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Human Regulatory B Cells Combine Phenotypic and Genetic Hallmarks with a Distinct Differentiation Fate

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Regulatory B cells (B-reg) produce IL-10 and suppress inflammation in both mice and humans, but limited data on the phenotype and function of these cells have precluded detailed assessment of their contribution to host immunity. In this article, we report that human B-reg cannot be defined based on a phenotype composed of conventional B cell markers, and that IL-10 production can be elicited in both the CD27\(^+\) memory population and naïve B cell subset after only a brief stimulation in vitro. We therefore sought to obtain a better definition of IL-10–producing human B-regs using a multiparameter analysis of B cell phenotype, function, and gene expression profile. Exposure to CpG and anti-Ig are the most potent stimuli for IL-10 secretion in human B cells, but microarray analysis revealed that human B cells cotreated with these reagents resulted in only \(\sim 0.7\%\) of genes being differentially expressed between IL-10\(^+\) and IL-10\(^-\) cells. Instead, connectivity map analysis revealed that IL-10–secreting B cells are those undergoing specific differentiation toward a germainal center fate, and we identified a CD11c\(^+\) B cell subset that was not capable of producing IL-10 even under optimal conditions. Our findings will assist in the identification of a broader range of human pro–B-reg populations that may represent novel targets for immunotherapy. *The Journal of Immunology, 2014, 193: 000–000.

The primary function of B cells is to undergo differentiation into plasma cells (PCs) and efficiently produce Abs, but B cells can also present Ags to T cells (1), secrete a range of potent immunomodulatory cytokines (2), and even retrain inflammatory responses by secreting IL-10 (3, 4). IL-10–secreting B cells (or regulatory B cells [B-regs]) are increasingly being recognized as an important component of host homeostasis that can protect against autoimmune pathology and limit inflammatory damage (5). B-regs have previously been shown to modulate T cell differentiation and limit disease in mouse models of multiple sclerosis (3), arthritis (6), and colitis (7), whereas functional and numerical defects in B-regs have also been reported in human autoimmune diseases including systemic lupus erythematosus (8) and multiple sclerosis (9). Indeed, long-term remission in patients receiving therapy for autoimmune diseases is associated with an increase in the number of IL-10–secreting B-regs in peripheral blood (10), and in solid organ transplant recipients, tolerant patients display a significantly increased B-reg frequency in comparison with patients who require immunosuppressive therapy to maintain stable graft function (11, 12). B-regs thus represent promising therapeutic targets for a wide range of disease indications, but a clearer definition of this population will be required before the development of effective B-reg–specific immunotherapies can begin in earnest.

Murine B-regs have been reported to exhibit a CD19\(^+\)CD1d\(^hi\) CD5\(^+\) phenotype in the spleen and peritoneal cavity (13), and may present as CD19\(^+\)CD21\(^+\)IgM\(^+\) splenic B cells either with or without expression of CD23 (5, 14, 15). In humans, CD19\(^+\)CD27\(^+\) CD24\(^-\)CD38\(^+\)CD5\(^+\) immature B cells isolated from peripheral blood have also been reported to display regulatory capacity (8), but other investigators have identified that IL-10\(^-\) B-regs in blood and spleen are enriched in the CD19\(^+\)CD27\(^+\)CD24\(^hi\) memory cell compartment (16). In this latter report, the authors observed that most of the cell-surface molecules assessed (IgM, IgD, CD1d, CD5, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD38, and CD40) were expressed at comparable levels in both IL-10\(^+\) and IL-10\(^-\) B cells (16). Similarly, we have previously described a B-reg subset that is present in both the memory (CD19\(^+\)CD27\(^+\)) and immature (CD24\(^hi\)CD38\(^hi\)) B cell populations in blood and spleen (17). Thus, no conclusive data are currently available to support the hypothesis that B-regs constitute a distinct cell lineage; no unique marker or set of markers can exclusively identify human B-reg, and efficient IL-10 production is their only known distinguishing feature. Detailed characterization of B-reg will therefore be required to facilitate a better understanding of their putative regulatory functions and to permit the future development of B-reg–targeted therapies.

IL-10 exerts potent anti-inflammatory effects and protects against tissue damage, whereas also enhancing the survival, proliferation, differentiation, and isotype switching of human B cells (18). In mice, B-regs can differentiate into Ab-secreting cells after a transient phase of IL-10 secretion, but it is uncertain whether a similar pathway...
also exists in humans (19). One common method of B-reg identification depends on intracellular staining of IL-10 protein, which requires cell fixation and permeabilization leading to cell death, thus preventing detailed characterization of these cells in functional assays. However, an alternative method of isolating IL-10+ B cells that allows the recovery of viable cells for further study has recently been described (20). We therefore used this new approach to conduct a detailed characterization of IL-10+ B cells with the aim of identifying novel populations of human B-reg. In this study, we demonstrate that after combined stimulation through TLR9 and the BCR, there was no significant difference in IL-10 secretion capacity between naive and memory B cells, and microarray analysis confirmed that just -0.7% of genes were differentially expressed between IL-10− and IL-10+ B cells when activated in vitro. However, we successfully identified a CD11c− B cell population that was enriched in IL-10-producing cells after stimulation, and connectivity map analysis (CMAp) (21) revealed that IL-10+ B cells were those specifically undergoing differentiation toward the germinal center (GC) B cell fate. Accordingly, after in vitro culture for 5 d, IL-10+ B cells were capable of differentiating into PCs, whereas IL-10− B cells were unable to efficiently generate PCs. Taken together, our data suggest that after TLR9/BCR costimulation, putative B-reg exhibit phenotypic and genetic hallmarks together with a distinct lineage fate that can be used to efficiently identify IL-10−producing human B cells ex vivo.

Materials and Methods

**Human B cell separation**

All blood samples and procedures in this study were approved by the National University of Singapore Institutional Review Board (approval NUS 1076) in accordance with the guidelines of the Health Sciences Authority of Singapore. Written, informed consent was obtained by the staff of the blood bank of the Health Sciences Authority (Singapore), in accordance with the Declaration of Helsinki. PBMCs were isolated from buffy coats obtained via the blood bank of the Health Sciences Authority (Singapore), using Ficoll-Paque density gradient centrifugation (GE Healthcare). B cells were isolated using the Dynabeads Untouched Human B-cells kit (Life Technologies) according to the manufacturer’s instructions. Isolated B cells were routinely >95% CD19+.

**Human B cell culture**

Human B cells were cultured at a density of 2.5×10^6 cells/ml in complete medium (RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin; Life Technologies) and were stimulated or not with 3 μg/ml CpG-B 2006 (InvivoGen) and 10 μg/ml Goat Anti-Human IgA + IgG + IgM (H+L; anti-Ig; Jackson ImmunoResearch Laboratories) for 48 h at 37°C, 5% CO2. For Ab production assays, B cells were cultured at a density of 6×10^6 cells/ml in complete medium and were stimulated with 6 μg/ml CpG-B 2006, 50 ng/ml IL-21, and Staphylococcus aureus Cowan I cells (1×; Pansorbin cells; Calbiochem) for 7 d at 37°C, 5% CO2.

**Isolation of naive, memory, and IL-10+ B cell populations**

IL-10−secreting B cells were isolated using the MACS IL-10 cytokine secretion assay detection kit (Miltenyi Biotec) according to the manufacturer’s protocol. B cells were then resuspended at 1×10^6 cells/ml in cold FBS containing 0.5% BSA and 2 mM EDTA (pH 7.2) before being stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies) and anti-CD19 AF700 Ab (BioLegend). CD19+ B cells were sorted into three separate populations using a BD FACSAria II 4-Laser Cell Sorter (BD Biosciences) (1), unstimulated IL-10− cells (2), stimulated IL-10− cells (3), and stimulated IL-10+ cells. Alternatively, the B cells were further stained with anti-CD27 Ab (BD Biosciences) and then sorted into CD27− naive and CD27+ memory cell subsets. The Abs used are listed in Table 1. Fluorescence-minus-one controls were used to compensate all flow cytometry data (22).

**RNA extraction**

Total RNA was extracted using miRNeasy Mini Kits (Qiagen). RNA Integrity Number was assessed by Agilent Bioanalyzer (Agilent Technologies). All RNA samples exhibited RNA Integrity Number ≥9.2.

Gene expression analysis

Target ssDNA was prepared from 100 ng total RNA using the Ambion WT Expression Kit (Ambion), combined with the Affymetrix GeneChip WT Terminal Labeling and Controls Kit (Affymetrix). A total of 5.5 μg fragmented target ssDNAs was hybridized to the Affymetrix GeneChip Human Exon 1.0 ST Arrays for 17 h at 45°C. The arrays were then washed and stained using the Affymetrix Fluidics Station 450 and scanned using GeneChip Scanner 3000. Array images were analyzed using Expression Console, and comparative analyses were carried out according to the instructions provided (Affymetrix). The raw intensity CEL files were read and processed using the robust multiple averaging methodology and the Bioconductor oligo package in R. The expression values were summarized to transcript cluster/gene level using the core probes. To identify the differentially expressed genes (DEGs) between the IL-10− and IL-10+ B cell populations, we used limma (23) with samples paired by donors. The p values were adjusted for multiple testing using the Benjamini-Hochberg procedure (24), and the genes with adjusted p < 0.05 were considered as differentially expressed. To map transcript clusters to genes, we used the Affymetrix NetAffx HuEx-1.0-st-v2 Annotation Release 22. Based on the microarray data, we then filtered out the transcript clusters with no gene assignment, or with gene assignment but no RefSeq ID, or with more than one gene assignment (considered as nonspecific). CMAp analysis was performed with genes differentially expressed between IL-10− and IL-10+ B cells using GSE12236, a Gene Expression Omnibus data set (http://www.ncbi.nlm.nih.gov/geo) generated from classical human B cell populations (naive, memory, GC B cells, and PCs). The CMAp scores are scaled dimensionless quantities that measure degree of enrichment of the gene set in the samples and indicate “closeness” of IL-10+ B cells to other B cell populations. All analyses were performed in R version 2.13.0 (http://www.R-project.org) with Bioconductor 2.1.2 (http://www.bioconductor.org) and enabled by Pipeline Pilot (www.accelrys.com). The gene expression data are publicly available under accession number GSE50895 in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50895).

**Quantitative PCR**

RNA (1 μg) was reverse transcribed using the High Capacity cDNA Re- verse Transcription Kit (Applied Biosystems) according to manufacturer’s instructions. Primers were designed using Primer3 and synthesized by ProLigo (Sigma-Aldrich). Primer sequences are provided in Supplemental Fig. 1. The quantitative PCR (qPCR) analyses were performed on a 7900HT Real-Time PCR System (Applied Biosystems) using the same forward and reverse primers with KAPA SYBR FAST ABI Prism qPCR kits (Kapa Biosystems). Reactions were run for 40 cycles using the following thermal profile; 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and then 60°C for 1 min. Data were collected in the SYBR channel, and the final cycle was followed by a thermal melt curve. Quantification of mRNA levels was performed using a comparative threshold cycle (Ct) method (25). Reactions were performed in triplicate, and all experiments were repeated with cells obtained from at least three different donors.

**ELISAs**

Supernatants were assayed for IL-10 and IgG using ELISA kits from eBioscience with standard protocols.

**Statistical analyses**

All experiments were performed using at least three different cell cultures or blood donors in independent experiments. Student t test and Mann–Whitney tests were used to assess normally distributed data and non-normally distributed data, respectively. Nonparametric one-way ANOVA with Dunn’s posttest or two-way ANOVA with Bonferroni posttest were used to assess differences between groups. The p values <0.05 were considered significant.

**Results**

**Kinetics of IL-10 secretion by human B cells upon TLR9/BCR costimulation**

We previously reported that the most potent method of inducing IL-10 production in human B cells is cotreatment with CpG-B and anti-Ig (17). We used this approach with B cells purified from human blood to observe that the proportion of IL-10+ B cells in culture was increased after only 24 h, reached maximum levels at 48 h, and had decreased by 72 h posttreatment (n = 7; Fig. 1A, left...
Accordingly, IL-10 protein levels in the culture media increased between 24 and 72 h (n = 5; Fig. 1A, right panel), with maximal IL-10 release being achieved within 48 h of B cell activation. In contrast, IL-10 protein levels were below the limit of detection in ELISA analyses of culture supernatants from unstimulated B cells incubated for the same times. The proportion of IL-10+ B cells detected 48 h after stimulation varied between donors (mean 7.4 ± 3.4%, range 1.5–15%), whereas the percentage of IL-10+ B cells in unstimulated cultures was only 0.6 ± 0.5% (n = 35; p < 0.0001; Fig. 1B). We therefore concluded that the most efficient method of purifying putative human B-regs would be to FACS-sort IL-10+ B cells from cultures of total B cells that had been stimulated with CpG-B and anti-Ig for 48 h.

**Human IL-10+ “B-regs” are present in both naive and memory B cell compartments**

Several different phenotypes have been reported to define B-regs in both mice and humans, and it is possible that several distinct subsets of B-regs exist in different tissues in vivo. We previously reported that after in vitro stimulation, B cell capacity to produce IL-10 is not restricted to either the memory or transitional subsets (17). We therefore FACS-sorted CD19+CD27− naive/transitional B cells and CD19+CD27+ memory B cells from healthy donors to test the capacity of each population to secrete IL-10 after 48-h stimulation with CpG-B and anti-Ig (Fig. 2A). No significant differences were observed in IL-10 concentration in the culture supernatants, and the percentage of IL-10–producing cells detected after stimulation was comparable between unsorted total B cells and sorted populations of naive and memory B cells (n = 5; Fig. 2B, 2C). In contrast, IL-10 protein concentrations were below the limit of detection in cultures of unstimulated B cells, which included only trace numbers of IL-10+ cells as assessed by flow cytometry. Taken together, these data indicate that naive and memory B cells exhibit similar capacities to secrete IL-10 in response to CpG-B and anti-Ig, thus confirming our previous observation that multiple B cell subsets are capable of producing IL-10 after short-term activation in vitro, although the mechanisms underpinning this activity remained unknown.

**FIGURE 1.** Kinetics of IL-10 secretion by human blood B cells. Purified B cells were cultured either in medium alone or were treated with CpG and anti-Ig in combination for the indicated times (A). The frequencies of IL-10+ cells were measured by flow cytometry using IL-10 secretion assays (left panel) and analysis of culture supernatants by ELISA (right panel). Bar graphs show mean ± SEM of IL-10+ B cell frequency (n = 7) or IL-10 protein concentration in the culture supernatants (n = 5). Significant differences were determined by one-way ANOVA. (B) The frequency of IL-10+ cells among total purified CD19+ B cells was substantially increased by cotreatment with CpG and anti-Ig for 48 h (left panel shows a representative example analysis, right panel shows grouped data from n = 35 healthy donors). Significant differences were determined using Student t test (*p < 0.001).
**IL-10**^+ human B cells exhibit a distinct gene expression profile

To better define the cellular characteristics that support IL-10 production by human B cells, we next conducted a microarray analysis of total B cells together with purified IL-10^- and IL-10^+ B cells sorted after stimulation with CpG-B and anti-Ig for 48 h (or unstimulated control B cells). Mean cell purities after sorting were 98 ± 2% for the unstimulated CD19^+ cells, 97 ± 3% for the stimulated IL-10^- B cells, and 96 ± 4% for IL-10^+ B cells (n = 21; Fig. 3A). Before proceeding with the microarray analysis, we first analyzed IL-10 mRNA levels in each of the B cell populations using real-time PCR (Supplemental Fig. 2A). As expected, IL-10 mRNA was significantly upregulated in stimulated IL-10^- B cells (48 ± 16-fold, p < 0.04; n = 3), whereas IL-10 mRNA was barely detectable in either unstimulated B cells or stimulated IL-10^- B cells. These data confirmed that our approach of combining an IL-10 secretion assay with high-purity cell sorting is an efficient method of enriching IL-10-secreting human B cells for further study.

We next used Affymetrix GeneChip Human Exon 1.0 ST Arrays with sorted B cell populations from five different donors to identify DEGs between IL-10^- and IL-10^+ human B cells. A total of 1008 transcript clusters were identified as being differentially expressed between the two sample groups, and 336 of these transcript clusters had no gene assignment according to the latest NetAffx annotation (9 had gene assignment but no RefSeq ID; 47 had multiple gene assignments and were considered to be nonspecific). The remaining 616 transcript clusters corresponded to a unique gene assignment and RefSeq ID, accounting for 3.5% of the 17,633 transcripts detected by the microarray. Within the DEGs, 103 transcripts were differentially expressed with fold change ≥1.5 (27 were overexpressed and 76 underexpressed in IL-10^+ versus IL-10^- cells; Fig. 3B). A heatmap profile of the 103 DEGs detected in the 5 healthy donors is shown in Fig. 3C, and the name, role, localization, RefSeq ID, and fold change for each gene is shown in Supplemental Fig. 4. Although the total number of DEGs detected with fold change ≥1.5 was relatively low, it is important to note that these data were generated using homogenous cell populations subjected to identical stimuli during culture. These data therefore indicated that B cell secretion of IL-10 is associated with differential expression of a select subset of genes that can be induced by stimulation with CpG-B and anti-Ig.
FIGURE 3. IL-10+ and IL-10− human B cells display distinct gene expression profiles. Human CD19+ B cells were obtained by negative selection from healthy adult peripheral blood and were then either left unstimulated or cultured in the presence of CpG and anti-Ig for 48 h. (A) Total unstimulated CD19+ B cells and populations of stimulated IL-10+ and IL-10− B cells were then purified by FACS sorting. Dot plots representative of n = 21 independent experiments are shown. (B) Expression of annotated gene transcripts (17,633 total) was highly comparable between IL-10+ and IL-10− B cells, but a select subset of DEGs was able to successfully distinguish these populations. Venn diagram shows total number and percentage of DEG transcripts with fold change ≥1.5 (red for IL-10+ B cells, green for IL-10− B cells). Transcripts expressed at similar levels by both IL-10+ and IL-10− B cells are shown in yellow. (C) Expression levels and designations of 103 DEG transcripts in total B cells are represented in a heat map showing expression fold change for each of the five healthy individuals tested. (D) RNA was prepared from FACS-sorted populations of activated IL-10− and IL-10+ B cells, and gene expression was quantified by qPCR (ΔΔCt method) in n = 3 separate healthy adult volunteers to validate the microarray data.
To validate the microarray transcriptional analysis, we next performed qPCR using activated IL-10+ and IL-10− B cells obtained from three new healthy donors who had not participated in the gene chip studies. We focused on genes coding for secreted proteins or products located at the plasma membrane, and selected a subset of 21 genes that were modulated in IL-10+ B cells (based on root mean square error <0.5). As shown in Fig. 3D, qPCR analysis confirmed the trend observed in the microarray data; expression of CD150 mRNA was increased 7.1 ± 5.8-fold and CD11c mRNA was decreased 7.0 ± 0.7-fold in stimulated IL-10+ B cells compared with IL-10− B cells. Whereas qPCR analyses indicated that differential expression of the genes AREG, IL-6, WFD2, and SLA did not reach the 1.5-fold threshold predicted by the microarray analysis, the trends observed in each case were consistent with our earlier data. The qPCR results thus confirmed the microarray data indicating that IL-10+ B cells and IL-10− B cells exhibit distinct transcriptional profiles when activated with potent IL-10–inducing stimuli.

Differential surface expression of integrin chains and signaling lymphocyte activation molecule family molecules by human IL-10–secreting B cells

Identification of surface markers that can delineate human B-regs would enable further characterization of these cells and facilitate therapeutic targeting of pro–B-reg subsets in future studies. Among the various membrane proteins already reported to identify stimulated IL-10+ B cells, we focused on assessing B cell expression of the integrin chains CD11c and β7, as well as members of the signaling lymphocyte activation molecule family, CD150 and CD229. Each of these surface markers has previously been reported to be expressed by human peripheral blood B cells (26), and the commercial availability of reagents directed against these molecules would enable other investigators to easily exploit their use for identification of human B-regs. First, we evaluated whether these markers could be used to define unique subsets of unstimulated B cells in PBMCs obtained from healthy donors. Expression of CD11c, CD150, CD229, and β7 was common among blood leukocytes, and each of these markers was readily detected within the CD19+ B cell population (Fig. 4A). Further analysis of unstimulated B cells revealed that the frequency of CD11c+ cells was relatively low (mean 8.5 ± 2.7%, range 4.3–14.7%), whereas CD150+ cells accounted for roughly half of the total B cell population (mean 52.6 ± 7.3%, range 36.9–64.5%), as did β7+ cells (mean 60.4 ± 12.6%, range 43.6–77.7%), with CD229 expression being detected on the majority of B cells (mean 84.5 ± 9.4%, range 62.7–95.2%; Supplemental Fig. 2B). In addition, differential expression of CD11c and CD150 could be used to define four distinct B cell subsets (CD11c+, CD11c−CD150+, CD150+, and double negative), and a similar analysis could also be conducted based on B cell expression of CD229 and β7 (β7+, β7+CD229+, CD229+, and double negative; Fig. 4B, 4C). Moreover, expression of CD229 and β7 was not uniformly distributed.

**FIGURE 4.** Surface expression of integrin chains and signaling lymphocyte activation molecule family molecules distinguish subsets of human B cells. (A) PBMCs were stained with anti-CD19, CD11c, CD150, CD229, and β7 for evaluation by flow cytometry. Dead cells were excluded by LIVE-DEAD staining, and the blood lymphocyte population was gated to determine the percentages of different B cell subsets. (B) Expression level and proportion of CD19+ B cells expressing CD11c, CD150, and CD229. (C) β7 and CD229 expression in subpopulations of CD19+ B cells as defined by expression of CD150. (D) Purified human B cells were stimulated or not with CpG and anti-Ig for 48 h before being stained with anti-CD19, IL-10, CD11c, CD150, CD229, and β7 for evaluation by flow cytometry. Surface marker expression by unstimulated B cells (blue line) and stimulated populations of IL-10+ (green line) and IL-10− B cells (red line) is shown together with isotype-matched control staining (solid gray). Representative data from n = 5 independent experiments are shown. (E) CD19+ CD11c− and CD19+ CD11c+ B cells were FAC-sorted to high purity from n = 7 healthy adult peripheral blood samples. (F) Purified B cells and the sorted populations were cultured for 48 h with or without CpG/anti-Ig costimulation, and IL-10 concentrations in the culture supernatants were determined by ELISA.
among human blood CD19+ B cells; in the CD11c+CD150− subset, expression of CD229 (mean fluorescence intensity [MFI] 913) and β7 (MFI 1566) was substantially higher than that observed within the CD11c+CD150+ population (CD229 MFI = 595, β7 = 679). Taken together, these data indicate that combinations of the surface markers CD11c, CD150, CD229, and β7 can be used to distinguish different populations of human blood B cells that may exhibit distinct expression patterns of costimulatory molecules (CD229) and tissue-homing receptors (β7).

We next evaluated whether B cell surface expression of CD11c, β7, CD150, and CD229 proteins could be specific to IL-10–secreting cells, as suggested by our earlier microarray analysis. In purified human B cells cultured with or without CpG-B/anti-Ig for 48 h, we observed that CD11c, β7, CD150, and CD229 were each upregulated after stimulation, except in the case of IL-10+ B cells, which failed to increase surface expression of CD11c (Fig. 4D). The differences detected in mRNA expression between activated IL-10+ and IL-10− B cells were thus confirmed at the protein level for CD11c, but not for CD150, CD229, or β7. These data suggested that the absence of CD11c may identify human B cells with the ability to produce IL-10.

Distinct genetic profile and CD11c− phenotype of IL-10–secreting human B cells

Having established that CD11c upregulation after stimulation is a feature of IL-10− B cells, but not IL-10+ B cells, we next investigated whether CD11c could be used to enrich for IL-10–secreting B cells before activation with CpG-B/anti-Ig. Human B cells were sorted based on differential expression of CD11c (Fig. 4E), and then stimulated or not with CpG-B/anti-Ig for 48 h. Quantification of cytokine secretion by ELISA revealed that only CD11c− B cells were capable of producing IL-10 (Fig. 4F). In contrast, B cells sorted based on the expression of CD150 or β7 exhibited comparable IL-10 concentrations in the culture supernatants irrespective of subset phenotype (data not shown), thus demonstrating that CD11c expression is a useful marker of B cell potential for IL-10 secretion before stimulation.

We next performed CMAP analysis to compare activated IL-10+ and IL-10− B cell gene sets with the expression profiles of classical human B cell populations (naïve, memory, GC B cells, and PCs) (27). CMAP scores facilitate comparisons between sets of transcriptional expression data and allow investigators to probe the ontogeny of human cell populations. Using this approach, we observed that the profile of IL-10+ B cells overlapped most closely with that of GC B cells, followed by naïve B cells, whereas enrichment of DEGs associated with IL-10 production was absent with that of GC B cells, followed by naive B cells, whereas enrichment of DEGs associated with IL-10 production was absent with that of naive B cells, followed by memory B cells, and PCs. PCs or memory cells depend on T cell help, which was absent in our system (28). Indeed, naïve B cells are known to differentiate into GC B cells after stimulation via the BCR, whereas their further differentiation into naive B cells are known to differentiate into GC B cells after stimulation via the BCR, whereas their further differentiation into plasma cells depends on T cell help, which was absent in our system (28). In summary, these results suggest that human IL-10+ B cells resemble GC B cells but require additional stimulation to differentiate into PCs or memory B cells.

**IL-10–secreting B cells differentiate into memory and GC cell populations**

Given our finding that IL-10+ B cells share characteristics of GC B cells, we next sought to determine the differentiation fate of these putative B-regs by stimulating total B cells for 48 h with CpG-B/anti-Ig, FACS sorting the IL-10+ and IL-10− fractions, and then culturing the cells for a further 5 d in the absence of further stimulation. In cultures of IL-10+ B cells, we observed enrichment of CD38+CD27+ GC B cells (mean 39 ± 4%) and depletion of CD38+CD27+ PCs (mean 8 ± 3%) relative to cultures of IL-10− B cells (Fig. 5A). Intriguingly, naïve B cells are known to differentiate into GC B cells after stimulation via the BCR, whereas their further differentiation into naive B cells are known to differentiate into GC B cells after stimulation via the BCR, whereas their further differentiation into plasma cells depends on T cell help, which was absent in our system (28). In summary, these results suggest that human IL-10+ B cells resemble GC B cells but require additional stimulation to differentiate into PCs or memory B cells.
CD27⁻ B cells, typically considered to represent naive cells, that were equally represented in cultures of IL-10⁺ and IL-10⁻ B cells (mean 38 ± 3 and 45 ± 12%, respectively; p = NS). We further observed that IL-10⁺ B cells produced significantly less IgG than did IL-10⁻ B cells when assessing the Ig content of the culture supernatants (Fig. 5C). Taken together, these data confirmed that IL-10⁺ B cells favor alternative differentiation fates to IL-10⁻ B cells.

Discussion
Pro-B-regs that produce IL-10 suppress inflammation in both mice and humans, but methods for the robust identification of these cells have so far remained elusive. In this report, we have used a multiparameter approach to uncover the phenotypic and transcriptional hallmarks of human IL-10⁻–secreting B cells (putative B-regs) and reveal that these cells are predisposed toward the GC B cell differentiation pathway.

In mice, CD1dⁱCD⁵⁺CD⁻¹⁹⁺ B cells contain an enriched population of IL-10⁻–secreting cells, but only ~10% of this population appears capable of producing IL-10 cytokine upon specific stimulation (13, 19). In humans, several groups have reported various different phenotypic definitions of putative B-regs (8, 16, 17), but currently no data are available that establish B-regs as a unique cell lineage. To date, the only known marker that clearly characterizes B-regs is efficient production of IL-10. In this study, we observed that both memory and naive B cells exhibit similar capacities to secrete IL-10. Strikingly, only a small fraction of the total B cell pool could produce IL-10 in response to stimulation (15% at most), despite the fact that all B cells express a BCR and can upregulate TLR9 to mediate efficient activation (29). This strongly suggests that B-regs have a unique signaling profile that enables them to secrete IL-10 after stimulation. To identify a unique surface marker and transcription factor signature for human B-regs, we performed a microarray analysis of gene expression in viable human B cells that were activated with the potent IL-10⁻–inducing stimuli CpG and anti-Ig for 48 h in vitro. Using this approach, we observed that only ~0.7% of the genes analyzed differentiated IL-10⁻ from IL-10⁻ B cells. To better facilitate the future identification of B-regs by other investigators, we focused our analyses on the DEGs that encoded cell-surface markers that could be easily exploited in other studies. Our data indicated that the integrin chains CD11c and β7, and costimulatory molecules CD150 and CD229, could be used to define distinct B cell subsets in healthy human blood. Contrasting with data from the mouse (30), none of these markers was sufficient to facilitate the enrichment of IL-10⁻ B cells for further study. However, we were able to identify that CD11c⁺ B cells do not secrete IL-10 in response to activation with CpG/anti-Ig, closely resembling recent data from other investigators showing that CD11c⁺ B cell frequency is increased in autoimmune disease in both murine models and human patients (31).

Van de Veen et al. (32) recently characterized human IL-10⁻–secreting B cells by means of whole-genome analysis, and consistent with this report, they also identified that both naive and memory B cells can secrete IL-10 after stimulation, thus indicating that the ability to produce IL-10 is not restricted to a single B cell subset. Whereas van de Veen used CpG only to activate B cells in their study, in this report, we used both CpG and BCR ligation together to induce maximal production of IL-10. Consequently, the only DEG identified by both our study and that of van de Veen et al. (32) was FCGR2B, a gene implicated in countering BCR-induced activation (33). Numerous other reports have also confirmed that B cell mode of activation determines the magnitude of the IL-10 response. For example, Blair et al. (8) stimulated transitional B cells for 72 h with CD40L-expressing CHO cells, Iwata et al. (16) stimulated B cells with a combination of CD40L and CpG/LPS for either 5 or 48 h, and in this report, we used a combination of CpG and BCR stimulation according to our previous study (17). Together, these data confirm that the balance of B cell subsets and the nature of the activating stimuli are important determinants of IL-10 production in the B cell compartment.

We used CMAP to identify that human IL-10⁺ B cells are undergoing differentiation toward GC B cells, and we were able to confirm this concept by maintaining IL-10⁺ B cells in culture and observing substantial enrichment of GC B cells in parallel with depletion of PCs after only 5 d (34), consistent with the reduced level of IgG production detected in the supernatants of the IL-10⁺ B cell cultures. In mice, B-regs differentiate into Ab-secreting cells after transient production of IL-10 (19), and in humans, IL-10 induces PC differentiation in vitro (35, 36), hence additional stimuli appear to be required to enable differentiation of IL-10⁺ B cells into efficient Ab-producing cells (perhaps including T cell–derived signals that would be absent in our B cell–only model). In mice, the transcription factor IRF4 has been shown to be required for generation of GC B cells (37), and in this study, IRF4 was one of the most significantly upregulated genes in IL-10⁺ B cells, hence IRF4 may also play a role in determining B cell fate in the human system.

The concept that regulatory T cells represent a distinct cell lineage remained controversial among immunologists until the discovery of the transcription factor FOXP3, which controls regulatory T cell development (38). Our study revealed that a distinct subset of transcription factors is differentially expressed in IL-10⁺ human B cells, but that none of these is exclusively expressed in IL-10⁺ B cells only. These data suggest that IL-10 secretion by B cells may not be regulated by a “B-reg”–specific genetic program. It has recently been suggested that B-regs could be derived from a subset of Ag-experienced B cells (39), and B cells specific for the major bee venom allergen exhibit increased expression of IL-10 in nonallergic beekeepers and can increase in frequency after treatment of allergic patients (32). However, in our purified B cell system, we observed that both memory and naive populations exhibit comparable IL-10 secretion after stimulation via BCR/TLR9, suggesting that the ability to produce this cytokine is not restricted to a pre-existing population of resting B cells. An alternative hypothesis for the generation of IL-10⁺ B cells might therefore be that modified BCR signaling resulting from ligation of CD40 (40) or IL-21 stimulation (41) promotes the differentiation of cells with the ability to produce IL-10.

In conclusion, our study clearly demonstrates that IL-10⁻–secreting B cells are a functionally distinct cell subset that is undergoing differentiation toward the GC B cell fate, and that CD11c⁻ B cells are enriched in the ability to produce IL-10 after stimulation. Although we were unable to identify a unique marker, or a set of markers, that is restricted to IL-10⁺ B cells, our combinatorial approach to the isolation of viable IL-10⁺ putative human B-regs will assist future studies of these cells and facilitate the preclinical testing of novel B-reg–targeted immunotherapies.

Acknowledgments
We thank Seri Munirah Mustafah and Ivy Low for cell sorting. Dr. Neil McCarthy of Insight Editing London provided writing assistance.
## Supplementary Figure S1

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Supplementary Figure 2A: RNA was prepared from FACS-sorted unstimulated total B-cells, and stimulated populations of IL-10- and IL-10+ B-cells (n=3). IL-10 mRNA levels were evaluated by qPCR and gene expression was analyzed by microarray. The ΔCt method was used to determine relative IL-10 expression level versus β2-microglobulin and GAPDH reference genes. Expression level of IL-10 mRNA is expressed relative to the background level of IL-10 mRNA detection in stimulated IL-10- B-cells.

Supplementary Figure 2B: PBMC from healthy individuals (n=22) were stained with anti-CD19, CD11c, CD150, CD229 and β7 for evaluation by flow-cytometry. Dead cells were excluded by Live-Dead staining. Percentage of the different subsets among CD19+ blood lymphocytes is shown.
Supplementary Figure S3: CD19+ cells exhibit differential expression of CD27 and CD38 in blood and tonsil. B cells purified from blood and then activated for 2 days with CpG + anti μ displayed reduced expression of CD27 but maintained CD38 expression during culture, whereas unstimulated control B cells exhibited only a minor reduction in CD27 levels after the 2 day incubation and otherwise resembled the phenotype of fresh B cells in unseparated PBMC.

When the phenotype of stimulated B cells was compared between the IL-10- and IL-10+ populations, we observed that the IL-10-producing subset displayed marginally higher expression of CD27. After 7 days culture in the absence of stimulation, purified B cells displayed down-regulation of CD38 followed by down-regulation of CD27 (control -). In contrast, B cell exposure to SAC + IL-21 + CpG (a regimen reported to differentiate B cells into plasma cells), resulted in up-regulation of CD38 and CD27 expression to very high levels (control +). These data clearly demonstrate that the sorted IL-10+ B cells exhibited a CD38 and CD27 expression profile distinct from that of IL-10- B cells.
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**Supplementary Figure 4**: Differentially expressed genes (DEG) with expression fold-change > 1.5, gene symbol, gene description, Ref Seq ID, fold-change and product localization is shown (PM: plasma membrane, S: secreted; C: cytoplasm; N: nucleus). Genes are shown as being either up-regulated or down-regulated in activated IL-10+ B-cells (red) versus activated IL-10+ B-cells (green).