

IL-10–overexpressing B cells regulate innate and adaptive immune responses

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Background: Distinct human IL-10–producing B-cell subsets with immunoregulatory properties have been described. However, the broader spectrum of their direct cellular targets and suppressive mechanisms has not been extensively studied, particularly in relation to direct and indirect IL-10–mediated functions.

Objective: The aim of the study was to investigate the effects of IL-10 overexpression on the phenotype and immunoregulatory capacity of B cells.

Methods: Primary human B cells were transfected with hIL-10, and IL-10–overexpressing B cells were characterized for cytokine and immunoglobulin production by means of specific ELISA and bead-based assays. Antigen presentation, costimulation capacity, and transcription factor signatures were analyzed by means of flow cytometry and quantitative RT-PCR. Effects of IL-10–overexpressing B cells on Toll-like receptor–triggered cytokine release from PBMCs, LPS-triggered maturation of monocyte-derived dendritic cells, and tetanus toxoid-induced PBMC proliferation were assessed in autologous cocultures.

Results: IL-10–overexpressing B cells acquired a prominent immunoregulatory profile comprising upregulation of suppressor of cytokine signaling 3 (SOCS3), glycoprotein A repetitions predominant (GARP), the IL-2 receptor α chain (CD25), and programmed cell death 1 ligand 1 (PD-L1).

Concurrently, their secretion profile was characterized by a significant reduction in levels of proinflammatory cytokines (TNF- α , IL-8, and macrophage inflammatory protein 1 α) and augmented production of anti-inflammatory IL-1 receptor antagonist and vascular endothelial growth factor.

Furthermore, IL-10 overexpression was associated with a decrease in costimulatory potential. IL-10–overexpressing B cells secreted less IgE and potently suppressed proinflammatory cytokines in PBMCs, maturation of monocyte-derived dendritic

cells (rendering their profile to regulatory phenotype), and antigen-specific proliferation *in vitro*.

Conclusion: Our data demonstrate an essential role for IL-10 in inducing an immunoregulatory phenotype in B cells that exerts substantial anti-inflammatory and immunosuppressive functions. (J Allergy Clin Immunol 2014;■■■:■■■–■■■.)

Key words: IL-10 overexpression, regulatory B cells, immune regulation, anti-inflammatory effects, immunoregulatory capacity

B lymphocytes display a unique role in immune response through the production of antibodies, representing the humoral arm of the adaptive immune response. In addition, B cells substantially contribute to the full magnitude and fate of the normal immune response through antigen presentation, cytokine secretion, and lymphoid tissue organization. Consequently, the importance of complex B-cell biology was recognized in altered/inadequate pathologic immune responses, such as (1) asthma and allergies, a chronic immune reactivity to innocuous antigens (allergens) in sensitized subjects; (2) autoimmunity, a lack of control of pathologic immune response to self-antigens; (3) antitumor immunity with insufficient immune response to tumor antigens; and (4) acceptance or rejection of transplanted organs.^{1,2}

A growing body of evidence has attributed an essential role for B cells in limiting excessive immune reactivity. IL-10–mediated immune regulation by B cells has been described in experimental models of infection, allergic inflammation, autoimmunity, tolerance, tumorigenesis, and organ transplantation.^{3–11} A functional role for human regulatory B cells was further supported by the finding that B cell–depleting therapy was associated with exacerbations of colitis and psoriasis induction and neutropenia in patients undergoing organ transplantation.^{12–14} Furthermore, altered numbers, function, or both of regulatory B-cell subsets in patients with chronic inflammatory and autoimmune diseases, as well as insufficient antitumor immunity, was associated with locally increased numbers of IL-10–producing B cells. Allergen-specific immunotherapy typically leads to suppression of IgE and upregulation of IgG₄ production, as well as increased IL-10 production, in allergen-specific T and B cells. These observations have stimulated scientific interest to study the role and capacity of regulatory B cells and their underlying immunosuppressive mechanisms.^{15–18}

Distinct human IL-10–producing B-cell subsets with an *in vivo* demonstrated role have been described. Recently, we reported that IL-10⁺ B regulatory 1 (Br1) cells that were enriched among CD25⁺CD71⁺CD73^{lo} B cells could potently suppress antigen-specific CD4⁺ T-cell proliferation.¹⁹ These Br1 cells were comprised of 2 temporally distinct but spatially linked immunosuppressive functionalities: increased IL-10 production and subsequent preferential IgG₄ secretion. These findings were demonstrated in phospholipase A2-specific B cells of beekeepers,

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Abbreviations used

APC:	Antigen-presenting cell
BLIMP-1:	B lymphocyte-induced maturation protein 1
Br1:	B regulatory 1
DC:	Dendritic cell
FITC:	Fluorescein isothiocyanate
GARP:	Glycoprotein A repetitions predominant
G-CSF:	Granulocyte colony-stimulating factor
IL-1Ra:	IL-1 receptor antagonist
IRF-4:	Interferon regulatory factor 4
MDDC:	Monocyte-derived dendritic cell
MIP:	Macrophage inflammatory protein
PD-L1:	Programmed cell death 1 ligand 1
PE:	Phycoerythrin
SOCS:	Suppressor of cytokine signaling
TLR:	Toll-like receptor
TLR-L:	Toll-like receptor ligand
TT:	Tetanus toxoid
VEGF:	Vascular endothelial growth factor
XBP-1:	X-box binding protein 1

representing a human antigen-specific *in vivo* model of tolerance upon high-dose antigen exposure. These findings particularly highlight a role for regulatory B cells in the development and maintenance of antigen-specific peripheral tolerance. The suppressive mechanisms of most regulatory B-cell subsets thus far described are, at least in part, IL-10 dependent.¹⁹⁻²²

IL-10 is a pivotal anti-inflammatory cytokine that protects the host from excessive tissue damage during host defense to pathogens and acts as one of the key molecules critically involved in the development and maintenance of immune tolerance and homeostasis.^{23,24} IL-10 deficiency leads to the development of spontaneous colitis in mice.²⁵ IL-10 suppresses the production of proinflammatory cytokines and chemokines, as well as antigen presentation.²⁴ In B cells IL-10 enhances survival, proliferation, and differentiation and modulates class-switch recombination through suppression of IL-4-induced IgE and induction of IgG₄.^{24,26,27}

Plasmid-driven IL-10 transfection was performed to reveal the role of IL-10 on the phenotype and functions of B cells. IL-10 overexpression was sufficient for acquisition of a notable immunoregulatory phenotype in B cells. In conjunction with secreted IL-10, these B cells further extend their immunosuppressive functions on both innate and adaptive immune responses.

METHODS

Human B cells were purified from PBMCs by means of negative selection with immunomagnetic separation and transfected either with control (backbone) plasmid (ctrl_tr) or pORF-hIL-10 to overexpress *IL-10* (IL-10_tr) or left nontransfected (non-tr). Plasmid-mediated gene transfer in B cells using nucleofection was efficient and resulted in IL-10 overexpression (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). *IL10*-transfected B cells have been cultured in medium alone before cytokine and immunoglobulin secretion was quantified with ELISA and a bead-based multiplex assay, gene expression with quantitative RT-PCR, and expression of surface molecules with flow cytometry. Preliminary experiments were performed to determine the optimal time for measurements, which were used as indicated in the figure legends. IL-10-overexpressing B cells were stimulated with Toll-like receptor (TLR) 9 ligand for 72 hours before transfection and then cocultured with (1) autologous PBMCs stimulated with either TLR ligands (TLR-Ls) to induce proinflammatory cytokine secretion or antigen for induction of specific proliferation or (2) monocyte-derived

dendritic cells (MDDCs) stimulated with LPS for their maturation capacity to address their suppressive potential.

A detailed description of the materials and methods used in this study is available in the **Methods** section in this article's Online Repository at www.jacionline.org.

Statistical analysis

Presented data are expressed either as individual values of each donor or as means $\pm$ SEMs. Statistical analysis was performed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, Calif), and paired *t* tests (if not stated differently) were used for assessment of statistical significance. Statistically significant scores are indicated on the graphs as asterisks, representing significantly changed values between samples having *P* values of at least less than .05. The grade of statistical significance is displayed in the figure legends.

RESULTS**IL-10-overexpressing B cells show decreased production of proinflammatory cytokines with increased IL-1 receptor antagonist and VEGF levels**

Under optimized transfection conditions (see Fig E1, A and B) and after 24 hours of culture in medium, IL-10_tr B cells upregulated *IL10* mRNA expression (approximately 3500-fold) and secreted IL-10 (approximately 100-fold) compared with levels seen in ctrl_tr B cells (Fig 1 and Fig 2, A, top left panels). The transfection procedure did not alter the stimulatory effect of TLR9-L on IL-10 induction compared with that seen in non-tr B cells (see Fig E1, C). However, lack of further increase in IL-10 production in IL-10_tr B cells stimulated with TLR9-L at the protein level was accompanied by significant downregulation of *IL10* gene transcription 24 hours after transfection (see Fig E1, D; similar data were observed after 48 hours [data not shown]). This implies that after reaching maximal production capacity, IL-10 might limit/downregulate its own expression through a negative feedback mechanism at the transcriptional level. High IL-10 production in IL-10-overexpressing B cells was accompanied by a significant decrease in production of the proinflammatory cytokines TNF- α , IL-8, and macrophage inflammatory protein (MIP) 1 α (Fig 1). In contrast, levels of the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1Ra) and vascular endothelial growth factor (VEGF) were significantly increased compared with those in control cells. Furthermore, there was a tendency for inhibition of MIP-1 β and interferon-inducible protein 10, whereas detectable levels of IL-6, IFN- γ , GM-CSF, and RANTES were not significantly changed (see Fig E2 in this article's Online Repository at www.jacionline.org). Additionally, although there was no measurable Semaphorin3A in *IL10*-transfected B cells after 48 hours, secreted levels of TGF- β were detectable but did not significantly change in relationship to IL-10 overexpression.

These data demonstrate that IL-10 overexpression suppresses proinflammatory cytokines and induces anti-inflammatory cytokines in B cells.

IL-10-overexpressing B cells acquire an immunoregulatory phenotype characterized by expression of CD25, GARP, PD-L1, and CD38

In IL-10_tr B cells there was a significant increase in mRNA expression of IL-2 receptor α (*IL2RA*, CD25) and glycoprotein A

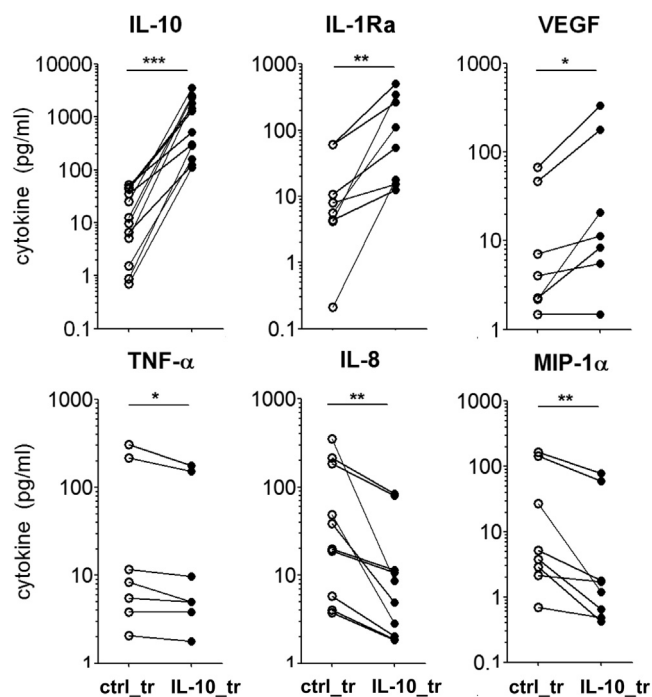


FIG 1. Overexpression of human IL-10 and profile of concurrent cytokine release from IL-10-overexpressing B cells. Freshly purified human B cells were transfected with pORF-hIL-10 (IL10_tr, solid circles) or pORF-mcs (ctrl_tr, open circles) and cultured in medium for 24 hours. Secreted human IL-10 and other cytokines, chemokines, and growth factors were quantified by using a bead-based multiplex cytokine quantification method. Each connected pair of dots represents individual donor results ($n = 12$ donors). * $P < .05$, ** $P < .01$, and *** $P < .001$, Wilcoxon matched pairs test.

repetitions predominant (GARP; *LRRC32*; Fig 2, A). Both molecules have been also described to be expressed on and functionally linked to regulatory T cells. In addition, mRNA expression of PD-L1 (*CD274*), a member of the PD-1/PD-L1 axis that promotes a tolerogenic phenotype in T cells, as well as OX40 ligand (*TNFSF4*), was significantly increased, whereas transcription of PD-L2 (*PDCD1LG2*) and inducible costimulator ligand (*ICOSLG*) was not altered in IL-10_tr B cells (Fig 2, B). In line with these, gene transcription of CD40, a molecule important for B-cell survival upon interaction with activated T cells, was also significantly enhanced.

Additional regulatory B cell-associated surface molecules were analyzed in parallel experiments. CD38 expression was significantly upregulated after IL-10 overexpression. A tendency toward increase was observed for CD5, whereas expression of CD1d and CD24 did not change (Fig 2, D). mRNA expression of CD71 (*TFRC*) and CD73 (*NT5E*), previously reported to be differentially expressed in Br1 cells, did not change following IL-10 transfection (see Fig E3 in this article's Online Repository at www.jacionline.org). Along with significantly downregulated CD27 expression on IL-10_tr B cells, expression of CD148 was overall low in both ctrl_tr and IL-10_tr B cells, suggesting that IL-10 overexpression is not sufficient to provide activated status of B cells but rather keeping them in a naive/transitional phenotype. At the same time, expression of CD48 and CD147 was high on both ctrl_tr and IL-10_tr B cells without showing any difference. Expression of different costimulatory molecules and HLA-DR was assessed

in IL-10_tr B cells to address their role in antigen presentation and costimulation. There was a significant decrease in CD80 expression and a similar trend in CD86 expression, whereas no change in HLA-DR and CD83 expression was observed (Fig 2, E).

Not surprisingly, *SOCS3*, as a direct IL-10-regulated gene, was significantly induced in IL-10-overexpressing B cells (Fig 2, A), with a similar trend observed in *SOCS1* expression (see Fig E3). This might account for the mechanism of downregulation in production of distinct proinflammatory cytokines by limiting their self-enhancing positive feedback regulation. At the same time, there was no significant effect on the gene transcription of IL-10 receptor α (*IL10RA*), and suggesting no direct negative feedback mechanism on the IL-10 receptor expression level, there was no difference in the transcription of TGF- β (*TGFB1*; see Fig E3).

Relative mRNA expression for *FAS*, Fas ligand (*FASLG*), and TNF-related apoptosis-inducing ligand (*TNFSF10*), as well as both mRNA and intracellular levels for granzyme B (*GZMB*), were measured to test the potential role of different apoptosis-related molecules in the mechanisms of suppression by IL-10-overexpressing B cells. There was no detectable mRNA expression for both *FASLG* and *GZMB*, nor was it detectable at the protein level. Although *TNFSF10* expression was not altered, there was significantly more *FAS* mRNA expressed in relation to IL-10 overexpression (Fig 2, C).

The effect of additional TLR9-L stimulation on the expression of immunoregulatory and apoptotic molecules in B cells after IL-10 overexpression indicates that molecules beyond IL-10 (and suppressor of cytokine signaling [SOCS] 3) are required for induction of Semaphorin3A secretion in B cells (the similar was true for secreted TGF- β ; see Fig E4, A, in this article's Online Repository at www.jacionline.org). On the other hand, TLR9-L stimulation downregulates mRNA expression of *TGFB1*, *FAS*, and *TNFSF10* (see Fig E4, B). This suggests that the TLR9-L pathway does not directly contribute to the apoptotic mechanisms mediated through Fas ligand or granzyme B (not detectable at mRNA level) or TNF-related apoptosis-inducing ligand (TRAIL).

These data suggest that on IL-10 overexpression, B cells acquire a regulatory phenotype characterized by upregulation of CD25, GARP, PD-L1, and SOCS-3 accompanied by reduced costimulatory potential.

Effect of IL-10 overexpression on B-cell maturation and immunoglobulin isotypes

The analysis of distinct surface markers and surface IgM and IgD molecules was performed to assess the activation status of IL-10-overexpressing B cells. Overexpression of IL-10 in B cells resulted in consistently reduced expression of CD19, CD27, and surface IgM, whereas IgD expression increased 36 hours after transfection (Fig 3, A).

After IL-10 overexpression in B cells, the expression profile of transcription factors demonstrated a significant increase in interferon regulatory factor 4 (IRF-4) and X-box binding protein 1 (XBP-1) but not PR domain zinc finger protein 1 (*PRDM1*) encoding B lymphocyte-induced maturation protein 1 (BLIMP-1). This indicates the potential for preferential maturation toward plasmablast phenotype (Fig 3, B). The transcription factor signature was accompanied by a significant

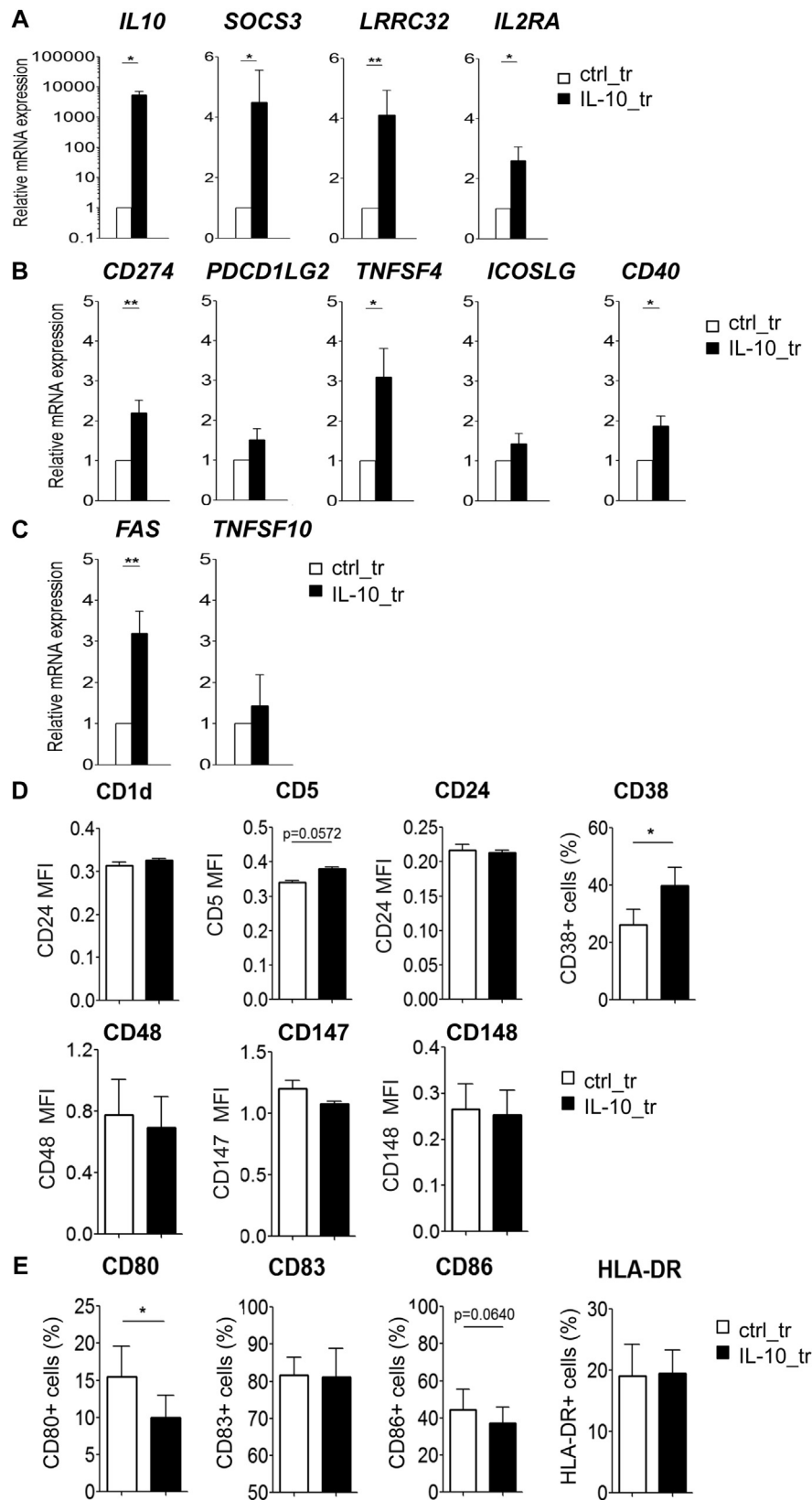


FIG 2. Expression profile of immunosuppression- and costimulation-related genes in IL-10-overexpressing B cells. Expression of immunoregulatory molecules (**A**), members of the B7 gene family (**B**), and common apoptosis-related molecules (**C**) was determined in transfected B cells by using quantitative RT-PCR after 24 hours, and expression of surface immunoregulatory molecules (**D**), costimulatory molecules, and HLA-DR (**E**) was determined by using flow cytometry 36 hours after transfection, respectively. The extent of transcription for target genes was calculated as $\Delta\Delta$ cycle threshold values relative to the elongation factor 1 α (EF1 α) housekeeping gene and presented when corresponding values for control transfected B cells were set as 1. Protein expression data are presented in graphs as either mean fluorescent intensities (MFI) or proportions of positive cells (in percentages) and depicted as a pair of dots linking each donor's data (n = 4-9 donors). * $P < .05$ and ** $P < .01$, paired t test.

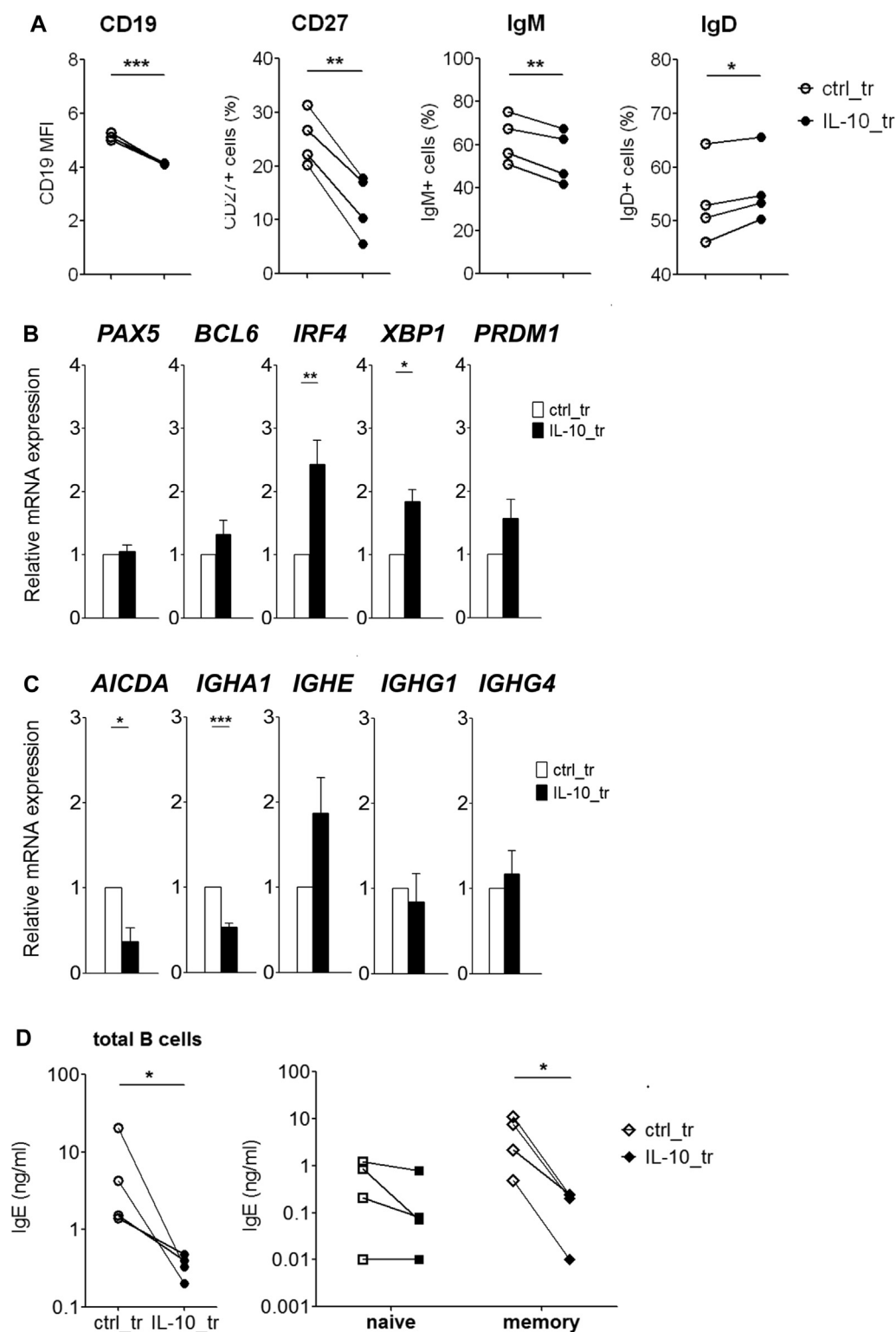


FIG 3. Surface marker phenotypes, transcription factor signatures, and profiles of IgE production in IL-10-overexpressing B cells. **A**, Expression of the B-cell surface molecules CD19 and CD27 and the surface immunoglobulins IgM and IgD was assessed in transfected B cells by means of flow cytometry 36 hours after transfection. **B** and **C**, mRNA levels for transcription factor genes (Fig 3, **B**) and activation-induced cytidine deaminase (*AID*) and immunoglobulin genes (Fig 3, **C**) were determined by using quantitative RT-PCR 24 and 72 hours after transfection, respectively ($n = 4-9$). **D**, Cell-culture supernatants of total B cells, sorted naive B cells ($CD19^+CD27^-$), or memory B cells ($CD19^+CD27^+$) transfected with pORF-hIL-10 or pORF-mcs and subsequently stimulated with TLR9-L were quantified for secreted IgE 10 days after transfection by using the same method ($n = 4$ donors). * $P < .05$, ** $P < .01$, and *** $P < .001$, paired t test.

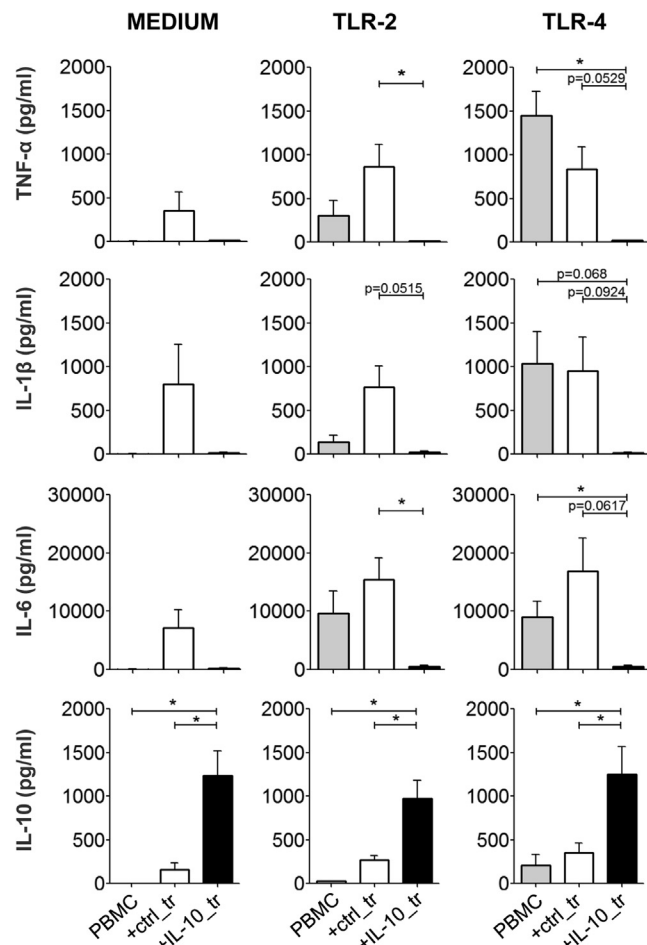


FIG 4. Cytokine secretion from PBMCs cocultured with TLR9-L-pretreated and IL-10-transfected B cells on TLR2-L or TLR4-L stimulation. Purified B cells were prestimulated with TLR9-L (72 hours), transfected either with pORF-hIL-10 or pORF-mcs, cocultured with autologous PBMCs (B cell/PBMC ratio, 1:4), and stimulated with TLR2-L or TLR4-L. Secreted cytokines, growth factors, and chemokines were quantified in cell-coculture supernatants by using a bead-based multiplex cytokine measurement 24 hours after stimulation ($n = 4$ donors). * $P < .05$, paired t test.

increase in HELIOS (*IKZF2*, 4.6-fold), but not AIOLOS (*IKZF3*) and IKAROS (*IKZF1*) expression (see Fig E3). Transcripts encoding activation-induced cytidine deaminase (*AICDA*), an enzyme crucial for class-switch recombination and somatic hypermutation, were significantly decreased in IL-10_tr B cells. The transcription levels of IgE, IgG₁, and IgG₄ genes were not found to be significantly altered, whereas IgA expression decreased (Fig 3, C). Naive (CD19⁺CD27⁻) and memory (CD19⁺CD27⁺) B cells were sorted, separately transfected to overexpress IL-10, and stimulated with TLR9-L for 10 days to address differences in immunoglobulin production. Among distinct isotypes measured, only the concentration of secreted IgE was significantly reduced, particularly in IL-10_tr memory B cells (Fig 3, D). A similar tendency was observed for the total B cells stimulated with TLR9-L before IL-10 overexpression (see Fig E5, A and C in this article's Online Repository at www.jacionline.org). In addition to increased *IL2RA* (CD25) gene transcription, TLR9-L prestimulation and subsequent IL-10 overexpression resulted in an increase in IgG₄ mRNA expression (see Fig E5, B).

IL-10-overexpressing B cells inhibit TLR2-L- and TLR4-L-induced cytokine secretion in PBMCs

PBMCs were cocultured with autologous TLR9-L-pretreated (72 hours) and IL-10_tr or ctrl_tr B cells (ratio, 1 B cell/4 PBMCs) and subsequently stimulated with TLR2 or TLR4 ligands to investigate the effect of IL-10-overexpressing B cells on early innate mechanisms of cytokine release. As expected, both TLR2-L and TLR4-L stimulation triggered the release of proinflammatory TNF- α , IL-1 β , IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), and IFN- γ , as well as IL-10, in PBMCs alone (Fig 4 and see Fig E6 in this article's Online Repository at www.jacionline.org). Interestingly, an increase in cytokine release was observed in unstimulated ctrl_tr B cell cocultures, which was further enhanced upon stimulation (especially by TLR2-L). The increase in cytokine release in ctrl_tr B cells cocultures without stimulation might be due to the presence of other B cell-produced mediators, which become suppressed or superseded by IL-10 overproduction in the case of IL-10-transfected B-cell cocultures. However, in IL-10_tr B cell cocultures the proinflammatory cytokines TNF- α , IL-1 β , IL-6, G-CSF, IFN- γ , and IL-8 were suppressed in both TLR2-L- and TLR4-L-stimulated conditions (59% to 95% of inhibition), as well as in unstimulated cocultures (54% to 98% of inhibition). These results demonstrate a potent role of IL-10-overexpressing B cells in limiting the production of proinflammatory cytokines elicited through TLRs.

IL-10-overexpressing B cells suppress the maturation of MDDCs and inhibit their cytokine release on LPS stimulation

Freshly purified B cells were prestimulated with TLR9-L for 72 hours, transfected to overexpress IL-10, and subsequently cocultured with autologous MDDCs at different B cell/MDDC ratios to test their effect on DC maturation. Maturation of MDDCs was induced with LPS for 36 hours and resulted in loss of CD14 expression and induction of CD80, CD83, and CD86. CD14 expression on CD11c⁺ MDDCs was less reduced on coculture with IL-10_tr B cells (Fig 5, A). Coculture with IL-10_tr B cells led to reduced expression of CD83 and CD86 with the same trend for CD80, although to a lesser extent, whereas PD-L1 was augmented (Fig 5, B). Additionally, in the supernatants of MDDCs cocultured with IL-10_tr B cells, there was a reduction in IL-12p70 levels, with the same direction tendency for G-CSF, TNF- α , and IFN- γ , whereas VEGF and IL-1Ra levels were increased (Fig 5, C). Levels of secreted GM-CSF and MCP-1 were comparable between IL-10_tr B cells and ctrl_tr B cell cocultures, although reduced compared with LPS-stimulated MDDCs alone. All significant effects were cell ratio dependent.

In conclusion, the data indicate that IL-10-overexpressing B cells can suppress differentiation of monocytes to DCs and limit DC maturation, rendering their phenotype into a regulatory one.

IL-10-overexpressing B cells potentially inhibit antigen-specific proliferation of PBMCs

The suppressive capacity of IL-10_tr B cells on antigen-specific proliferation was analyzed in cocultures with autologous PBMCs in response to tetanus toxoid (TT) stimulation. Although nontransfected B cells did not alter antigen-induced proliferation, control transfected B cells significantly inhibited

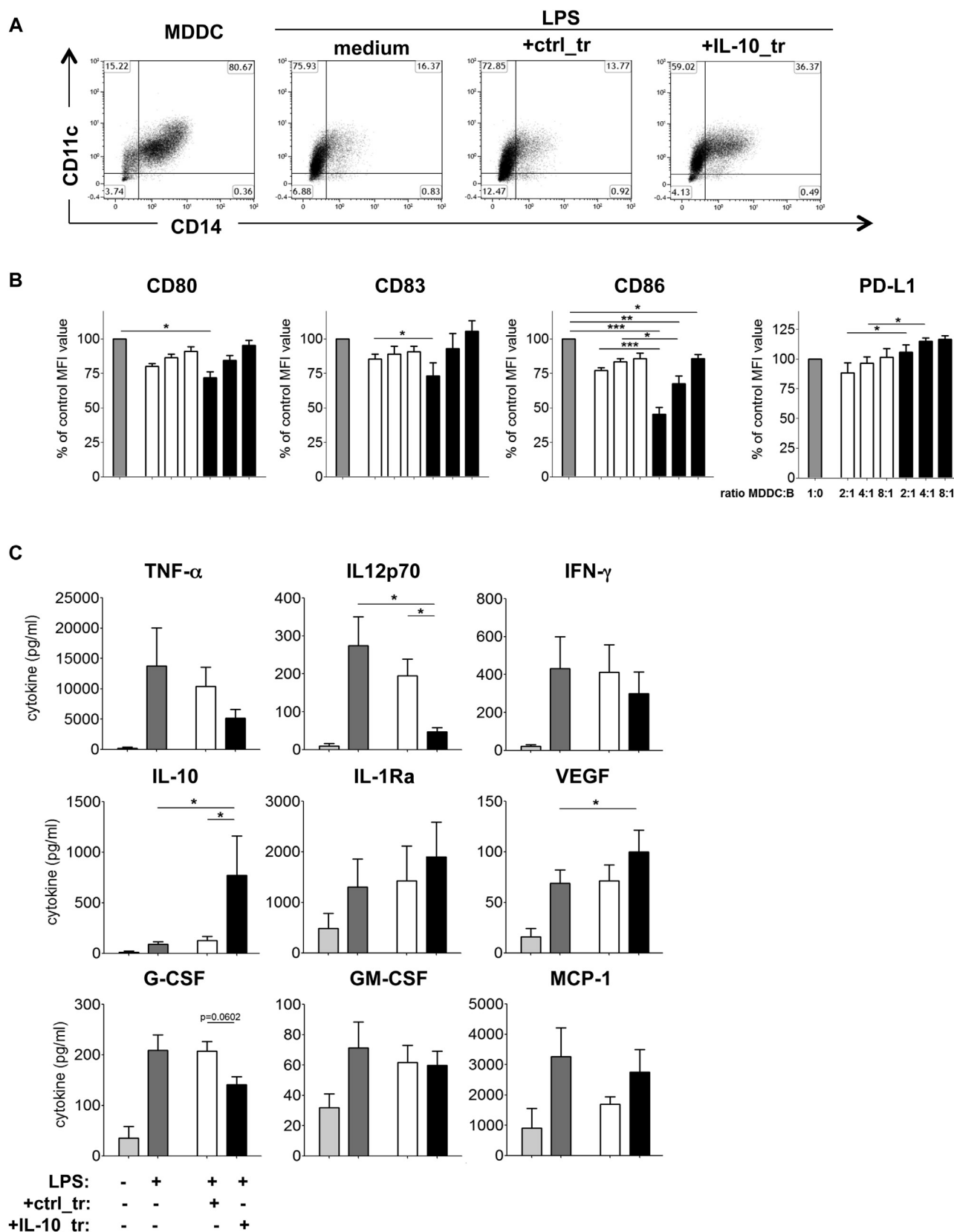


FIG 5. MDDC maturation, expression of costimulatory molecules, and cytokine release on coincubation with TLR9-L-pretreated and IL-10-transfected B cells and stimulation with TLR4-L. B cells were pretreated with TLR9-L for 72 hours, transfected with pORF-hIL-10 (hIL-10_trB) or pORF-mcs (ctrl_trB), and coincubated with MDDCs (B cell/MDDC ratios, 1:2, 1:4, or 1:8). Cocultures are subsequently stimulated with TLR4-L for 36 hours. **A** and **B**, Expression of CD14 and CD11c (Fig 5, A) and costimulatory molecules (Fig 5, B) was assessed by using flow cytometry. A representative dot plot of CD14/CD11c expression and normalized percentage values for CD80, CD83, CD86, and PD-L1 relative to control sample (stimulated MDDCs set as 100%) are presented. **C**, Cytokine secretion was quantified in cell-coculture (ratio, 1:2) supernatants by using a bead-based multiplex cytokine quantification ($n = 4$ donors). * $P < .05$, paired t test.

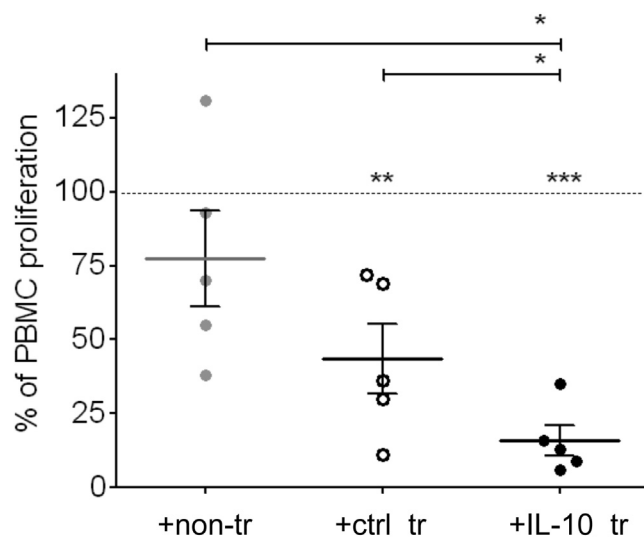


FIG 6. Antigen-specific proliferation of PBMCs coincubated with IL-10-overexpressing B cells. B cells were transfected to overexpress IL-10 and cocultured with PBMCs after stimulation with TT for 5 days (B cell/PBMC ratio, 1:4). Tritiated thymidine was added to cultures for the last 8 hours of culture. Cells were harvested, and the proportion of incorporated thymidine was quantified. Results are presented as proliferation relative to the TT-treated PBMC sample (proliferation control set as 100) and expressed as a percentage ($n = 5$ donors). * $P < .05$, ** $P < .01$, and *** $P < .005$, paired t test.

TT-induced PBMC proliferation by 56%. IL-10_{tr} B cells suppressed antigen-specific proliferation of PBMCs by 86% (Fig 6). These findings provide evidence for strong suppressive capacity of IL-10-overexpressing B cells toward antigen-specific responses.

DISCUSSION

Under physiologic conditions, IL-10 is often secreted together with other soluble and/or surface immune mediators that, alone or in combination, can affect the pure IL-10-mediated effects (positively, negatively, or synergistically). TLR9 stimulation induces IL-10 production in PBMC-derived human B cells but is accompanied by relatively high amounts of proinflammatory IL-6.¹⁹ In the present study our aim was to investigate the direct effects of solely IL-10 overexpression in B cells on their phenotype and immunoregulatory potential.

Human primary B cells are highly resistant to most gene transfer techniques. Nucleofection was described as the most efficient nonviral method to introduce plasmid DNA into human pre-B cells and murine B cells.^{28,29} Accordingly, we established efficient plasmid-mediated transfer of the *IL10* gene into human peripheral B cells using nucleofection and consequently demonstrated high overexpression of functional IL-10. IL-10 transfection into B cells led to rapid and substantial IL-10 release and transcription of *SOCS3*, a direct IL-10-responsive gene. This is in accordance with a previously described finding that the ability of IL-10 to inhibit gene expression in monocytes is associated with rapid induction of *SOCS3* synthesis and inhibition of JAK-STAT-dependent signaling.³⁰

Overexpression of IL-10 in B cells induced an anti-inflammatory phenotype characterized by low secretion of the proinflammatory mediators TNF- α , IL-8, and MIP-1 α , whereas levels of anti-inflammatory IL-1Ra and VEGF were significantly

increased. These findings are in line with those of other reports studying the direct effects of IL-10 on gene expression in monocytes and macrophages of both human and mouse origin.³¹⁻³³ In this context IL-10 has been ascribed a crucial role in inhibiting transcription elongation of the *TNFA* gene and enhancing recruitment of LPS-induced nuclear factor κ B to the promoter of the *IL1RA* gene.^{34,35} Therefore IL-10 seems to exert similar effects on cytokine secretion in both B lymphocytes and cells of monocyte/macrophage lineage. However, IL-10-mediated regulation of a costimulatory phenotype might differ between these 2 cell types. Although IL-10_{tr} B cells exhibit downregulation of surface CD80, with no change in CD83 expression (and mediate downregulation of both molecules in MDDC), IL-10 was reported to drive an increase in CD80 and a decrease in CD83 expression on monocytes.³¹ Furthermore, high IL-10 expression in B cells at the same time reduced CD80 expression and upregulated PD-L1 expression, suggesting overall lower costimulatory capacity. A similar phenotype on dendritic cells (DCs) has already been suggested to promote a regulatory environment for T-cell activation.³⁶

Previously described human regulatory B-cell subsets with an *in vivo*-demonstrated role include 3 types of IL-10-producing B cells. IL-10⁺CD19⁺ B lymphocytes enriched among CD24^{hi}CD38^{hi} cells, which could suppress the differentiation of T_H1 cells in healthy subjects, have been found to be functionally impaired in patients with systemic lupus erythematosus.²⁰ B10 and B10pro cells predominantly found within CD24^{hi}CD27⁺ B cells were shown to negatively regulate monocyte cytokine production *in vitro*.²¹ In a recent study by our group, IL-10⁺ Br1 cells were enriched among CD25⁺CD71⁺CD73^{lo} B cells, which could potentially suppress antigen-specific CD4⁺ T-cell proliferation. Furthermore, these Br1 cells selectively upregulate IgG₄ production.¹⁹

IL-10-overexpressing B cells acquired an immunoregulatory surface phenotype characterized by augmented transcription of *LRRC32*, *IL2RA*, and *CD274* genes as early as 24 hours after transfection.^{37,38} Interestingly, all 3 molecules were described to be upregulated on regulatory T cells, and CD25 and PD-L1 were highly expressed in IL-10⁺ Br1 cells.¹⁹ IL-10_{tr} B cells were distinct from IL-10⁺CD19⁺ regulatory B cells (enriched among CD24^{hi}CD38^{hi} cells) in their low CD24 levels and a decrease in CD19 expression while retaining high CD38 expression.²⁰ Similar to B10 and B10pro cells, IL-10_{tr} B cells significantly downregulated surface CD27.²¹ A trend for higher CD5, but not CD1d expression, was observed along with IL-10 overexpression in B cells, which partially resembled the phenotype of mouse regulatory CD1d^{hi}CD5⁺ IL-10-producing cells.²² These data suggest that solely IL-10 might have influence on direct or indirect induction of CD25, CD38, and CD5 in human IL-10-producing B cells, whereas higher expression of CD24, CD71, and CD73 might require additional signals beyond IL-10 (eg, TLR9-L). Nevertheless, the effect of additional TLR9 stimulation to IL-10 overexpression, that might induce certain molecules playing a role in additional suppressive capacity, cannot be completely ruled out. In the present study IL-10-overexpressing B cells demonstrated a similar pattern of surface molecules that partially overlap with different types of human regulatory IL-10⁺ B cells. Other types of secreted stimuli and/or surface molecules, such as IL-6 receptor blocking,³⁹ B cell-activating factor of the TNF family,⁴⁰ or phorbol ester/ionomycin, as a general stimuli that might have been used to induce IL-10 remain to be further elucidated.

Early changes in the expression of transcription factors included the upregulation of *IRF4* and *XBPI1*, but not *PRDM1*, as a characteristic of memory B cells in transition before terminal differentiation to plasma cells.⁴¹ In addition, we observed upregulation of *IKZF2* gene encoding HELIOS, a hematopoietic-specific transcription factor involved in the regulation of B-lymphocyte development, in IL-10-overexpressing B cells. This transcription factor has also been demonstrated to be expressed in a subset of regulatory T cells.⁴²⁻⁴⁴

IL-10-overexpressing B cells significantly downregulated surface IgM expression while upregulating surface IgD, which in parallel to significantly less CD19 and CD27 expression suggests a “naïve/transitional” B-cell phenotype. Taken together, high IL-10 expression in B cells supports possible lines of B-cell fate, one resembling immature transitional-like B cells and the other resembling IgG/IgE-switched plasmablasts. Secreted immunoglobulin levels demonstrate downregulation of IgE production, particularly in the memory B-cell compartment, as a consequence of high IL-10 production. These data suggest that, similar to IL-10-secreting CD4⁺CD25⁺ T regulatory cells, IL-10-overexpressing B cells (also acquired CD25) might directly contribute to suppression of allergic diseases through downregulation of IgE.¹⁷ An additional increase in total IgG₄ secretion when IL-10 is combined with TLR9 stimulation might be one of the mechanisms of action contributing to successful allergen-specific immunotherapy.¹⁹

General suppressive functions of IL-10-overexpressing B cells include direct effects on antigen-specific proliferation and the cytokine profile of PBMCs. PBMCs cocultured with IL-10_{tr} B cells and stimulated with either TLR2-L or TLR4-L produce remarkably less proinflammatory cytokines, indicating potent anti-inflammatory activity of IL-10_{tr} B cells. These effects might be mediated, at least in part, through direct or indirect IL-10 actions through *SOCS3* and *IL1RA* and decreased costimulatory capacity for antigen presentation by B cells. Furthermore, IL-10_{tr} B cells remarkably reduced the overall costimulation potential of MDDCs, through inhibition of cytokine release and decrease in costimulatory molecules expression, impacting central role of antigen presenting cells (APCs) in initiation and shaping of the adaptive immune responses. In combination with the induction of PD-L1 on MDDCs, these APCs are rendered toward a regulatory phenotype.⁴⁵ This might account for the indirect mechanism of potent inhibition of antigen-specific responses by professional APCs in the presence of IL-10-secreting B cells.¹⁹ In addition, there was no evidence that IL-10 overexpression enhanced the expression of common apoptosis-related molecules in B cells, suggesting that immunoregulatory actions of B cells are not directly mediated through apoptosis induction.

Taken together, solely high IL-10 expression in B cells is sufficient to rapidly induce a complex B regulatory phenotype characterized by an increase in multiple immunoregulatory factors. In turn and in conjunction with secreted IL-10, these grant B cells the capability of exerting a remarkable and comprehensive “package” of suppressive effects regulating different aspects of the immune response. In this article, their immunoregulatory profile of actions is demonstrated to be directed against (1) early activation of nonspecific immunity, by inhibition of proinflammatory cytokine release elicited through TLRs in APCs, reduction in maturation of professional APCs and their costimulatory capacity, and (2) specific immunity

through limiting memory T-cell responses and decreasing IgE production.

Therefore, human IL-10-overexpressing B cells represent regulatory B cells with a prominent immunoregulatory phenotype, capable to exert potent anti-inflammatory functions and modulation of the immune response, contributing to a tolerance-inducing environment. These data elucidate our understanding of the capacity of solely IL-10 on B-cell physiology and regulatory B-cell profile generation, which is applicable as a therapeutic strategy for substantial immune modulation of pathologic human immune responses in the fields of allergy and asthma, chronic infections, autoimmunity, organ transplantation, and tumor immunity.

Key messages

- IL-10 alone has a prominent role in inducing a complex immunoregulatory phenotype in B cells.
- The IL-10-overexpressing B-cell immunoregulatory phenotype resembles features of regulatory T cells through surface expression of GARP and CD25 and intracellular *SOCS3* and similarly can potentially inhibit the antigen-specific T-cell response.
- IL-10-overexpressing B cells provide less proinflammatory cytokines and more anti-inflammatory IL-1Ra.
- IL-10-overexpressing B cells are capable of instructing professional APCs toward a regulatory phenotype.

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METHODS

Isolation of PBMCs and B cells

Human PBMCs were isolated from heparinized peripheral blood or buffy coats from healthy adult volunteers by using density gradient centrifugation on Ficoll (Biochrom, Berlin, Germany). Cells were then washed and resuspended in RPMI 1640 medium (Biochrom) supplemented with 10% heat-inactivated FCS and antibiotics (penicillin, streptomycin, and kanamycin), MEM vitamin, L-glutamine, nonessential amino acids, and sodium pyruvate (Life Technologies, Carlsbad, Calif), which are referred to here as complete RPMI medium. From PBMCs, non-B cells were labeled with a cocktail of biotin-coupled antibodies against CD2, CD14, CD16, CD36, CD43, and CD235a and anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by magnetic separation (AutoMACS, Miltenyi Biotec). Therefore B cells were purified as an untouched fraction (negative selection). The purity of isolated B cells was assessed by using flow cytometry with anti-CD19 antibody and routinely resulted in greater than 96% CD19⁺ cells. For some experiments, pre-enriched B cells were labeled with anti-CD27 antibody, and CD27⁻ (primarily naive B) and CD27⁺ B cells (primarily memory B) were sorted with the FACSria II cell sorter (BD Biosciences, Franklin Lakes, NJ).

Plasmids

An expression plasmid containing the human IL-10 gene construct pORF-hIL-10 or its backbone control, pORF-mcs (both from Invitrogen, Life Technologies), were introduced in electrocompetent bacteria and propagated under ampicillin selection at 37°C overnight. Plasmid DNA MaxiPreps EF kits (Qiagen, Hilden, Germany) were used for purification of plasmid DNA. DNA concentration was determined with the NanoDrop 2000 (Wilmington, Del) and adjusted to 1 mg/mL.

Transfection of B cells

Freshly purified B cells were transfected with either pORF-hIL-10 (IL-10_{tr}) or pORF-mcs (ctrl_{tr}) plasmid vectors by using the nucleofection method recommended for human B cells (Human B Cell Nucleofection Kit and Nucleofector, Amaxa; Lonza Cologne AG, Köln, Germany). Then cells were washed, centrifuged at 250 g for 8 minutes, resuspended in complete RPMI medium, and used for the experiments. For the optimization of the transfection protocol, B cells were transfected with a commercial pmxGFP construct (Lonza), and transfection efficiency was assessed along with cell death exclusion (7-aminoactinomycin D [7-AAD]) by using flow cytometry 24 hours after transfection. Transfection conditions were tested, varying B-cell numbers and quantity of either pmxGFP (Fig E1, A), pORF-hIL-10, or pORF-mcs plasmid vectors (Fig E1, B). The transfection protocol was optimized to use 2 to 5 × 10⁶ B cells and 5 to 10 μg of plasmid DNA per transfection. It typically resulted in approximately 50% green fluorescent protein-positive cells among approximately 75% of viable cells (pmxGFP), as well as significant and plasmid amount-dependent IL-10 release after 24 hours (IL-10_{tr}). For some experiments, B cells were stimulated with 1 μmol/L synthetic phosphorothioate B type TLR9-L CpG 2006 (Microsynth GmbH, Balgach, Switzerland) for 72 hours before transfection.

Preparation of MDDCs

PBMCs were seeded in plates at a density of 5 × 10⁶/cm². Monocytes were obtained as a fraction of adherent PBMCs after 2 hours, and the rest of the cells were washed away with prewarmed complete RPMI medium. A combination of 10 ng/mL GM-CSF (PeproTech, London, United Kingdom) and 50 ng/mL IL-4 (Novartis, Basel, Switzerland) was used to induce differentiation of monocytes to dendritic cells (MDDCs) over a period of 7 days. Differentiation was confirmed by means of downregulation of CD14 expression and retained high expression of CD11c.

Cell cultures and cocultures

All B-cell culture conditions were set in a format of 1 × 10⁶ cells/mL in complete RPMI medium. Cell-culture supernatants were collected for secreted cytokine quantification and cell lysates for gene transcription

determination after 24 and 72 hours. Furthermore, cells were harvested for protein surface expression measurement after 36 hours. For the coculture studies, B cells were stimulated with 1 μmol/L synthetic phosphorothioate B type TLR9-L CpG 2006 (Microsynth GmbH) for 72 hours before transfection.

Freshly isolated and IL-10-transfected autologous B cells were cocultured with target cells at different cell number ratios, keeping target cells at a fixed density of 1 × 10⁶/mL in complete RPMI medium, to test the effect of IL-10-overexpressing B cells on TLR-Ls induced cytokine secretion from PBMCs, maturation of MDDCs, and antigen-specific proliferation of PBMCs. Cocultured cells were left rested for 2 hours before appropriate stimuli were added. For maturation of MDDCs, cocultured cells were stimulated with 50 ng/mL TLR4-LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St Louis, Mo) for 36 hours. Cell-culture supernatants were collected for secreted cytokine quantification, and cells were harvested for surface marker expression analysis after 24 and 36 hours, respectively. For induction of cytokine secretion from PBMCs, cocultures were incubated in complete RPMI medium with addition of either 100 ng/mL lipoteichoic acid from *Staphylococcus aureus* (LTA-SA; InvivoGen, San Diego, Calif) or 50 ng/mL LPS. Supernatants were taken for quantitative cytokine measurement after 24 hours. Antigen-specific stimulation of PBMCs in cocultures was elicited by 0.8 IU of TT antigen (Novartis, Switzerland) for 5 days. Cells were then pulsed with 1 μCi of tritiated thymidine per well (DuPont, Wilmington, Del) for the last 8 hours of cell culture and harvested, and the amount of incorporated labeled nucleotide was measured in the LKB β plate reader (GE Healthcare, Fairfield, Conn).

Flow cytometry

After isolation or cell culture, cells were harvested, centrifuged at 300 g for 8 minutes at 4°C, and resuspended in ice-cold PBS. Cells were then incubated with the fixable viability dyes eFluor450 or eFluor780 (eBioscience, San Diego, Calif) in PBS for 30 minutes on ice and washed with staining buffer (0.5% BSA and 2 mmol/L EDTA in PBS). For the expression of surface markers, cells were stained with the following antibodies: CD19–fluorescein isothiocyanate (FITC), CD80–FITC, HLA-DR–ECD (Beckman Coulter, Fullerton, Calif), IgD–FITC, IgM–PerCP/Cy5.5, CD27–phycoerythrin (PE), CD83–PC5, CD86–PE, CD274–FITC (BD Biosciences, Franklin Lakes, NJ), CD5–PC7, CD11c–Pacific Blue, CD14–PE–Cy7, CD19–APC/Cy7, CD19–BV510, CD24–BV421, CD27–FITC, CD38–PerCP/Cy5.5, CD38–PC7, CD48–FITC, CD147–AF647, CD148–PE (BioLegend, San Diego, Calif), and CD1d–PE (eBioscience, San Diego, Calif) in staining buffer for 30 minutes on ice. Cells were washed once, resuspended in 0.5% paraformaldehyde/PBS, and measured. Corresponding isotype control antibodies were used as negative controls. Samples were analyzed with a Gallios Flow Cytometer (Beckman Coulter) or sorted with FACSria II (Becton Dickinson). Data were analyzed with Kaluza software (Beckman Coulter).

RNA isolation and reverse transcription

RNA was extracted from cell lysates with RNeasy kits (Qiagen) modified to additionally include a DNase digestion step. Reverse transcription was performed with reverse transcription reagents (Fermentas, St Leon-Rot, Germany) by using random hexamers, according to the manufacturer's recommendations.

Real-time PCR

cDNA samples were amplified with target gene-specific primers (Microsynth) and SYBR green PCR master mix (Bio-Rad Laboratories, Hercules, Calif), according to the manufacturer's protocol, on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif). The Δ cycle threshold method was used to obtain quantification of gene transcription relative to the housekeeping gene elongation factor 1α (EF1α). Subsequently, the comparative ΔΔ cycle threshold method was performed to obtain relative quantification among different samples when values for control transfected samples were set as 1. The following primer pairs were designed according to sequences reported in GeneBank and used as follows: activation-induced cytidine deaminase (*AICDA*), forward

5'-AGAGGCGTGACAGTGCTACA-3' and reverse 5'-TGAGCGGAGG AAGAGCAAT-3'; AIOLOS (*IKZF3*), forward 5'-AAAGTGCAGCGTTTT GAATG-3' and reverse 5'-AGCTGTAAAGGATTTCAGGCT-3'; BCL-6 (*BCL6*), forward 5'-AGACCGTCCATACCGG-3' and reverse 5'-CGCAAGT GAAGTCGCA-3'; BLIMP-1 (*PRDM1*), forward 5'-AGCTGACAATGAT GAATCTCA-3' and reverse 5'-GTGAAATGTTAGAACGGTAGAG-3'; CD40 (*CD40*), forward 5'-TTGGGGTCAAGCAGATTGCTA-3' and reverse 5'-GCAGATGACACATTGGAGAAGA-3'; CD71 (*TFRC*), forward 5'-GG CTACTGGGCTATTGTAAAGG-3' and reverse 5'-CAGTTTCTCCGAC AACTTTCTCT-3'; CD73 (*NT5E*), forward 5'-ACTGGGACATTG GGTTTTGA-3' and reverse 5'-CCTCACTTTCTGAGCGATGAGT-3'; elongation factor 1 α (EF1 α), forward 5'-CTGAACCATCCAGGCCAAAT-3' and reverse 5'-GCCGTGTGGCAATCCAAT-3'; FAS (FAS): forward 5'-CTTTTCGTGAGCTCGTCTCTGA-3' and reverse 5'-CCCCAGAAG CGTCTTTGAAC-3'; HELIOS (*IKZF2*), forward 5'-TCACCCGAAAGG GAGCACT-3' and reverse 5'-CATGGCCCTGATCTCATCTT-3'; GARP (*LRR32*), forward 5'-GCCCTGTAAGATGGTGGACAAG-3' and reverse 5'-CAGATAGATCAAGGGTCTCAGTGTCT-3'; IgA (*IGHA1*), forward 5'-CGCTGGCCTTCACACAGAA-3' and reverse 5'-CGCCATGACAACAGA CACA-3'; IgE (*IGHE*), forward 5'-ACACATCCACAGGCACCAAA-3' and reverse 5'-TTGCAGCAGCGGGTCAA-3'; IgG₁ (*IGHG1*), forward 5'-CTCTCAGCCAGGACCAAGGA-3' and reverse 5'-GGTGGGCATGTGT GAGTTTTG-3'; IgG₄ (*IGHG4*), forward 5'-ACC^C_ATGGTCACC GTCTCTCA-3' and reverse 5'-GGGACCATATTTGGACTC-3'; inducible costimulator ligand (ICOS-L; *ICOSLG*), forward 5'-CTCCGCCGCAC CAT-3' and reverse 5'-CTACCATCGCTCTGACTTCCTTCT-3'; IKAROS (*IKZF1*), forward 5'-TTCCGTGATCCTTTTGAGTGC-3' and reverse 5'-CTCGCGTTATGTGCGACGA-3'; IL-10 (*IL10*), forward 5'-GTGATGCCC CAAGCTGAGA-3' and reverse 5'-CACGGCCTTGCTCTGTTTT-3'; IL-10Ra (*IL10RA*), forward 5'-ACAGTTGGCAGTGTGAACCTA-3' and reverse 5'-GTAGCTGAATCTTCCCGAGGA-3'; CD25 (*IL2RA*), forward

5'-AAACTCTAGCCACTCGTCCTG-3' and reverse 5'-ACTTGTTCGTTG TGTTCGA-3'; IRF-4 (*IRF4*), forward 5'-AACGCCTTACCCTTCG-3' and reverse 5'-CCCGGTAGTACAGGCA-3'; PAX-5 (*PAX5*), forward 5'-CTGATCTCCCAGGCAAACAT-3' and reverse 5'-TTGCTCATCAAGGT GTCAGG-3'; PDL1 (*CD274*), forward 5'-ACTGGGACATTCCGGG TTTTGA-3' and reverse 5'-CCTCACTTTCTGAGCGATGAGT-3'; PDL2 (*PDCD1LG2*), forward 5'-GCATAATAAGATGGCTCCCAGAA-3' and reverse 5'-AAAGAGGGAAGTGAACAGTGCTATC-3'; SOCS-1 (*SOCS1*), forward 5'-CCCTGGTTGTTGTAGCAGCTT-3' and reverse 5'-CAAC CCCTGGTTTGTGCAA-3'; SOCS-3 (*SOCS3*), forward 5'-CTTCAGCAT CTCTGTCGGAAGA-3' and reverse 5'-GCATCGTACTGGTCCAGGA ACT-3'; TGF- β (*TGFB1*), forward 5'-AAATTGAGGGCTTTTCGCCTTA-3' and reverse 5'-GAACCCGTTGATGTCCACTTG-3'; TRAIL (*TNFSF10*), forward 5'-TGAAGCAGATGCAGGACAAGT-3' and reverse 5'-AGTCTTTCTAACGAGCTGACGG-3' and XBP-1 (*XBPI*), forward 5'-AG GAGTTAAGACAGCGC-3' and reverse 5'-GAGTCAATACCGCCAG-3'.

Quantification of cytokines and immunoglobulin isotypes

Cell-culture supernatants were quantified for IL-10, TGF- β and Sema-phorin-3A content by using specific ELISAs (IL-10 and TGF- β from R&D Systems, Minneapolis, Minn; Semaphorin-3A from USCNC Life Science, Wuhan, China). Other secreted cytokines and immunoglobulins were quantitatively determined by using the fluorescence-immunocoupled bead-based multiplex assays Bio-Plex Hu Cytokine panel, 17-Plex Group I or 27-Plex Group I, or Bio-Plex Pro Human Isotyping Panel (all from Bio-Rad Laboratories), according to the manufacturer's instructions, respectively. Fluorescent signals were read and analyzed with the Bio-Plex 200 System (Bio-Rad Laboratories).

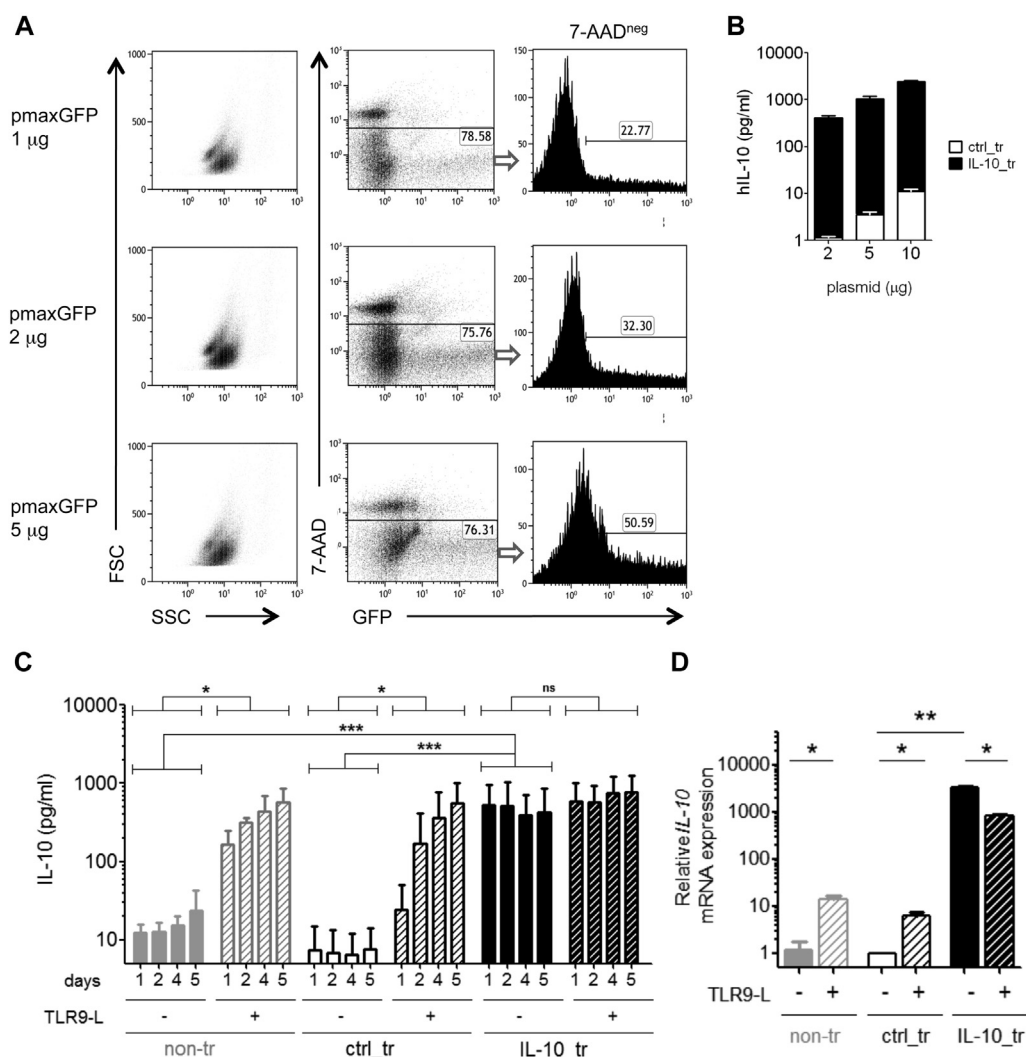


FIG E1. Optimization of B-cell transfection efficiency and kinetics of IL-10 expression. Freshly purified human B cells were transfected with indicated amounts of either pmaxGFP construct or pORF-hIL-10 (IL-10_tr B) or pORF-mcs (ctrl_tr B) plasmid and cultured in medium for 24 hours. **A** and **B**, Transfection efficiency was determined by using flow cytometry and presented as the proportion of live green fluorescent protein (GFP)-positive cells (Fig E1, A) and secreted IL-10 (Fig E1, B) in cell-culture supernatants of transfected cells measured by using a specific ELISA. After transfection, B cells were either cultured in medium only or stimulated with 1 µmol/L TLR9-L for 5 days. **C** and **D**, Secreted IL-10 was quantified in supernatants (Fig E1, C), and IL-10 mRNA expression was quantified in cell lysates by using quantitative RT-PCR 24 hours after transfection (Fig E1, D). Both types of transfected samples were additionally compared with nontransfected B cells under corresponding conditions (n = 4 donors). **P* < .05, ***P* < .01, and ****P* < .001, paired *t* test.

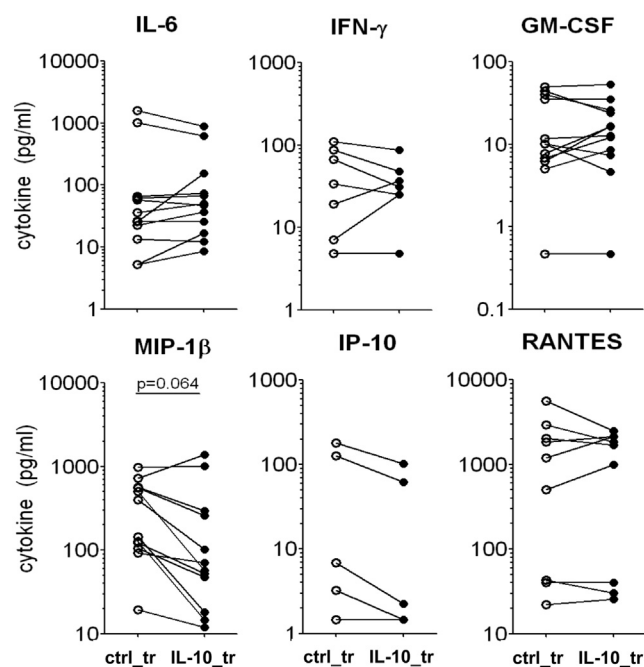


FIG E2. Extended profile of cytokine secretion from IL-10-overexpressing B cells. Transfected B cells were cultured in medium for 24 hours. Secretion of cytokines, chemokines, and growth factors was quantified by using bead-based multiplex cytokine measurement ($n = 12$ donors).

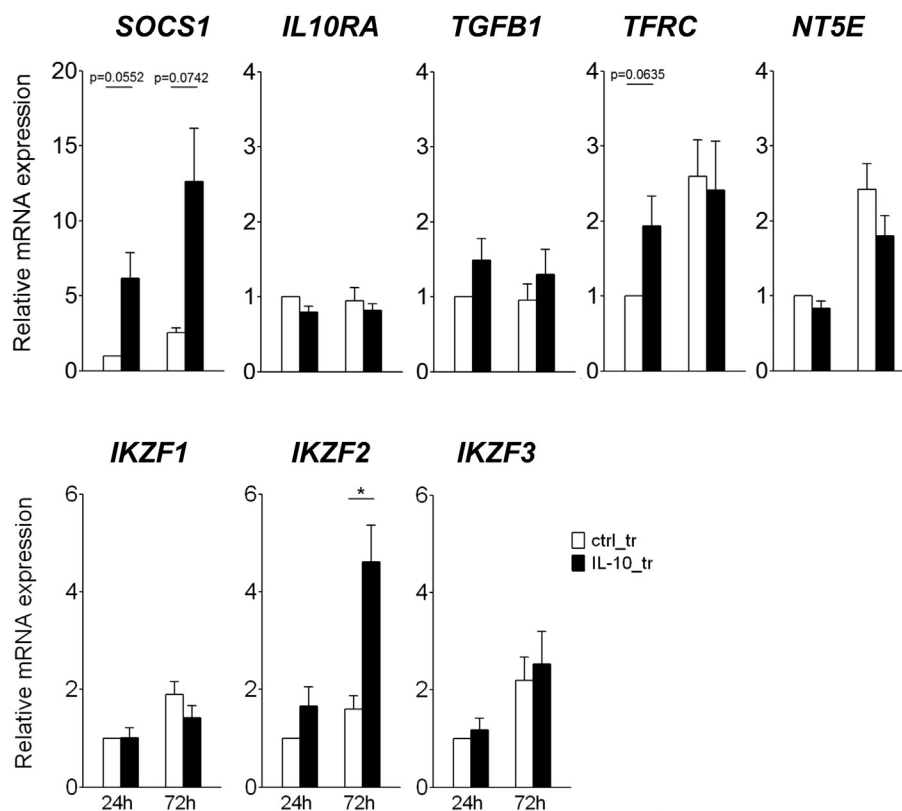
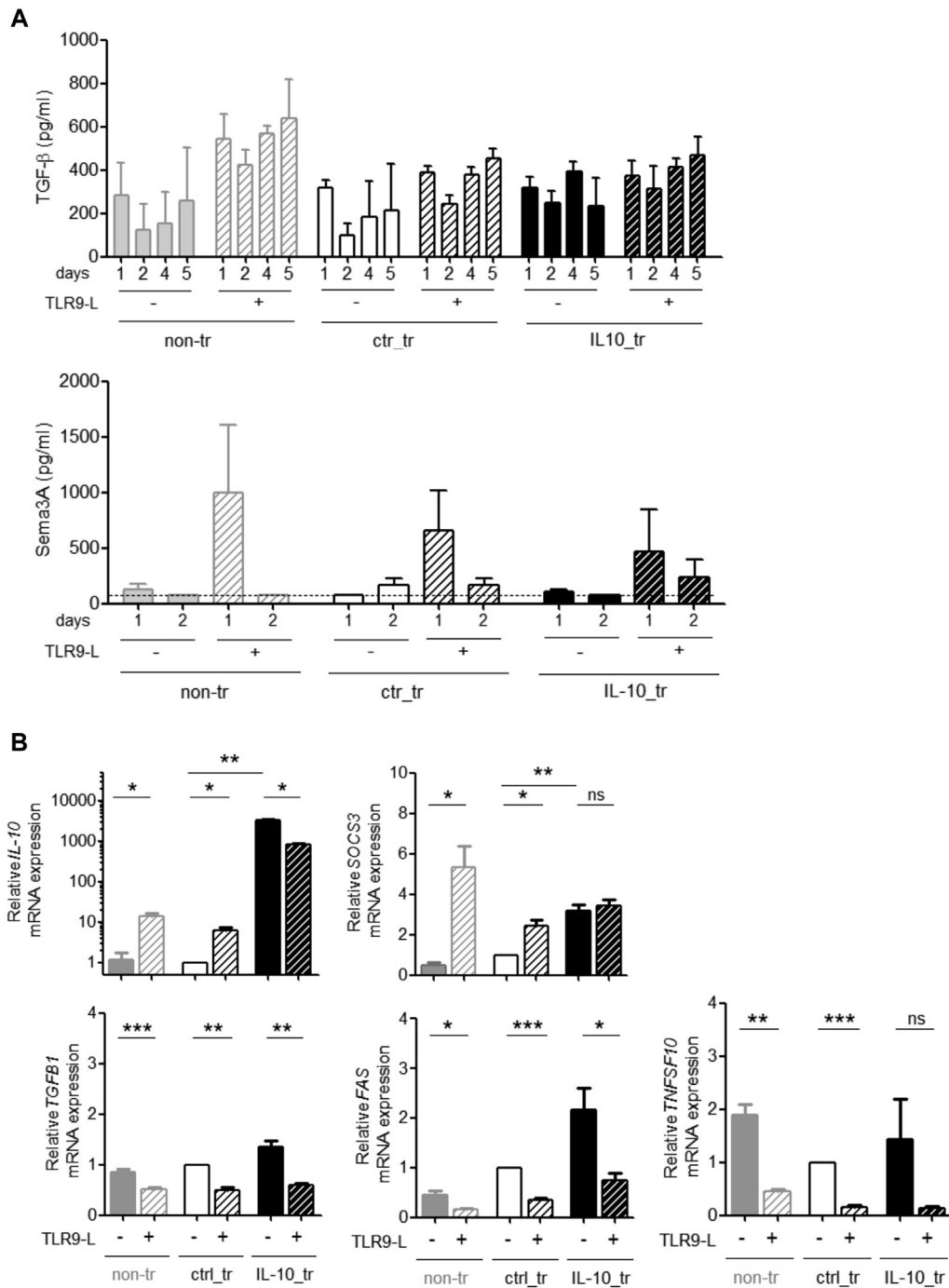


FIG E3. Extended profile of gene expression in IL-10-overexpressing B cells. Gene expression was determined by using quantitative RT-PCR in transfected B cells 24 hours after transfection ($n = 4-9$ donors). * $P < .05$, paired t test.



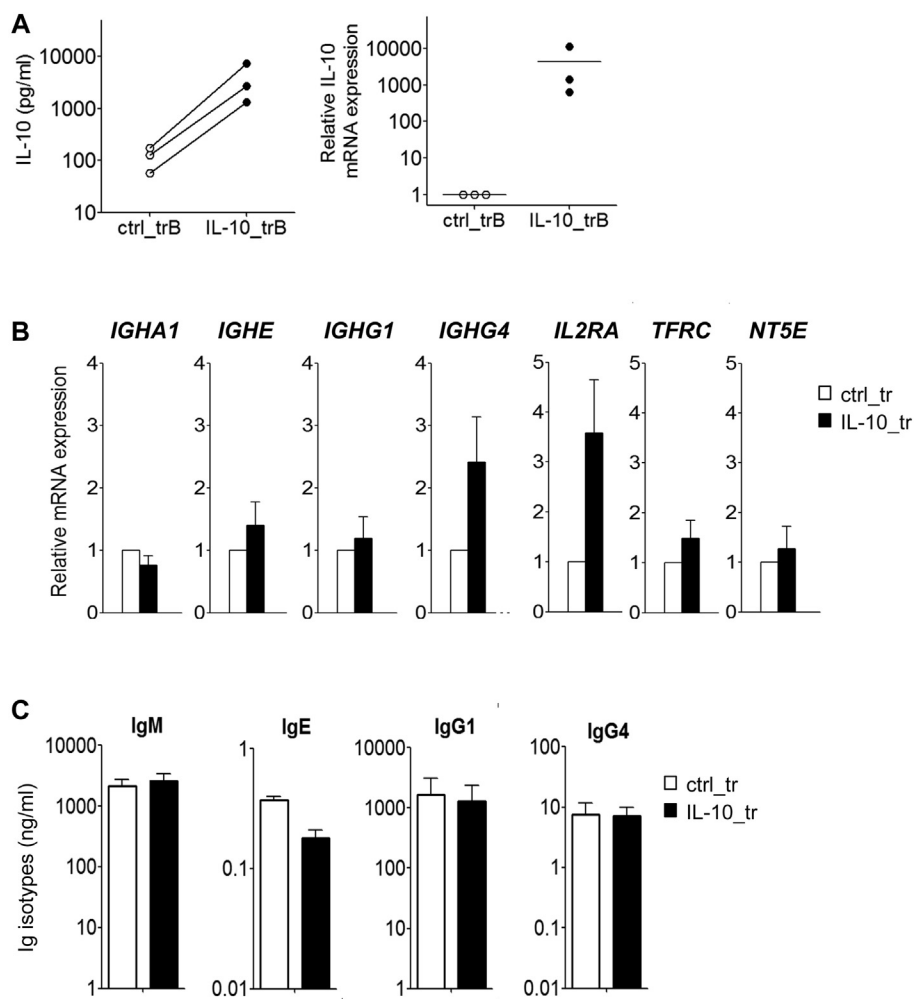


FIG E5. Phenotype of TLR9-L-pretreated IL-10-overexpressing B cells. B cells were preconditioned with CpG for 72 hours, transfected, and cultured in medium. **A**, Twenty-four hours after transfection, cell-culture supernatants or cell pellets were quantified for secreted IL-10 and relative IL-10 mRNA expression by using specific ELISA and quantitative PCR, respectively. **B**, In addition, relative gene expression for the immunoglobulin isotypes CD25, CD71, and CD73 was determined ($n = 3$ donors). **C**, In parallel samples cell-culture supernatants were collected 7 days after transfection and quantified for protein secretion by using bead-based multiplex immunoglobulin isotype measurement ($n = 2$ donors).

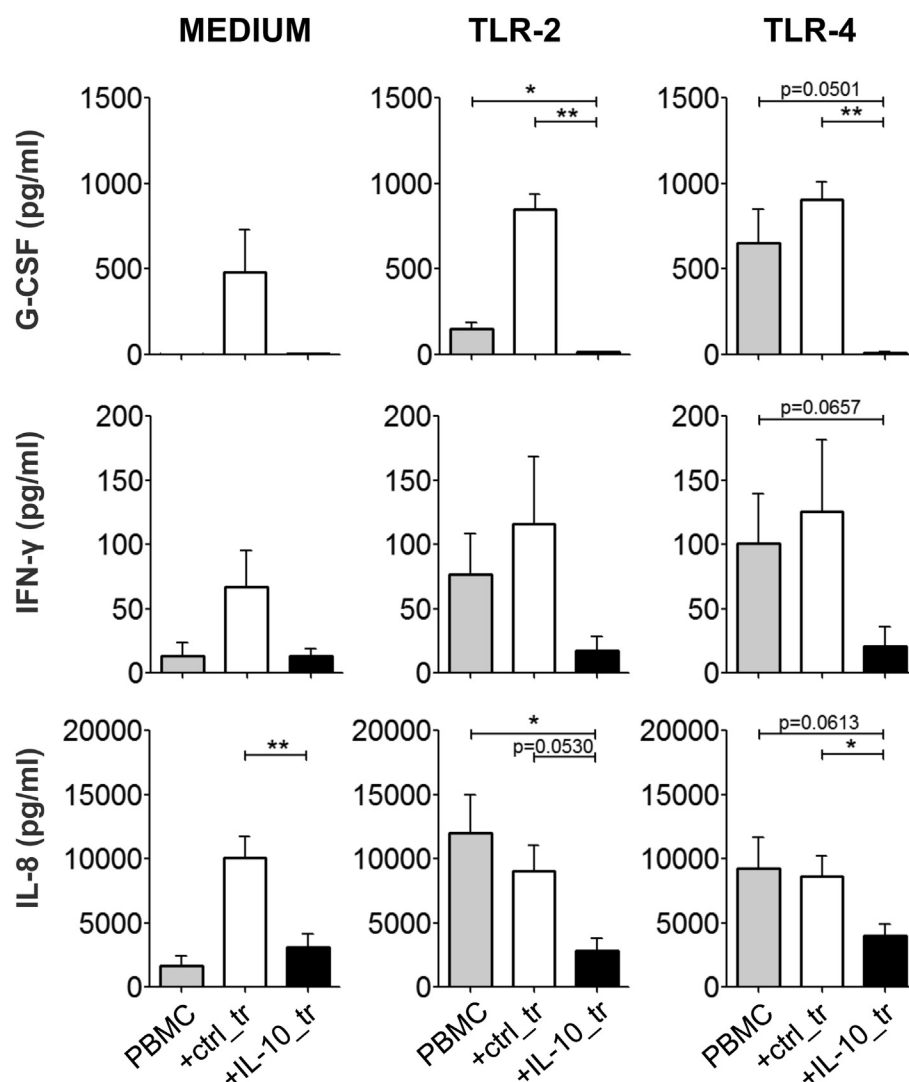


FIG E6. Extended profile of cytokine secretion from PBMCs coincubated with TLR9-L-pretreated and IL-10-transfected B cells on TLR2-L or TLR4-L stimulation. Secreted cytokines, growth factors, and chemokines were quantified in cell-coculture supernatants by using bead-based multiplex cytokine measurement 24 hours after stimulation ($n = 4$ donors). * $P < .05$ and ** $P < .01$, paired t test.