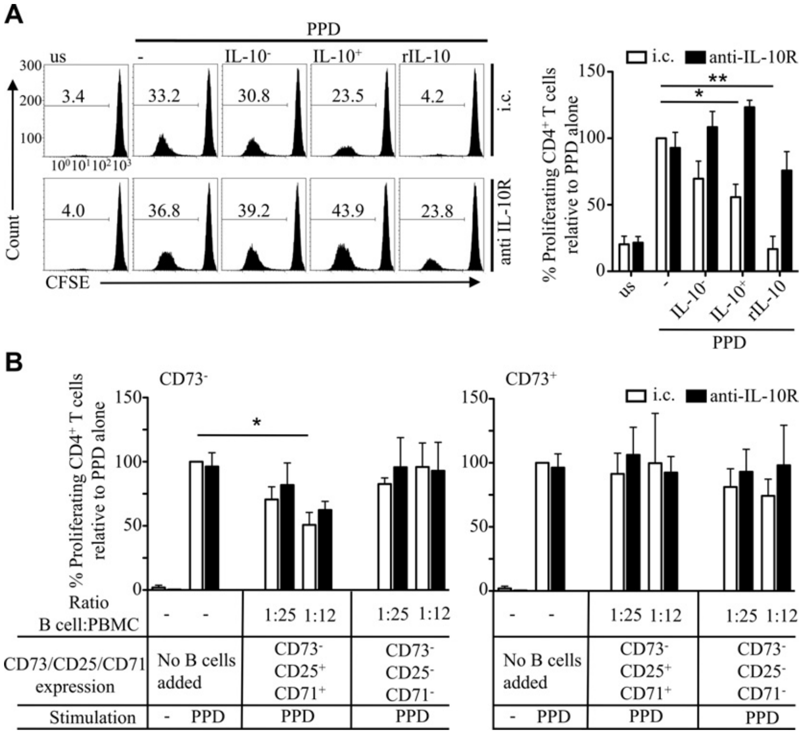

FIG 3. B_R1 cells suppress antigen-specific proliferation. **A,** Cocultures of carboxyfluorescein succinimidyl ester *(CFSE)*–labeled PBMCs with IL-10$^+$ or IL-10$^-$ B cells (ratio, 1 B cell to 25 PBMCs) with blocking anti–IL-10R or isotype control *(i.c.)* mAbs. rIL-10 was used as a control. Histograms show a representative example. Numbers indicate the percentage dividing CD4$^+$ cells. **B,** Cocultures of PBMCs with CD73, CD25, and CD71+/− B cells. Combined data from 4 independent experiments are shown. $*P < .05$ and $**P < .01$.

mRNA expression was significantly increased (median increase, 30.8-fold) in PLA-specific B cells (Fig 5, *B*). B cells from beekeepers and patients with bee venom allergy before and after SIT were stimulated for 3 days with CpG and stained for IL-10 and PLA binding to their surface to measure IL-10 protein production. The mean frequency of IL-10$^+$ cells was 1.8-fold higher in PLA$^+$-gated than in PLA$^-$-gated B cells from beekeepers. PLA$^+$ B cells in allergic patients had relatively low frequencies of IL-10$^+$ B cells (1.8% to 2.9%), which significantly increased (5.5% to 13.9%) after 110 days of bee venom SIT (Fig 5, *C*). In line with the flow cytometric data, secreted IL-10 levels in these samples were 2-fold higher in PLA$^+$ than in PLA$^-$ cells (see Fig E5 in this article's Online Repository at www.jacionline.org).

Additional *in vivo* evidence for the role of B_R1 cells in allergen tolerance that links IL-10 to IgG_4 was obtained by measuring of PLA-specific IgE and IgG_4 levels in serum of beekeepers, patients with bee venom allergy, and allergic subjects who received 110 days of bee venom SIT. Allergic subjects who received SIT showed a significantly lower (100-fold) PLA-specific IgE/IgG_4 ratio when compared with nontreated allergic subjects. This difference was even greater (1000-fold) when compared with that seen in nonallergic beekeepers. In both cases this was due to substantially increased levels of specific IgG_4 (Fig 5, *D*).

DISCUSSION

This is the first study in which human inducible IL-10–producing B cells (designated as B_R1 cells) were purified based on their secretion of IL-10. Gene arrays were performed, and novel molecules that characterize B_R1 cells were identified. We demonstrated a potent suppressive capacity of IL-10–producing B cells on antigen-specific CD4$^+$ T-cell activation, and more importantly, we observed a striking shift toward production of anti-inflammatory IgG_4 antibodies in these cells.

Despite accumulating data describing immunoregulatory functions of IL-10–producing B-cell subsets in murine models, there is still little known about the function and phenotype of human Breg cells. There are indications that distinct B-cell subsets have immunoregulatory functions in human subjects.[29,30,32] Human CD24^{hi}CD38^{hi} B cells were shown to suppress the differentiation of T_H1 cells through provision of IL-10 and possibly also through CD80- and CD86-mediated signaling.[32,33] In another study a CD24^{hi}CD27$^+$ human B-cell subset was identified that contained a majority of IL-10–producing B cells and was found at higher frequencies in patients with autoimmune disease compared with healthy control subjects.[33]

A fraction of approximately 1% to 4% of B cells secreted IL-10 in response to TLR9-L stimulation. These cells were not restricted to a single population of B cells because both CD27$^-$ (primarily naive) and CD27$^+$ (primarily memory) B cells secreted similar amounts of IL-10. Unlike the described CD24^{hi}CD38^{hi} Breg cells that produced IL-10 in a CD40 ligand–dependent manner,[32] the frequency of B_R1 cells was equally distributed among CD24^{int}CD38^{int}, CD24^{hi}CD38$^-$, and CD24^{hi}CD38^{hi} B cells. Furthermore CD5$^+$ and IgM$^+$IgD$^-$ cells were enriched among IL-10$^+$ B cells. By using whole-genome expression analysis and flow cytometry, we could identify 4 surface markers that were significantly differently expressed on B_R1 cells compared with IL-10$^-$ B cells. B_R1 cells showed increased expression of CD25, CD71,

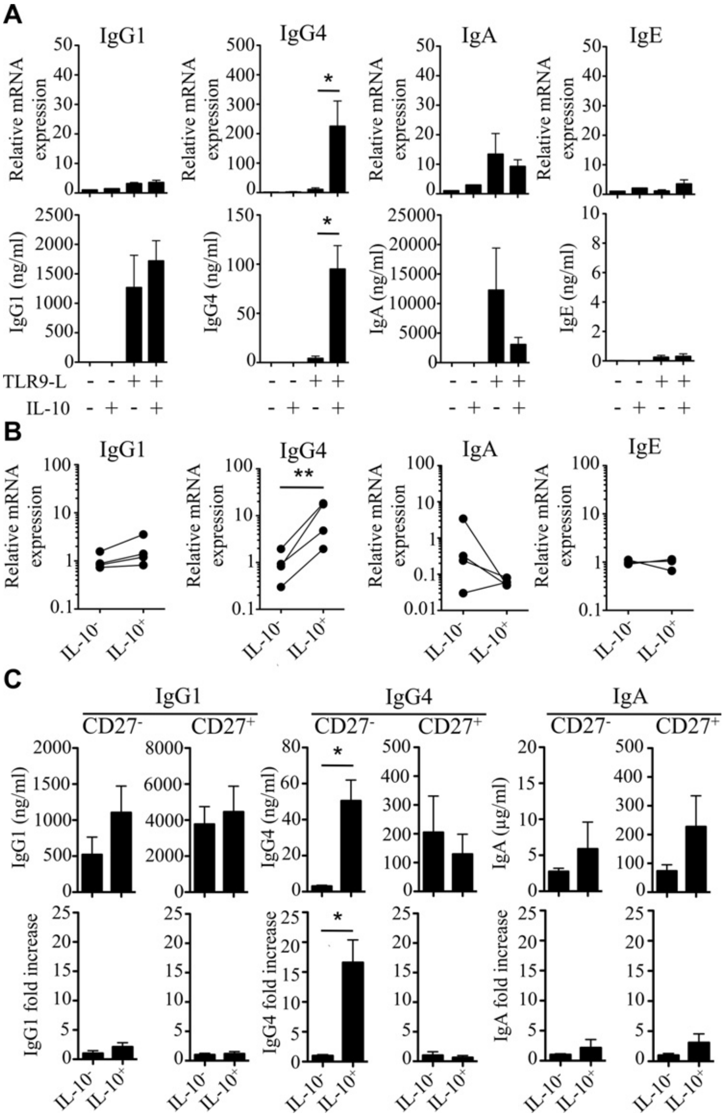

FIG 4. Immunoglobulin production by B_R1 cells. **A,** IgG_1, IgG_4, IgA, and IgE mRNA expression (relative to unstimulated cells) in total B cells. *Lower panel*, IgG_1, IgG_4, and IgA levels measured in supernatants (n = 5, 3 independent experiments). **B,** IgG_1, IgG_4, IgA, and IgE mRNA expression (average IL-10^- expression = 1) in IL-10^- and IL-10^+ B cells. *Connected dots* represent individual donors. **C,** Secreted IgG_1, IgG_4, and IgA measured from IL-10^- and IL-10^+ cells derived from $CD27^-$ and $CD27^+$ B cells (n = 4). Fold increase is relative to the average of IL-10^-. IgE protein was not detectable. *$P < .05$ and **$P < .01$.

and CD274, whereas CD73 expression was lower. Some of these molecules are known to play a role in immune regulation. CD25 is expressed at high levels on natural Treg cells and contributes to their suppressive effect through cytokine deprivation–mediated apoptosis of $CD4^+$ effector cells.[34,35] Interestingly, CD25 expression appears to be essential for IL-10 expression by $CD4^+$ T cells and thereby plays an important role in the generation of T_R1 cells.[36] CD71 is the major transferrin receptor, which is involved in the cellular uptake of iron and the regulation of cell growth. CD25 and CD71 are both known activation markers of T and B cells, and their increased expression might reflect an increased activation state.[37] CD274, also named PD-L1, is an inhibitory costimulatory molecule that counteracts T-cell activation through binding to PD-1 on T cells.[38] PD-L1 can inhibit T-cell responses by promoting both the induction and maintenance of inducible Treg cells.[39] CD73, alternatively named ecto-5′-nucleotidase, is a cell-surface enzyme expressed on many cell types, including T and B cells. CD73 converts adenosine monophosphate to adenosine, which has potent immunosuppressive effects. CD73 expressed on Treg cells might be responsible for part of their immunosuppressive effects.[40] On the other hand, B_R1 cells express low levels of surface CD73, and therefore adenosine-mediated immune suppression is unlikely to play an important role in suppression of T-cell responses.

IL-10^+ cells potently suppressed antigen-specific proliferation of $CD4^+$ T cells. A ratio of 1 B cell to 25 PBMCs led to 50% suppression of the proliferative T-cell response. IL-10^- B cells could also weakly suppress proliferation. A possible explanation might be that the sensitivity of the IL-10 secretion assay was not sufficient to detect cells that produce minute amounts of IL-10 that might remain in the IL-10^- fraction and render them slightly suppressive. Kinetics of IL-10 induction could also play a role because some cells might not secrete IL-10 at the time of sorting

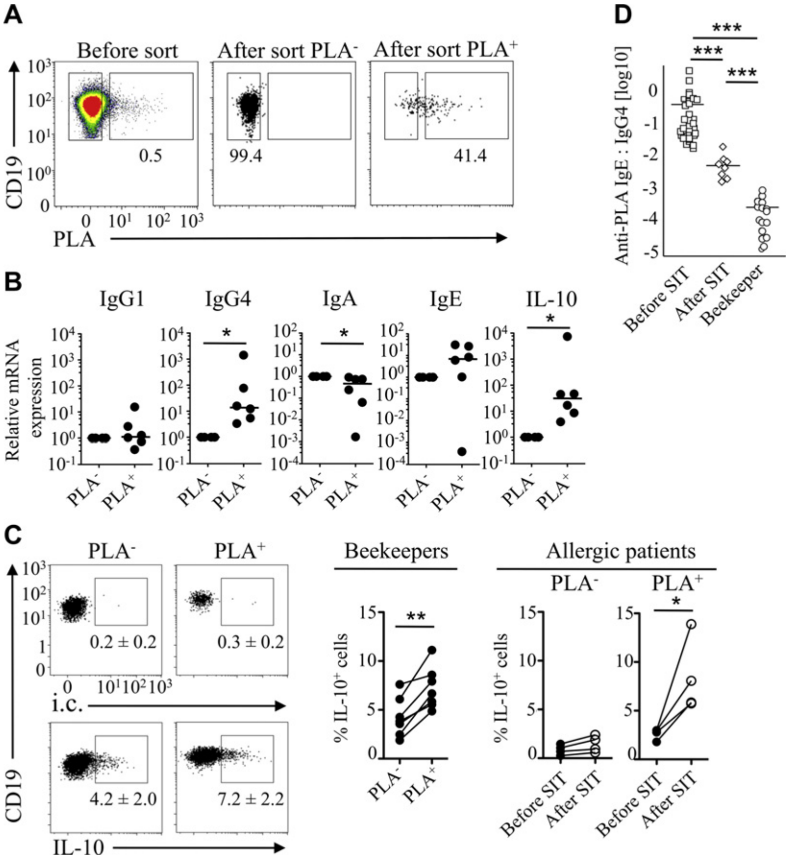

FIG 5. Allergen-specific B cells from tolerant subjects upregulate IL-10 and IgG_4. **A,** Enrichment of PLA-specific B cells from beekeeper-derived peripheral $CD19^+$ cells. **B,** Direct *ex vivo* analysis of immunoglobulin and IL-10 mRNA expression (relative to PLA^- cells, n = 6). *Horizontal bars* indicate medians. **C,** IL-10 and PLA staining in B cells from beekeepers (n = 7) or patients with bee venom allergy (n = 4) after 3 days of TLR9-L stimulation. *Numbers in dot plots* indicate means ± SDs. **D,** PLA-specific serum IgE/IgG_4 ratio. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

but could do so later. The suppressive effect observed in our experiments is completely reversible through blocking of IL-10R. $CD73^-CD25^+CD71^+$ cells sorted from resting B cells could similarly suppress T-cell proliferation. The strength of the *in vitro* suppressive effect can differ from that of $IL\text{-}10^+$ sorted cells because not all $CD73^-CD25^+CD71^+$ cells produce IL-10 on activation. Control populations ($CD25^-CD71^-$ or $CD73^+$ cells) were not suppressive at these ratios. A slight blocking effect in the $CD73^-CD25^+CD71^+$ Breg cell–mediated suppression was observed in the presence of anti–IL-10R mAb compared with an isotype control, suggesting a partial involvement of IL-10 in the suppression. However, this effect was small and not statistically significant. It has to be noted that the $CD73^-CD25^+CD71^+$ B-cell population does not include all of the $IL\text{-}10^+$ B cells. Therefore $CD73^-CD25^+CD71^+$ expression can be used to identify a distinct population of Breg cells that does not include all IL-10–producing B cells. Furthermore, not all $CD73^-CD25^+CD71^+$ B cells produce IL-10. Therefore it is possible that $CD73^-CD25^+CD71^+$ B cells can apply additional mechanisms to suppress T-cell proliferation. The identification of the mechanisms involved will require a significant research effort because there can be many factors that play a suppressive role besides IL-10. For example, the surface markers that were used for cell sorting might play a role similar to the suppressive role for CD25 by means of IL-2 deprivation that has been described in Treg cells.[41] In addition, blocking of CD80 and CD86 has been shown to reverse Breg cell–mediated suppression of T-cell responses.[32] Furthermore, these cells express PD-L1, and this interaction might also play a role, as demonstrated in Treg cells.[8] Together, these data demonstrate that B_R1 cells are highly potent suppressors of T-cell responses. This is especially striking when compared with T_R1 and Treg cells, which suppress approximately 50% of antigen-specific proliferation at a ratio of 1 suppressor cell to 2 or 4 responder cells, respectively.[8,42]

In the experiments investigating the link of B_R1 cells to immunoglobulin production, we found that IgG_4 production is specifically confined to $IL\text{-}10^+$ B_R1 cells. TLR9 stimulation induced production of IgG_1, IgG_4, and IgA primarily in $CD27^+$ B cells, whereas simultaneous stimulation with TLR9 and IL-10 induced specific upregulation of IgG_4, particularly in $CD27^-$ B cells, without affecting IgG_1 and IgA production. These findings combined with the fact that TLR9 stimulation induces IL-10 in a subset of B cells raised the question of whether $IL\text{-}10^+$ cells are more prone to produce IgG_4 than $IL\text{-}10^-$ B cells. There was approximately 10-fold increased IgG_4 mRNA in $IL\text{-}10^+$ B_R1 cells compared with their $IL\text{-}10^-$ counterparts. When sorting B_R1 cells from $CD27^-$ and $CD27^+$ subsets, it emerged that IgG_4 production was specifically upregulated in $CD27^-IL\text{-}10^+$ B cells, suggesting that naive B_R1 cells are the main precursors of IgG_4-producing cells.

Induction of allergen-specific IgG_4 is a hallmark of successful peripheral tolerance induction, as observed in allergen SIT. Our data demonstrate that PLA-specific B cells from beekeepers mainly express IgG_4. PLA-specific and non–PLA-specific B cells were isolated from peripheral blood and directly analyzed without *in vitro* culture. The increased IgG_4 expression in PLA-specific B cells indicates that these cells mainly represent circulating IgG_4-switched PLA-specific memory B cells. Interestingly, IL-10 mRNA expression was also significantly higher in these cells, suggesting that *in vivo* circulating PLA-specific B cells in beekeepers have increased IL-10 production. Furthermore, when stimulated *in vitro* with TLR9-L, PLA-specific cells showed higher IL-10 secretion than non–PLA-specific B cells. This increased frequency of IL-10^+ cells among PLA-specific B cells was not observed in patients with bee venom allergy. However, after SIT, the frequency of PLA-specific IL-10^+ B cells significantly increased to the same level seen in beekeepers. PLA-specific IgG_4 was detected at high concentrations in sera of nonallergic beekeepers, which showed greater than 1000 times lower PLA-specific IgE/IgG_4 ratios than sera from allergic subjects. Allergen SIT and high-dose allergen tolerance has been linked to increased serum IgG_4 and IL-10 production from T cells.[13] Here we demonstrate that allergen-specific B-cell IL-10 production is increased during allergen SIT. Our data demonstrate that particularly $CD27^-$IL-10^+ B cells are precursors of IgG_4-producing cells. In addition, the ongoing immune response of memory B cells contributes to IgG_4 production. We found increased IgG_4 expression and an increased frequency of IL-10^+ cells among PLA-specific B cells in bee venom–tolerant subjects. This suggests that in these subjects there exists a PLA-specific IgG_4-switched memory B-cell compartment that retains high IL-10 expression and might play a role in maintenance of tolerance. Whether these cells derive from B_R1 cells requires further investigation.

Interestingly, *Schistosoma mansoni* infection in children correlates with increased IL-10 production in response to parasitic antigens and decreased atopy.[43] Furthermore, *S mansoni* infection mediates protection against experimentally induced anaphylaxis. This protective effect was dependent on IL-10–producing B cells.[24] In addition, in line with our findings, high levels of IgG_4 antibodies are detected in helminth-infected subjects.[44-46]

In conclusion, the current findings demonstrate an essential role for B cells in the induction and maintenance of immunologic tolerance. The present study demonstrates 2 ways to directly purify and analyze these cells in human subjects according to IL-10 secretion and surface CD25, $CD71^{high}$, and $CD73^{low}$ expression. By analyzing CD27, it was observed that both naive and memory subsets of B_R1 cells can express IL-10 on TLR9-L stimulation. B_R1 cells act on T-cell responses by interfering with $CD4^+$ T-cell activation, and they contribute to peripheral tolerance through production of noninflammatory IgG_4 antibodies. We demonstrated significant *in vivo* regulation of these cells by isolating major allergen-specific B_R1 cells in high-dose allergen-exposed beekeepers, as well as *in vivo* induction of these cells after allergen SIT of subjects with bee venom allergy. Therefore B_R1 cells bring together 2 important arms of immune tolerance, namely playing a role as suppressive cells and directing the humoral response toward IgG_4, which together characterize a healthy immune response to allergens.

Key messages

- **Human IL-10–secreting B_R1 cells potently suppress antigen-specific T-cell responses.**
- **Human B_R1 cells can be isolated based on IL-10 secretion and surface expression of CD25, CD71, and CD73.**
- **IgG_4-producing antibody-forming cells essentially develop from B_R1 cells.**
- **IgG_4-producing and IL-10^+ B_R1 cells increase in allergen SIT and high-dose venom exposure in nonallergic beekeepers.**

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