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ORIGINAL ARTICLE

IL-10 induces TGF-β secretion, TGF-β receptor II upregulation, and IgA secretion in B cells

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ABSTRACT. Background: **Interleukin-10 (IL-10) is a pleiotropic cytokine, which has both regulatory and stimulatory effects on different immune cell types. Different studies have reported the importance of IL-10 and Transforming growth factor-beta (TGF-β) in the regulation of B cell class switching the production of immunoglobulin A (IgA); however, the underlying mechanisms remain to be fully elucidated. The objective of this study was to investigate the TGF-β response during B stimulation of human B cells by IL-10. Methods**: **Pan B cells of healthy donors were negatively purified by a magnetic cell separation technique. B cells were cultured with multimeric CD40 ligand (mCD40L) and IL-10 for two and seven days. After harvesting in specific days, TGF-β receptor II and surface IgA expression was determined by flow cytometry, while IgA and TGF-β secretion was assessed by enzyme-linked immunosorbent assay. Results**: **B cells endogenously expressed TGF-β receptor II and after 48 hours cultivation with mCD40L or mCD40L plus IL-10, both the expression of this receptor and the production of TGF-β were significantly increased. Notably, TGF-β levels following stimulation with mCD40L and IL-10 were higher than those produced by B cells stimulated with mCD40L alone. Furthermore, at day 7 and following IL-10 stimulation, there was a significant rise in the amount of IgA secretion by class-switched plasma cells, which was higher than stimulation with mCD40L alone. Conclusion**: **Our findings suggest that IL-10 can modulate TGF-β production and TGF-β receptor expression in mCD40-activated human B lymphocytes.**

Key words: immunoglobulin A, interleukin-10, transforming growth factor-beta, class switching

INTRODUCTION

The human transforming growth factor-beta (TGF-β) is a member of TGF-β superfamily comprised of 33 distinct but structurally similar proteins [1]. TGF-β1, TGF-β2, and TGF-β3 are three highly homologous isoforms of TGF-β in humans among which TGF-β1 is the prototypic member of the superfamily. Each TGF-β is secreted as a precursor, which forms a latent homodimer complex that has to be activated for eliciting biological effects [2]. First, TGF-β precursor is proteolytically processed and latency-associated peptide (LAP) in the N-terminal domain remains noncovalently associated with the C-terminal bioactive dimer. LAP prevents the binding of TGF-β to its specific cell surface receptor and activation process. Then proteolysis of LAP by proteases such as plasmin, Matrix metalloproteinase 2 (MMP2) and MMP9 or the action of binding proteins such as thrombospondin 1 (THBS1) generates mature TGF-β [3, 4].

TGF-β family members trigger their biological effects through assembling a hetero-tetrameric receptor complex of type I and type II dual specificity kinase receptors. Two type I receptor components are the main signal propagators while two type II receptor components are activators [1]. They share a receptor complex and signal in a similar way but their expression levels differ depending on the target tissue [5]. Formation of heteromeric complexes starts with binding of TGF-β1 to

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TBRII (TGF-β receptor 2)—due to its higher affinity than TBRI—which subsequently leads to the recruitment of TBRI (activin-like receptor kinase 5 (ALK 5)). TBRII is a constantly active kinase capable of binding TGF-β1 on its own, whereas TBRI kinase is only activated by transphosphorylation by TBRII through complex formation [6, 7].

TGF-β is produced by both immune and stromal cell types. The protein is an important regulator involved in a range of biological processes of different major cell behaviors and activities, including cell growth, differentiation, migration, adhesion, apoptosis, immune functions, extracellular matrix production, angiogenesis, hematopoiesis, wound repair, organogenesis, and mammalian embryogenesis [8-10]. Furthermore, TGF-β modulates differentiation, proliferation, and function of majority of lymphocytes. In each cell type and each context with different types of cytokines, different sets of genes and signaling responses are repressed or activated in response to TGF-β stimulation, leading to different cellular responses in each cell and its context [11].

Interleukin-10 (IL-10) is known as of the most important immune regulative cytokines. It was first identified as an immune response down regulator due to its ability to inhibit the production of many cytokines [12]. In addition, IL-10 is a pleiotropic cytokine affecting multiple biological functions. Although it has a regulatory effect on immune cells such as T cells, dendritic cells, and macrophages, it is not always inhibitory and can also promote B cell activation and NK cell proliferation [13]. It is reported that IL-10 increases human B cell survival and, when combined with IL-4, is considered an important cofactor in B cell proliferation [14]. Exogenous IL-10 promotes the differentiation of activated human B cells into IgM or IgG secreting plasmablasts and, TGF-β, exogenous IL-10 leads to IgA secretion [15]. B cell-derived IL-10 not only suppresses immune cell functions through paracrine mechanisms, but can also induce the differentiation of IL-10-secreting B cells into plasmablasts that secrete IgM and IgG in both autocrine and paracrine fashion [16]. IL-10 and TGF-β are both pleiotropic cytokines and in several contexts have similar and related roles. Stimulation of IgM and IgD bearing B cells with anti-CD40 antibody together with TGF-β or IL-10 can induce class switch recombination from IgM to IgA [17, 18]. The objective of this study was to investigate the regulation of TGF-β response during B cell stimulation by IL-10.

MATERIAL AND METHODS

Subjects and cells

Cells were obtained from healthy donors without any complications or immunologic defects. Human peripheral blood mononuclear cells were isolated with the use of standard Ficoll-Hypaque gradient method. Pan B cells were purified by negatively Pan B Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany). Negative purification was performed using 10 µL per 10^6 total cells of a cocktail of biotin-conjugated monoclonal anti-human antibodies against CD2, CD3, CD4, CD14, CD15, CD16, CD34, CD56, CD61, CD235a (Glycophorin A), and FceRIa for 10 min at 4 °C. After washing, 20 µL of Anti-Biotin MicroBeads were added per 10^6 total cells for 20 min at 4 °C. Beads and labeled cells (NK cells, T cells, dendritic cells, monocytes, granulocytes, platelets, and erythroid cells) were removed by LS column (Miltenyi, Biotec, Gladbach, Germany) and untouched Pan B cells processed fresh at the time of the experiment. Purity was routine >90% tested by Fluorescence-activated cell sorting (FACS). All the participants gave their consent, and the project has been reviewed and approved by the ethics committees of the Iran University of Medical Sciences (IUMS), Tehran, Iran.

Cultivation with multimeric human recombinant CD40L

$CD19^+$ B cells were seeded in 96-well plates in a final volume of 2×10^5 cells/200 µl/well in triplicate. Human B cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Paisley, Scotland), supplemented with 10% fetal bovine serum (Lymphosep, Biosera), penicillin (100 IU), streptomycin (100 ug/mL) (Biosera, Ringmer, East Sussex, UK) and Glutamax (Gibco, In-vitrogen Company). Then recombinant human IL-10 (15 ng/mL; R&D System, Abingdon, Oxon, UK), which was previously reconstituted by adding phosphate-buffered saline to the lyophylized powder and stored in 10 mL working aliquots, was added. The culture medium was supplemented with multimeric human recombinant CD40 ligand (mCD40L), 300 ng/mL from Miltenyi Biotec according to manufacturer's instructions (To form a multimer form; recombinant CD40L pre incubated at room temperature with cross-linking antibody for 30 minutes) alone and with recombinant human IL-10. The cells and supernatants were harvested after 48 hours and seven days of incubation at 37 °C in humidified 5% CO_2.

Quantification of supernatant levels of TGF-β and IgA

Cell culture supernatants were collected after 48 hours and seven days upon stimulation with/out IL-10, and TGF-β and IgA concentrations were assayed respectively in duplicate using commercially available enzyme-linked immunosorbent assay (ELISA) kit according to manufacturers' instructions: human TGF-β1 DuoSet ELISA kit (R&D systems, Minneapolis, MN, USA) and IgA human uncoated ELISA kit (Invitrogen, Waltham, MA, USA). The concentration of cytokines was calculated according to standard curves. A standard curve was obtained at each experiment to simultaneously quantify TGF-β and IgA concentrations.

Flow cytometric analysis

For surface staining, 1×10^5 cells were resuspended in 100 µl flow cytometry staining buffer. Harvested cells after two and seven days were incubated with Allophycocyanin (APC)-labeled anti-TGF beta Receptor II (clone REA903, Miltenyi Biotec),

phycoerythrin (PE)-labeled anti-CD210 (IL-10 receptor or IL-10R) (clone REA239, Miltenyi Biotec), PE-labeled anti-IgA (clone: IS11-8E10, Miltenyi Biotec) antibodies at optimal concentration for 20 min at 4 °C in the dark. All isotype control antibodies were purchased from Miltenyi Biotec to detect unspecific staining.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Iran University of Medical Sciences (IUMS), Tehran, Iran. Cases were recruited by obtaining a written informed consent.

Statistical analysis

The statistical analysis was performed using the SPSS software package, version 22 (SPSS Inc., Chicago, IL, USA). Values were expressed as frequency (number and percentage), mean (±SD) and median (IQR, presented as a range with 25th-75th percentiles) as appropriate. The Shapiro-Wilks test was used to estimate whether data were normally distributed. Parametric and nonparametric analyses were performed based on the findings of this test. A *P*-value of 0.05 or less was considered significant.

RESULTS

IL-10 receptor I expression

To determine whether IL-10 receptor I (IL-10R1) was expressed in all healthy individuals, we examined the IL-10R1 expression on pan B cells after MACS separation by flow cytometry before stimulation. *Figure 1* shows the representative flow cytometric histograms of IL-10R1 expression on B cells. We found that IL-10-R1 is expressed by >95% of B cells (range 90-98%) in all the participants, although it is shown at measured levels of only a few hundred per cell [19, 20].

TGF-β receptor II assay

Given that TGF-β receptor II (TBRII) but not TBRI is constantly activated and has an ability to bind to TGF-β1, we analyzed the expression of TBRII in B cells to directly assess the effect of IL-10 stimulation on TGF-β receptor expression. The median (IQR) of endogenous TBRII expression on B cells was 14.0 (6.29-15.95) *(figure 2A)*. We also observed that after 48-hour cultivation with mCD40L or mCD40L plus IL-10, B cell expression of TBRII increased significantly [28.4 (22.2-44.0), $P < 0.001$ and 30.4 (26.3-45.8), $P < 0.001$, respectively]. Although IL-10/mCD40L improves TBRII expression level as compared with isolated mCD40L stimulation, the difference was not statistically significant ($P = 0.489$). Of note, an increase in the stimulation dosage of IL-10 has a negative effect on B cell TBRII surface expression level *(figure 2C)*.

TGF-β secretion assay

In order to quantitate total TGF-β1 level in media conditioned by cultured pan B cells, we used a commercially available ELISA kit. The median (IQR) of the total TGF-β1 level under treatment with mCD40L and IL-10 was higher than mCD40L alone in supernatant [692 (IQR 637-756) ng/ml *versus* 593 (IQR 484-746) ng/ml, $P = 0.095$] *(figure 2B)*. A dose-independent relationship was obtained repeatedly at TGF-β1 concentration from 15 to 100 ng/ml of IL-10 stimulation *(figure 2C)*. Because of apoptosis of B cells in the absence of necessary stimulators, in unstimulated cells cultured with medium alone there was no detectable TGF-β (data not shown).

IgA secretion and expression assay

We next studied how IL-10 influences B cell differentiation into surface IgA^+ B cell and IgA secretion. Following seven days' incubation in the presence and

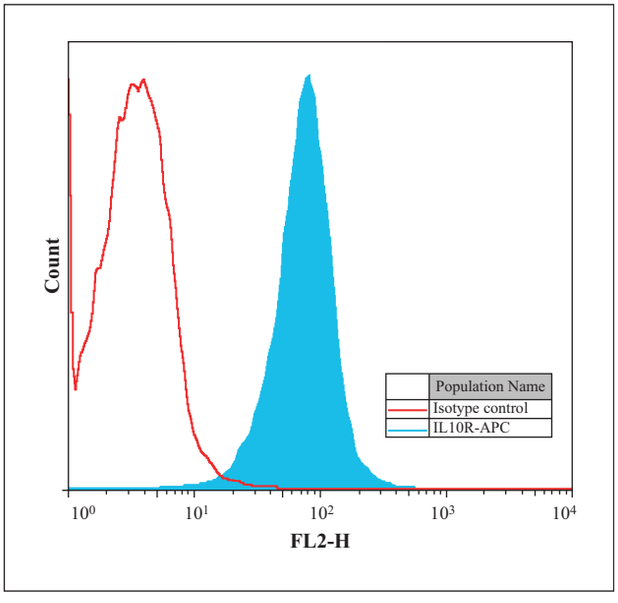

Figure 1
Representative flow cytometry histograms for analysis of IL-10 receptor (IL-10R) expression on B cells.

absence of rIL-10, IgA secretion and surface IgA^+ B cells expression by mCD40-activated freshly isolated B cells were examined. IL-10 stimulation gave rise to a higher amount of surface IgA^+ B cells than cultures stimulated with mCD40L alone [10.25 (IQR 5.67-12.8) *versus* 2.05 (IQR 1.72-3.25), $P = 0.003$]. Moreover, the level of IgA in the presence of IL-10 was higher than cultures stimulated with mCD40L alone [400 (IQR 115-850) ng/ml *versus* 210 (IQR 65-335) ng/ml, $P = 0.001$]. These results indicate that IL-10 has an essential role in differentiation and/or secretion of IgA *(figure 3)*.

DISCUSSION

In this study, we aimed to evaluate TGF-β secretion and TGF-β receptor expression following the stimulation of $CD19^+$ B cells of healthy individuals with mCD40L and rIL-10 + mCD40L and proposed/postulated that the presence of rIL-10 and mCD40L in the culture medium of these cells results in TGF-β upregulation. We showed that nearly all peripheral isolated B lymphocytes express IL-10 receptor. Additionally, we demonstrated that TGF-β concentration in the supernatants of $CD19^+$ B lymphocytes treated with mCD40L alone is significantly lower than that of with IL-10 and mCD40L. Finally, we investigated the effect of B cell stimulation by mCD40L with or without IL-10 on TGF-β production and endogenous expression of TGF-β receptor II.

It is well documented that IL-10 could be produced by various cell types of the microenvironment such as monocytes, $CD8^+$ T cells, TH2 cells, and different subsets of B cells [16, 21]. Our data proposed whether IL-10 is produced endogenously by cells located in the germinal center or is added in an exogenous fashion, it significantly exerts its effect on B lymphocyte and TGF-β secretion. TGF-β has a potent role in Ig class switching after B cell activation. During this process, TGF-β directs IgA production by triggering germline Igα transcription genes [22]. It is well reported that CD40 engagement induces B cell endogenous TGF-β expression and facilitates sequential DNA recombination of Sμ>Sγ and Sγ>Sα [23]. Furthermore, CD40-CD40L interaction initiates/induces cellular responses such as an increase in the antigen uptake by B cells, major histocompatibility complex class II(MHC II) upregulation or secretion of proinflammatory cytokines which results in the class switching of the four types of immunoglobulins [14, 24].

IgA 1 and IgA 2 are two subtypes of IgA in humans that are encoded by two distinct Cα1 and Cα2 genes.

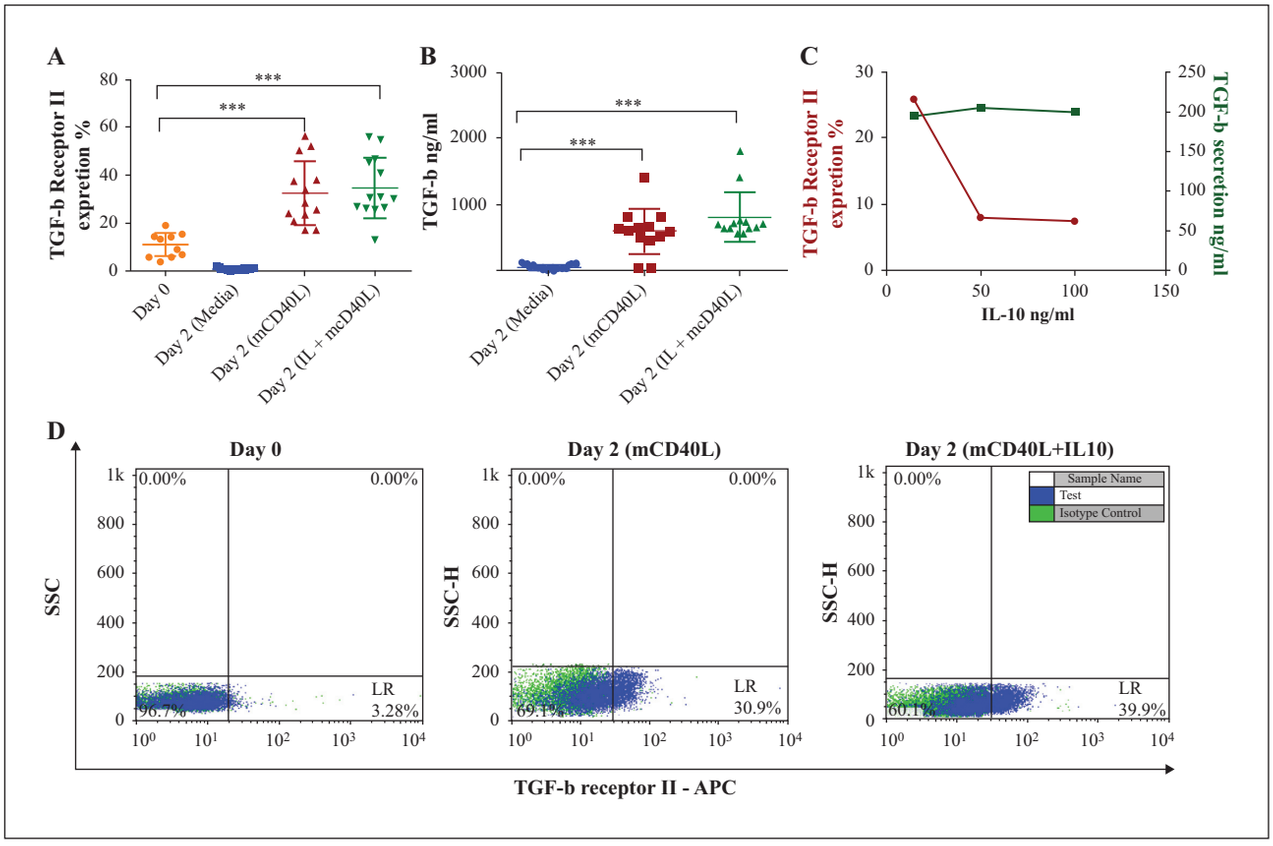

Figure 2

Induction of TGF-β production and TGF-β receptor II expression in B lymphocytes negatively separated with MACS. **(A)** TGF-β receptor II expression and **(B)** Total TGF-β level were measured after 48 hours of B cells (2×10^5 in 200 µl) treated with multimeric human recombinant CD40L (mCD40L) (300 ng/mL) plus recombinant IL-10 (15 ng/mL) (IL-10 + mCD40L) and without any treatment (media) from 15 healthy donors. **(C)** B cells were incubated with various concentrations of recombinant IL-10 for two days. There is not a dose-dependent response in TGF-β receptor II expression by IL-10 between 15 and 100 ng/ml but a dose-dependent relationship was obtained repeatedly at TGF-β1 concentration. **(D)** Representative expression of TGF-β receptor II on B lymphocytes at days 0 and 2 after negative MACS separation. Quadrant markers were positioned to define B cells that express TGF-β receptor II (lower right [LR]).

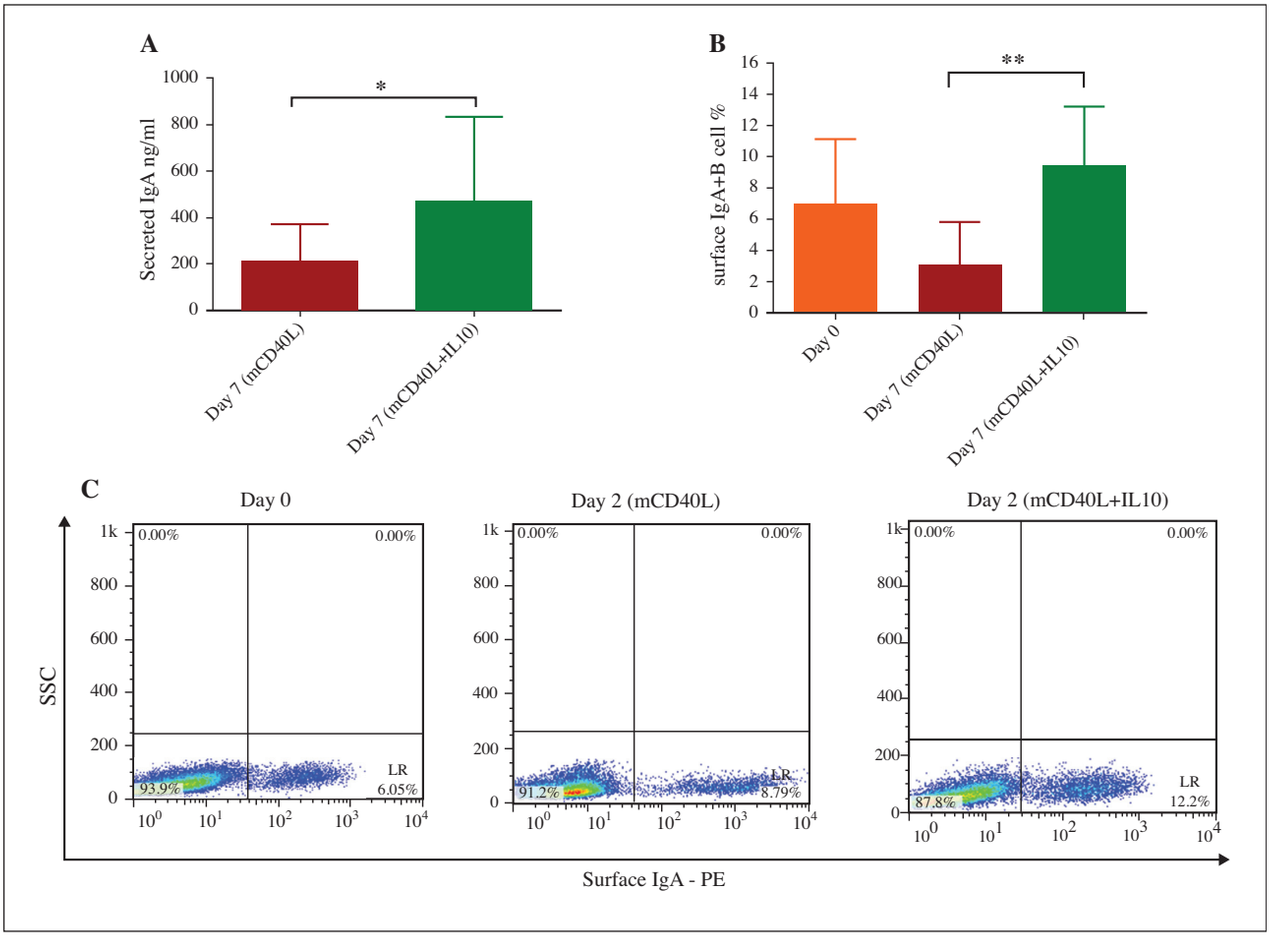

Figure 3
(A) IgA secretion and **(B)** surface IgA$^+$ B cells were measured by ELISA and Flow cytometry at days 0 and 7 from B cells negatively separated by MACS and cultured in multimeric human recombinant CD40L (mCD40L) (300 ng/mL) alone or in the presence of IL-10 (15 ng/mL). **(C)** Representative expression of surface IgA on B lymphocytes after negative MACS separation. Cell surface expression of IgA is presented on a 4-decade log scale as dot plots of the correlated x-axis and y-axis fluorescence. Quadrant markers were positioned to define surface IgA$^+$ B cells (lower right [LR]).

They have seemingly the same receptor-binding profile, but different distribution in body. IgA 1 is mostly present in the circulation while IgA 2 is particularly abundant at mucosa and sites of microbiota colonization. B cells acquire IgA secretion through class switch recombination, the process by which the IgH constant region μ (Cμ) substitutes with Cα [25]. Studies have shown that TGFb induces Iα1-Cα1 and Iα2-Cα2 germline transcription; however, VHDJH-Cα1 and VHDJH-Cα2 mature transcription occurs by the engagement of costimulatory signals like CD40 [26]. Furthermore, IL-10 contributes in the events leading to IgA isotype selection; however, studies regarding the effect of IL-10 in CSR of each IgA isotype are incomplete [17].

Studies regarding the effect of IL-10 on class switching toward IgA are controversial. While some studies have shown that IgA secretion in sIgD$^+$ CD40L-Bcells is contingent on the presence of IL-10 [14, 17], Zan *et al.* reported that only TGF-β and, not IL-10, is sufficient to mediate switching to IgA in CD40L activated B cells [23]. They investigated TGF-β and IL-10 effects on IgA secretion by neutralizing them, and demonstrated that only by blocking TGF-β, IgA secretion is ablated [23]. However, none of the mentioned studies discussed the reasons and probable mechanisms by which IL-10 affected their experiments. With that being said, one of the major points of the present study was to accent the role of IL-10 in modulating post-activation functions of B cells. We have shown that adding IL-10 to the cultures treated with mCD40L, increases the TGF-β concentration and TBRII expression compared with cultured with mCD40L alone. Zan *et al.* also demonstrated that IgA secretion was higher when CD40L cells were treated with IL-10. They cultured CD40L cells with IL-10 and TGF-β separately and once together. Furthermore, the synergistic effect of these two cytokines was also well depicted when the percentage of cells with surface IgA increased upon treatment with both IL-10 and TGF-β [23]. These results are consistent with our observation that IL-10 would amplify TGF-β expression and functions. Of note, according to their experiments, using neutralizing antibody, anti-TGF-β hampers IgA secretion even in the presence of IL-10. Therefore, they indicated that IL-10 is not a substitute for TGF-β and that TGF-β was the substantial cytokine which upon blocking, the whole process of IgA production can be affected. While in the present study we have argued that IL-10 might play its role through regulating TGF-β expression and its receptor, this could be explained by the fact that neutralizing TGF-β not only blocks TGF-β

functions but also would cease effect of IL-10 in IgA class switching indirectly. In addition, we conducted our experiment on human $CD19^+$ B cells while Zan *et al*. used CL-01 B cells, a monoclonal model of B cells bearing surface IgM/D which mimic human germinal center differentiation. It has been shown that B cell stimulation with CD40L causes autocrine secretion of IL-10, so it can shape a positive loop for an increase of TBRII expression and TGF-β secretion on B cell.

TGF-β receptors are comprised of two transmembrane subunits called TBRI and TBRII. Signaling through these receptors is initiated by ligand binding to TBRII, which then recruits TBRI to the complex and propagates downstream cascade. Hematopoietic cells including macrophages and B cells express TGF-β receptor I and II [22], which can be upregulated by *in vitro* stimulation [27]. In this respect, we demonstrated that when B cells are stimulated through their CD40 antigen, TGF-β receptor expression was increased. We also tested the cellular effects of IL-10 by exposing B cells previously harvested with CD40L to desired amounts of exogenous IL-10 and measured the expression of TBRII. The results showed an increase in TBRII concentration more than that of when treated with CD40L alone. However, the difference was not significant. In contrast, Cottrez and Groux have shown that addition of IL-10 to resting and activated human $CD4^+$ T cells significantly enhances TBRII expression in these lymphocytes, suggesting the modulatory effect of IL-10 in TGF-β responses [28].

IL-10 is known as pleiotropic cytokine acting in both paracrine and autocrine manners either to suppress the inflammation or to regulate the differentiation of an activated B cell respectively [16]. Originally called cytokine synthesis inhibitory factor (CSIF), IL-10 is considered to amplify the humoral response by triggering DNA replication in B lymphocytes activated by CD40 antigen or BCR and the consequent secretion of IgG, M, and A [14]. Binding IL-10 to its specific receptor upon secretion is essential. IL-10R is a heterotetramer complex comprised of two IL-10R1 and two IL-10R2 subunits. While IL-10R1 serves as the binding subunit, IL-10R2 is responsible for the signal transduction of the complex. IL-10R1 is expressed at the basal level on most hematopoietic cells but can be upregulated upon activation [29]. In order to confirm that IL-10R1 is expressed on B lymphocytes of all the cases presented in this study and to make sure none were IL-10R1-deficient, we analyzed IL-10R1 expression on pan B cells of every individual. Our flow cytometer analyses showed that IL-10R1 was approximately expressed by all B lymphocytes of each participant with the minimum amount of receptors per cell.

The association between IL-10 and TGF-β with various clinical manifestations in different diseases has been shown [30-32]. Both IL-10 and TGF-β play an important role in inhibiting effector cells and skewing immune responses to tolerogenic states. Other than their cooperative function on IgA production in B cells, it is plausible that IL-10 can negatively regulate Th1, Th17, dendritic cells, and other effector cells in the microenvironment by upregulation of TBRII through the same proposed mechanism. Collectively, our findings suggest that IL-10 can modulate TGF-β production and TGF-β receptor expression in mCD40-activated human B lymphocytes, but provide no clue on the underlying molecular basis. Further studies on the genomic, transcriptional, or signaling levels may provide better insights on how IL-10 upregulates TGF-β responses.

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