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ORIGINAL ARTICLE

Evaluation of effective factors on IL-10 signaling in B cells in patients with selective IgA deficiency

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Abstract. Background: **Selective IgA deficiency is the most prevalent form of primary immunodeficiencies. The pathogenesis of the disease is still unknown. Several studies have suggested a defect in B cell responses to IL-10; however, the main reason for this defect has not been reported. Elucidating IL-10 signaling defects and their correlation with clinical manifestations could be helpful for better understanding and treatment of the disease. Methods**: **In this study, 15 SIgAD patients and 15 age- and sex-matched healthy controls were included. Surface expression of transforming growth factor β receptor II (TGF-β RII), IL-10R and IgA was assessed by flow cytometry in human purified B cells before and after stimulation by IL-10. Protein expression of STAT3, p-STAT3 and SOCS3 was measured by Western blotting analysis. TGF-β and IgA secretion was evaluated by ELISA. Finally, the measurement of B cell apoptosis was performed by flow cytometry. Results**: **The TGF-βRII expression level was decreased after stimulation with IL-10 in patients compared with controls. Notably, the TGF-β level were higher after stimulation with mCD40L and IL-10 in the control group as compared to stimulation with mCD40L alone. The IgA^+ B cell percentage and IgA secretion levels were significantly increased in controls as compared with SIgAD patients. The relative concentration of the total STAT3 was decreased as compared with controls. Conclusion**: **The defect in IgA production in SIgAD patients could be due to inadequate B cell responses to IL-10 stimulation that probably originate from defective regulation of IL-10-mediated TGF-b 'symbol' production TGF-β response by IL-10. Furthermore, it is suggested that the absence of STAT3 protein baseline expression could impair cytokine-mediated signaling such as thatinduced by IL-!0 and IL-21.**

Key words: selective IgA deficiency, B cell, IL-10, STAT3, TGF-β, apoptosis

INTRODUCTION

Selective immunoglobulin A deficiency (SIgAD) is the most prevalent form of primary immunodeficiencies. The majority of SIgAD patients are asymptomatic, but some of them show various clinical manifestations such as recurrent sinopulmonary infection, allergy, autoimmunity and malignancy [1]. The pathogenesis of the disease is still unknown, but some studies indicate that patients with SIgAD might have defects in one of the processes of IgA class switch recombination (CSR), production and secretion of antibody or survival of IgA^+ memory B cells and IgA producing plasma cells [2-4]. The differentiation of B lymphocytes into IgA producing plasma cells requires appropriate signaling induced by the cytokine network. Cytokine profile could be dysregulated due to aberrant interactions of B cells, T cells or dendritic cells. Transforming growth factor-β (TGF-β) is the main cytokine in IgA CSR that functions concomitantly with other interleukins such as IL-2, IL-4,

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IL-5, IL-6 and IL-10 [5, 6]. IL-10 is the most important regulatory cytokine of the immune system. Despite its inhibitory effects on pro-inflammatory cytokine secretion or T cell activation, IL-10 can lead to B cell activation and NK cell proliferation [7]. IL-10 increases the survival of B cells and serves as an important cofactor in B cell proliferation [8]. Both IL-10 and TGF-β are pleiotropic cytokines with similar roles in some immunological processes. Stimulation of IgM- and IgD- producing B cells with anti-CD40 and TGF-β or IL-10 may induce CSR from IgM toward IgA production [9, 10].

IgA CSR and IgA secretion occurs through TGF-β-related mechanisms. Studies on mouse models have demonstrated that selective lack of TGF-β receptors on B cells could be involved in defects in IgA production [11, 12]. There is a possibility that a defect in upregulation of TGF-β cytokine and its receptor might lead to IgA deficiency and that IL-10 could be a precursor for TGF-β II receptor (TBRII) and CSR signaling [13]. Several studies have reported a defect in B cell responses to IL-10 in SIgAD patients [14-16]; however, the main reason for this defect has not been clarified. Given that the pathogenesis of this disease is not fully understood, elucidating IL-10 signaling defect(s) and its correlation with clinical manifestations could help for better diagnosis and treatment of affected individuals. The aim of this study was to investigate the IL-10 signaling pathway in B lymphocytes of SIgAD patients and its effect on IgA CSR.

MATERIAL AND METHODS

Patients

In this study, 15 SIgAD patients and 15 age- and sex-matched healthy controls were included. Symptomatic patients with SIgAD who were referred to Children's Medical Center Hospital (Tehran, Iran) and diagnosed by specialist physicians were included in the study. The diagnosis of SIgAD was based on European Society for Immunodeficiencies criteria including undetectable serum IgA or less than 0.07 g/L but normal serum IgG and IgM, exclusion of secondary causes of hypogammaglobulinemia, normal IgG antibody response to all vaccinations and no T-cell defect [17]. To ensure that transient forms of SIgAD are excluded from the study, patients over 4 years old were recruited. For the control group, healthy age- and sex-matched individuals were enrolled. The patients were divided into two groups of mild and severe: patients with severe infections (*e.g.*, bloodstream, CNS, and deep-seated infections such as osteomyelitis and arthritis), autoimmunity or malignancy were categorized in the severe group and others were considered as mild patients. This study was approved by the Ethics Committee of the Tehran University of Medical Sciences, and informed consent was obtained from all patients or their legal guardians.

B cell isolation

Human peripheral blood mononuclear cells were isolated using standard Ficoll-Hypaque gradient method. Pan B cells were purified negatively by a Pan B Cell Isolation Kit (Miltenyi Biotec, Germany). Negative purification was performed using 10 µL per 10^6 total cells of a cocktail of biotin-conjugated monoclonal antihuman antibodies against CD2, CD3, CD4, CD14, CD15, CD16, CD34, CD56, CD61, CD235a (Glycophorin A) and FCERIA according to manufacturer's protocol. Untouched pan B cells were freshly isolated at the time of the experiment. The purity of >90% was obtained routinely by fluorescence-activated cell sorting.

B cell stimulation

$CD19^+$ B cells were seeded in 96-well plates in a final concentration of 2×10^5 cells/200 µl per well in triplicate. Isolated B cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Scotland), supplemented with 10% fetal bovine serum (Lymphosep, Biosera), penicillin (100 IU), streptomycin (100 ug/mL) (Biosera, UK) and Glutamax (Gibco, Invitrogen, USA). The culture medium was supplemented by multimeric human recombinant CD40 ligand (mCD40L) (300 ng/mL, Miltenyi Biotec) alone and with recombinant human IL-10 (15 ng/mL; R&D Systems, UK). To form a CD40L multimeric form, monomeric CD40L was incubated with a cross-linking antibody for 30 minutes to create a native multimeric form of CD40L.

Quantification of supernatant levels of TGF-β and IgA

Supernatants were collected from cell culture after 48 hours and 7 days upon stimulation with and without IL-10, and, subsequently, TGF-β and IgA concentrations were assayed using commercially available enzyme-linked immunosorbent assay (ELISA) kits, namely, total human TGF-β1 DuoSet ELISA kit (R&D systems) and IgA human uncoated ELISA kit (Invitrogen). The concentration of cytokines was calculated based on 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 B cells were resuspended within 100 µl flow cytometry staining buffer. After 2 and 7 days, harvested cells were incubated by allophycocyanin-labelled anti-TBRII (clone REA903, Miltenyi Biotec), phycoerythrin (PE)-labelled anti-CD210 (IL-10 receptor or IL-10R) (clone REA239, Miltenyi Biotec) and PE-labelled anti-IgA (clone IS11-8E10, Miltenyi Biotec) antibodies at the optimal concentration for 20 minute at 4 °C in the dark. All isotype control antibodies were purchased from Miltenyi Biotec to detect unspecific staining.

Apoptosis assay

To evaluate spontaneous or induced apoptosis of B cells in SIgAD patients and healthy control, fluorescein

isothiocyanate (FITC) Annexin V Apoptosis Detection Kit with PI (Biolegend, USA) was used. In each tube, 50,000 events were collected. Expression of annexin V in unstimulated B cells was measured after 6 days in the medium alone. For the measurement of induced apoptosis, purified B cells were cultured and stimulated with mCD40 and IL-10. B cells were pelleted by centrifugation and resuspended in binding buffer and stained with annexin V-FITC for 15 minute at room temperature in the dark. Then, B lymphocytes were analyzed by flow cytometry.

Western blotting analysis

The expression levels of STAT3, pSTAT3 and SOCS3 were measured using Western blotting analysis. Briefly, Pan B cells were unstimulated or stimulated with CD40L and IL-10 for 15 minutes and then harvested by RIPA buffer containing protease inhibitor cocktail, phosphatase inhibitor cocktail and PMSF (Santa Cruz, Sc-24948). A total of 25 μg of protein was loaded onto a 8% polyacrylamide gradient gel at 100 v and electrotransferred onto polyvinylidene difluoride membranes for 70 minutes at 100 v in 20% methanol transfer buffer. Membranes were subsequently incubated with polyclonal antibodies against STAT3 (Stat3α 86 kDa and Stat3β 79 kDa), p-STAT3, SOCS3 (25 kDa) and β-actin (45 kDa) as a housekeeping protein. They were then incubated with horseradish peroxidase-conjugated (HRP) labelled goat anti-rabbit. The signal was detected by chemiluminescence using the electrochemiluminescence (ECL) Plus™ Western Blotting detection system and evaluated by densitometry (Image J software).

Antibodies: Phospho-Stat3 (Tyr705) antibody, Cell Signalling Technology, Cat#9131. Stat3 antibody, D3Z2G, Cell Signaling Technology, Cat#12640. Anti-SOCS3 antibody abcam, Cat#16030. Anti-β Actin antibody abcam, Cat#8227. Goat Anti-Rabbit IgG H&L (HRP) Abcam Cat#205718.

Statistical analysis

The statistical analysis was performed using SPSS software package, version 22 (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk test was used to estimate whether data were normally distributed. Independent sample *t*-test was performed for analysing data with normal distribution, while Mann–Whitney *U* test was used for data that are not normally distributed. To evaluate quantitative expressions of SOCS3, STAT3 and p-STAT3 proteins compared with β-actin housekeeping protein, Image J software was used. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of patients

Fifteen symptomatic SIgAD patients and 15 controls (nine males and six females per group) were investigated in this study. The median (interquartile range [IQR]) age of patients at the time of the study was 14 (5-29) years. The median (IQR) age at the onset of symptoms was 4 (2-7) years, and the median (IQR) age at the time of diagnosis was 8 (4-23) years. Nine patients (60%) were results of a consanguineous marriage. Demographic and immunological charac-

Table 1
Demographic and immunological characteristics of SIgAD patients.

Patient	Age (years)	Sex	Consanguinity	First presentation	IgG (mg/dl)	IgA (mg/dl)	IgM (mg/dl)	IgE (mg/dl)
1	14	M	Yes	Pneumonia	843	0	82	0
2	12	M	No	Oral plague	1460	7	121	4
3	29	F	Yes	Motion disorder	2090	<7	263	1
4	9	M	Yes	None	854	4	51	7
5	28	M	Yes	Pneumonia	1440	<3	40	1
6	10	F	No	Cough	1179	<7	159	33
7	12	F	No	Diarrhea	2000	0	52	75
8	22	F	No	Shortness of breath	480	0	240	220
9	10	M	Yes	Diarrhea	1201	2	67	29
10	17	M	No	Recurrent infections	1655	5	97	15
11	39	M	Yes	Sinusitis	1813	0	50	32
12	9	M	Yes	Cough	1333	3	222	170
13	5	F	Yes	Recurrent infections, Sinusitis	12.86	0.3	49	75
14	16	M	Yes	Cough	1186	4	134	38
15	9	M	No	Common cold	1809	4	181	1113

Abbreviations: M: male; F: female; mg/dl: milligram/deciliter.
IgA normal range: 4-10 years: 41-297 mg/dl; 11-13 years: 44-395 mg/dl; adults: 70-400 mg/dl.
IgG normal range: 1-11 years: 650-1410 mg/dl; adults: 800-1700 mg/dl.
IgM normal range: 1-11 years: 55-210 mg/dl; adults: 50-370 mg/dl.
IgE normal range: 4-9 years: up to 90 IU/ml; 10-15 years: up to 200 IU/ml; adults: up to 100 IU/ml.

Table 2
Clinical manifestation in SIgAD patients.

Parameter	Number of patients (%)
Recurrent infection	6 (40%)
Upper respiratory infections	10 (66%)
pneumonia	7 (46%)
Sinusitis	8 (53%)
Otitis	4 (26%)
Autoimmune diseases	1 (6.6%)
Allergy	7 (46%)
Oral ulcer	2 (13.3 %)
Diarrhea	2 (13.3 %)
Recurrent urinary tract infections	1 (6.6%)
Eyes infection	3 (20 %)
Neurologic disorder	2 (13.3 %)
Dermatologic disorder	4 (26%)
Thyroid disorder	1 (6.6%)
Enteropathy	1 (6.6%)
Seizure	1 (6.6%)
Rickets	1 (6.6%)

teristics of SIgAD patients are summarized in *table 1*. The most common clinical manifestations among patients included recurrent infections, allergy, sinusitis, pneumonia, otitis media and recurrent infections (*table 2*). Nine patients (60%) were classified as mild phenotype and six (40%) patients were grouped as severe phenotype.

Expression of IL-10R1 and TBRII

We evaluated IL-10R1 expression using flow cytometry after isolation of B cell with magnetic-activated cell sorting (MACS). Almost all B cells of participants expressed IL-10R1 (>90%) (*figure S1*) and no significant difference was observed between controls [median (IQR) = 91% (89-96)] and patients [median (IQR) = 93% (85-94)]. TBRII is constantly expressed, unlike TBRI, and it can bind to TGF-β1 [18]. Baseline expression of this receptor on B cells in controls was higher [median (IQR) =14% (6.2-15.9)] than in the patients [median (IQR) = 8.7% (3.7-11)] ($P = 0.06$). After stimulation with mCD40L, TBRII expression was significantly increased both in controls [median (IQR) = 28.4% (22.2-44)] and in patients [median (IQR) = 22% (7.6-39)]. In addition, after stimulation of B cells with mCD40L + IL-10, TBRII expression was significantly increased both in controls [median (IQR) = 30.4% (26.3-45.8)] and in patients [median (IQR) = 22.4% (9.3-33)] than media in control [median (IQR) = 12.2% (7.5-14)] and patients [median (IQR) = 9.5% (5.8-12.4)] (*figure 1A, B, D*). This increase in TBRII expression was not significant between stimulation with mCD40l only and mCD40L + IL-10 (*figure 1A, 1B, D*). However, stimulated TBRII expression by mCD40L + IL-10 was significantly lower in the patients than controls ($P = 0.03$) (*figure 1C*). When the effect of stimulant's concentration on TBRII expression was evaluated, we observed that TBRII expression was decreased in high concentrations of IL-10 (15–100 ng/ul).

TGF-β secretion

The concentration of TGF-β was significantly increased in both controls [median (IQR) = 593 pg/ml (484-746)] (P = 0.01) and patients [median (IQR) = 585 pg/ml (792-1200)] (P = 0.004) after 48 hours of B cell stimulation by mCD40L .In addition, the concentration of TGF-β was significantly increased in both controls [median (IQR) = 692 pg/ml (637-756)] (P = 0.001) and patients [median (IQR) = 654 pg/ml (600-1392)] (P = 0.02) after 48 hours of stimulation by mCD40L + IL-10 (*figure 2*). The evaluation of the effect of IL-10 on TGF-β secretion showed that IL-10 significantly increased TGF-β secretion in the controls (P = 0.01), but this increase was not significant in patients ($P = 0.97$). Serum levels of TGF-β were also measured in both controls [8.1 ng/ml (IQR = 4.2-20)] and patients [8.4 ng/ml (IQR = 12-19)], showing no significant difference ($P = 0.69$) (*figure 3A*). The effect of IL-10 concentration on TBRII expression and TGF-β secretion was evaluated in three control cases. Although TGF-β secretion was not altered in response to the augmentation of IL-10 concentration, the surface expression of TBRII was decreased in higher levels of IL-10 concentration (*figure 3B*).
Comparison of TGF-β secretion between patients with consanguineous and non-consanguineous parents did not show a significant difference when treated with mCD40L or with mCD40L + IL-10. In the same condition, the comparison of TGF-β secretion between patients with mild and severe phenotypes did not show a significant difference.

Surface expression of IgA

The effect of IL-10 on the differentiation of B cell to IgA^+ B cells was evaluated. Prior to stimulation with IL-10, the percentage of IgA^+ B cells was measured. Then, surface IgA level was measured again 7 days after B cell stimulation with or without IL-10. The expression of surface IgA before stimulation was significantly higher in control group [median (IQR) = 7.4 pg/ml (2.8-10.5)] compared with SIgAD patients [median (IQR) = 0.55 pg/ml (0.28-0.78)] ($P < 0.001$).
The percentage of IgA^+ B cells did not increase significantly in both controls ($P < 0.62$) and patients ($P < 0.59$) 7 days after isolated stimulation with mCD40L. However, after stimulation with mCD40L + IL-10, the percentage of IgA^+ B cells was significantly increased in the control group [median (IQR) = 10.2 pg/ml (5.6-12.8)] ($P = 0.01$), but this increase was not significant in patients [median (IQR) = 0.1pg/ml (0.3-2.5)] ($P = 0.32$) (*figure 4A, B*). Stimulation of cells with only IL-10 significantly increased IgA^+ B cell percentage in the control group ($P = 0.01$), but the increase was not significant in patients ($P = 0.41$). As shown in *figure 4C*, the difference was significant between patients and controls in all treatments.

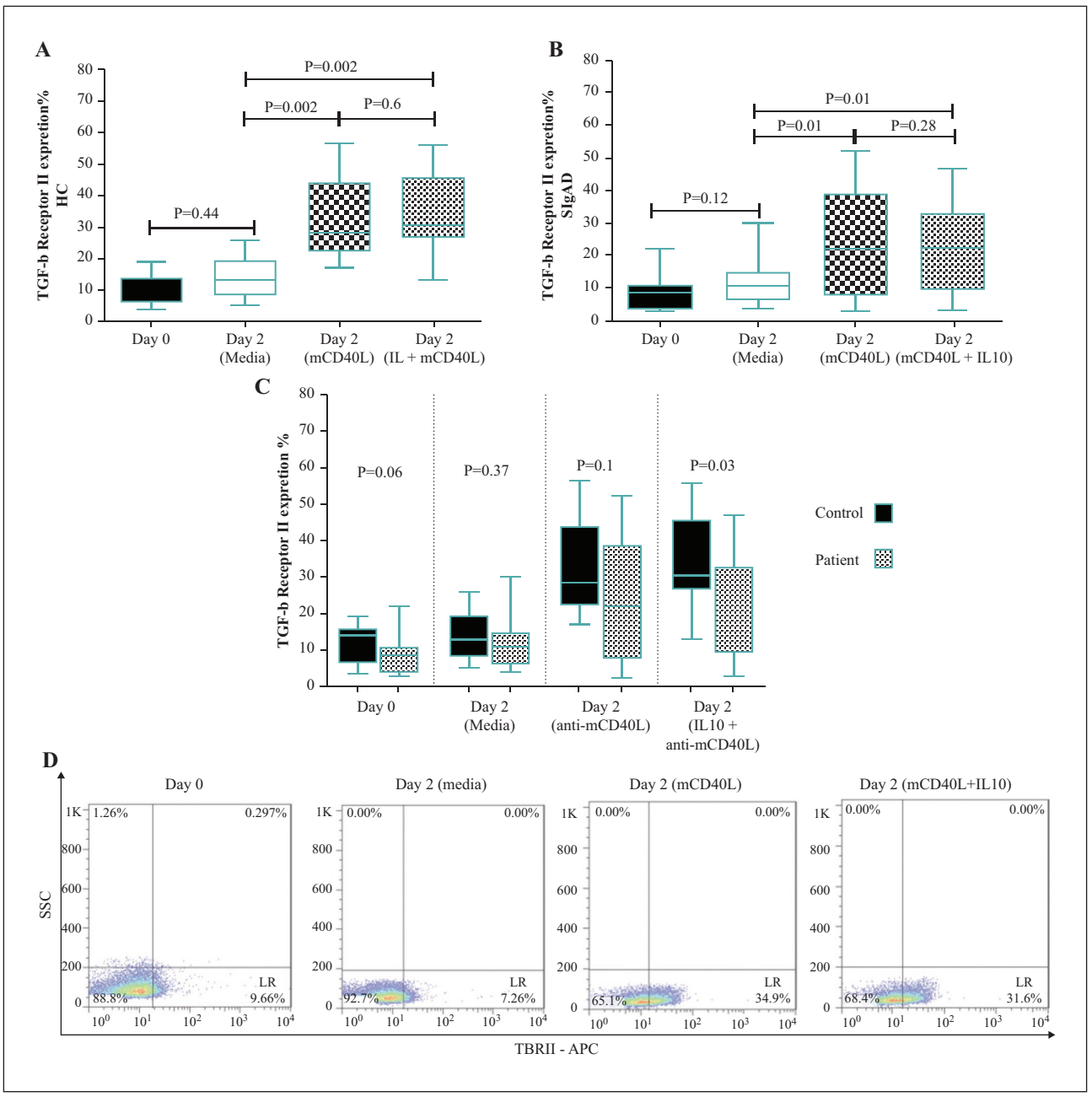

Figure 1

Expression of TBRII on B cells in controls (A) and patients (B) before stimulation and after culture in the presence of mCD40L alone, media or a combination of mCD40L and IL-10 for 24 h. (C) Comparison of TBRII expression in controls and patients before stimulation and after culture in the presence of mCD40L alone, media or a combination of mCD40L and IL-10 for 24 h. (D) Representative flow cytometric pattern for TBRII expression from patient data (LR: lower right).

IgA secretion

After 7 days of B cell stimulation by mCD40L, the concentration of secreted IgA in both patients [median (IQR) = 70 ng/ml (210-325)] and controls [median (IQR0 = 585 ng/ml (795-1200)] was assessed and no significant difference in stimulated cells compared with wells containing only media was seen. But stimulation with mCD40L + IL-10 has significantly increased the concentration of secreted IgA in both patients [median (IQR) = 400 ng/ml (135-845)] ($P = 0.002$) and controls [median (IQR) = 654 ng/ml (600-1392)] ($P = 0.02$) (*figure 5A, B*). The concentration of IgA in all treatments showed a statistical significance difference between patients and controls (*figure 5C*).

When IgA secretion was evaluated between patients with related and non-related parents, no significant difference was observed. Also, no statistical difference was observed between mild and severe phenotypes for IgA secretion (data not shown).

B cell apoptosis

Apoptosis of stimulated B cells in healthy controls [median (IQR) = 53 (49-70)] demonstrated a significant decrease compared with spontaneous apoptosis of non-switched B cells in these individuals [median (IQR) = 92 (86-95)] ($P = 0.05$). Similarly, apoptosis of stimulated B cells in SIgAD patients [median (IQR) = 43 (24-60)] was significantly lower compared

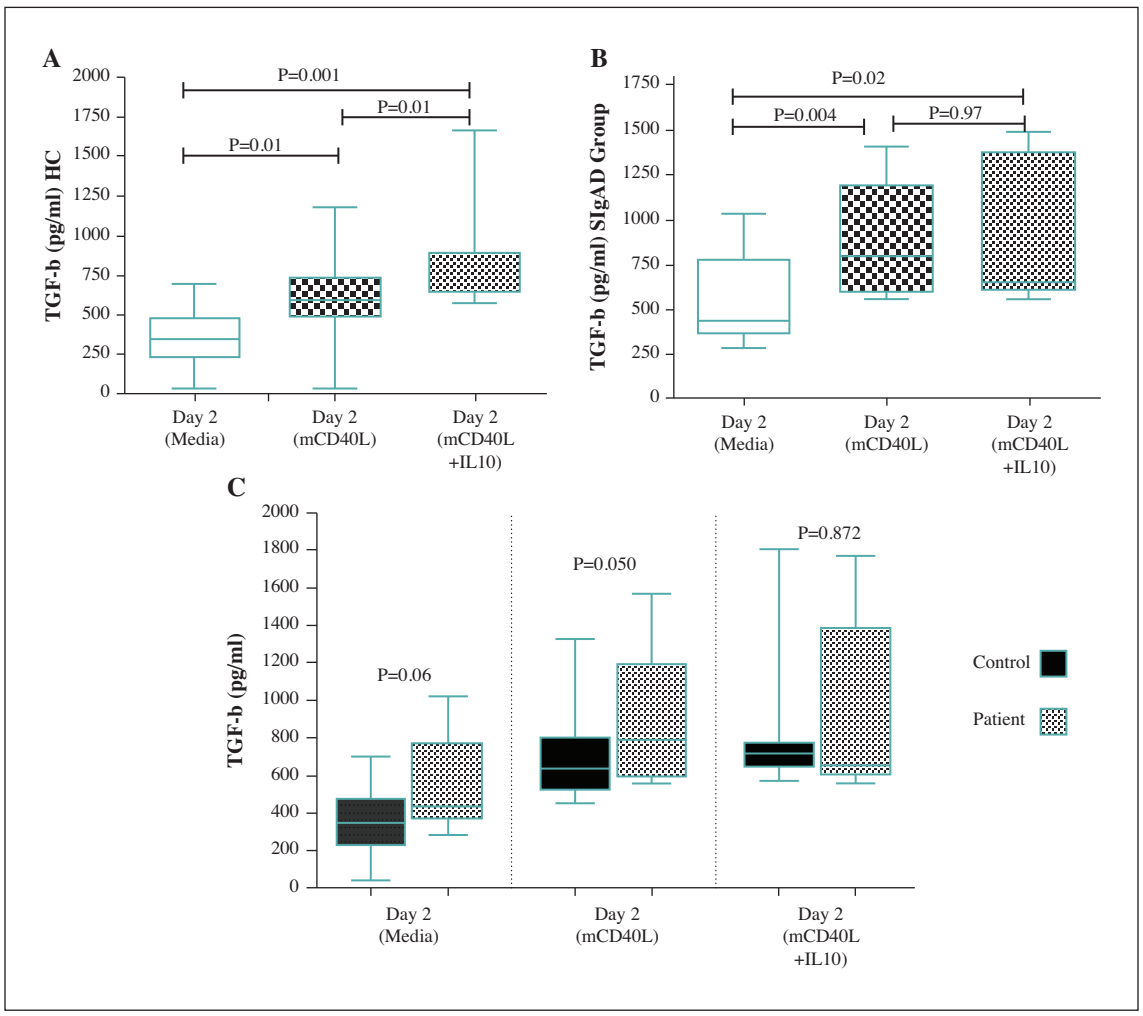

Figure 2

Concentration of TGF-β secretion by B cells in control (A) and patients (B) after cultured in the presence of mCD40L alone, media or a combination of mCD40L and IL-10 for 24 h. Comparison of TGF-β secretion by B cells in study groups when treated with mCD40L and IL-10 (C).

to their spontaneous apoptosis of B cells [median (IQR) = 92 (86-95)] ($P = 0.006$). Comparisons of apoptosis between patients and controls showed no significant difference (*figure 6*). Furthermore, no significant difference was observed in early apoptosis of stimulated cells ($P = 0.23$), late apoptosis of stimulated cells (P = 0.91), total apoptosis of stimulated cells (P = 0.78), early apoptosis of unstimulated cells

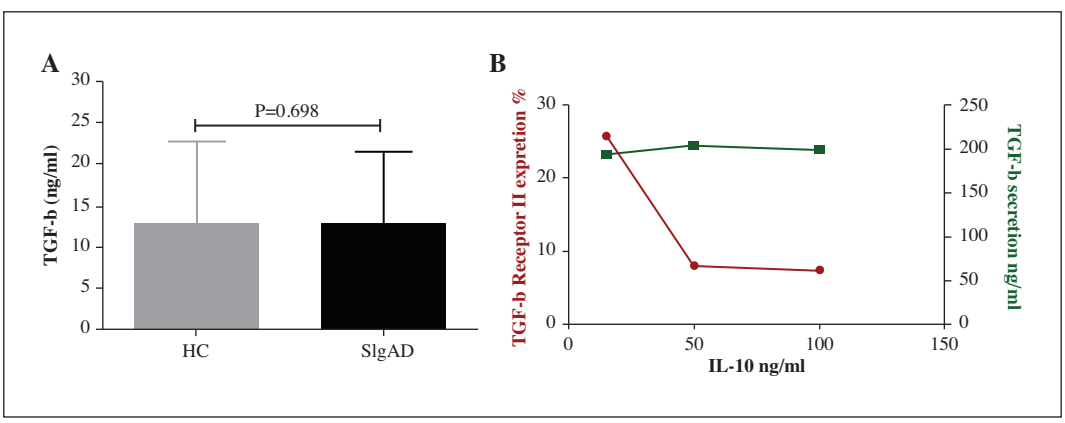

Figure 3

(A) Comparison of serum TGF-β levels in patients and controls. (B) Effect of IL-10 concentration on TBRII expression and TGF-b secretion was evaluated in three control cases. Isolated B lymphocytes cultured in the presence of mCD40L (300 ng/mL) and IL-10 (20, 50 and 100 ng/mL) for 24 h. At high concentrations of IL-10, TBRII expression was decreased (red line) and TGF-β secretion (green line) was not altered.

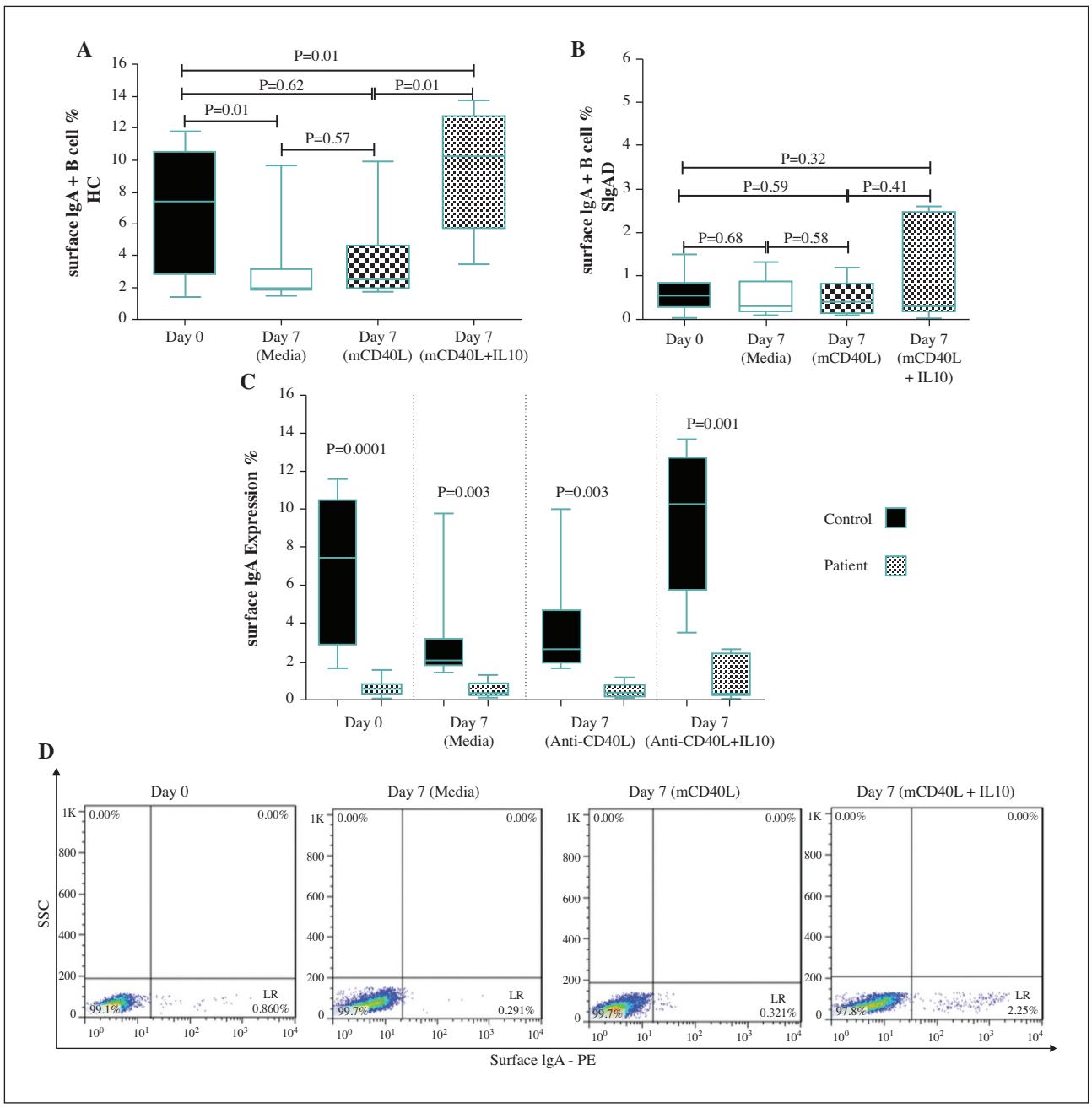

Figure 4

Percentage of IgA^+ B cell differentiation in control (A) and patients (B) in isolated B lymphocytes before stimulation and after culture in the presence of mCD40L alone, media or a combination of mCD40L and IL-10 for 7 days. (C) Comparison of IgA^+ B cell percentages in controls and patients before stimulation and after cultured in the presence of mCD40L alone, media or a combination of mCD40L and IL-10. (D) Representative flow cytometric pattern for detection of IgA expressing lymphocytes from patient data (LR: lower right).

($P = 0.08$), late apoptosis of unstimulated cells (P = 0.90) and total apoptosis of unstimulated cells (P = 0.90) (*figure 6*) (*table S1*).

STAT3, p-STAT3 and SOCS3 protein expression

The expression of p-STAT3 after 15 minutes of stimulation with mCD40L + IL-10 showed no significant difference between patients [median (IQR) = 0.63 (0.37-0.95)] and controls [median (IQR) = 0.7 (0.38-1.1)]. Also, the expression of p-STAT3 showed no significant difference between patients [median (IQR) = 0.25 (0.1-0.4)] and controls [median (IQR) = 0.5 (0.3-0.6)] after 15 minutes in the unstimulated condition. The basal expression of total STAT3 was significantly lower in patients [median (IQR) = 0.6 (0.38-0.92)] compared with controls [median (IQR) = 1.1 (0.87-1.7)] ($P = 0.01$) after 15 minutes in the unstimulated condition. Moreover, the expression of total STAT3 after 15 minutes of stimulation with mCD40L + IL-10 was significantly lower in patients [median (IQR) = 0.64 (0.49-0.89)] compared with controls [median (IQR) = 1.1 (0.66-1.9)] ($P = 0.04$) (*figure 7*). The expression of SOCS3 protein after 15 minutes of stimulation with mCD40L + IL-10 showed no significant difference between patients [median (IQR) = 0.63 (0.42-1.17)] and controls [median (IQR) = 0.5 (0.65-0.35)] ($P = 0.22$). In comparison

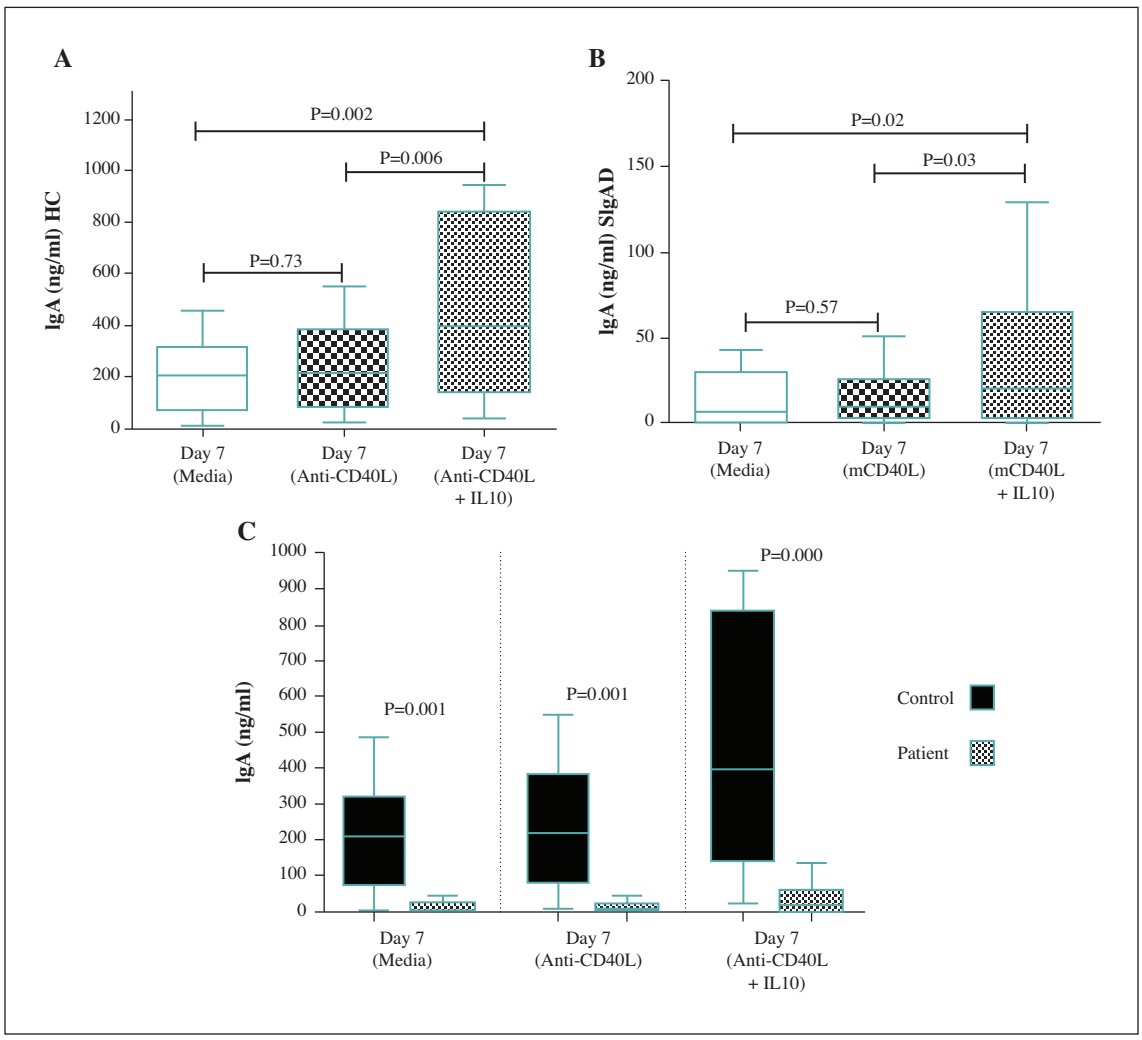

Figure 5

The concentration of secretory IgA in controls (A) and patients (B) by isolated B cells after 7 days of stimulation with mCD40L alone, media and combination of mCD40L and IL-10 (C) Comparison of IgA secretion in patients and controls by isolated B cells after 7 days of stimulation with mCD40L alone, media or a combination of mCD40L and IL-10.

between patients with related parents and non-related parents, no statistical difference was observed. The same applies for comparison between mild and severe phenotypes.

DISCUSSION

IL-10 is known as a pleiotropic cytokine acting through autocrine and paracrine to regulate inflammation and B cell differentiation, respectively [19]. IL-10, previously known as cytokine synthesis inhibitory factor, is considered as the amplifier of humoral response by inducing DNA replication in B lymphocytes, which are activated by CD40 antigen or B cell receptor [8]. It has been demonstrated that IL-10 has an important role in the proliferation and survival of B cells [20]. Preliminary studies have demonstrated that human naïve IgD^+ B lymphocytes produce IgA in response to IL-10 [9], as anti-IL-10 resulted in a significant decrease in circulating IgM and IgA production [21]. Considering the importance of IgA production in mucosal immunity and intestinal microbiota tolerance [22], biallelic IL-10 and IL-10 receptor deficiency in the human present with severe early-onset (infantile) inflammatory bowel disease [23]. Hence, we hypothesized that SIgAD patients might have a defect in IL-10 production and have an antagonist for IL-10 at the same time. Nevertheless, studies have shown that the mononuclear cells of SIgAD patients could produce IL-10 after stimulation with *Staphylococcus aureus* Cowan (SAC) and phytohemagglutinin (PHA), and no defect in IL-10 production was seen in these patients [24, 25]. In addition, IFN-γ production, which is known as IL-10 antagonist [26], was also observed to be the same in SIgAD compared with normal cells. Antibodies against IL-10 were detected in a small group of healthy controls, but they were not observed in the serum of 13 SIgAD patients [25]. According to these studies, it appears that there is no defect in IL-10 production or the presence of antibodies against IL-10 in these patients.

Despite different studies' reports on the low number of IgA^+ B cells in SIgAD patients [27], we and another study have shown normal and functional machinery of IgA antibody synthesis in SIgAD patients by *in vitro* stimulation of IgA^+ B cells [25]. We found that IL-10 contributes to a significant increase in IgA^+ B cell population in control groups, although this increase was not statistically significant in SIgAD patients. Given that some studies have also reported the

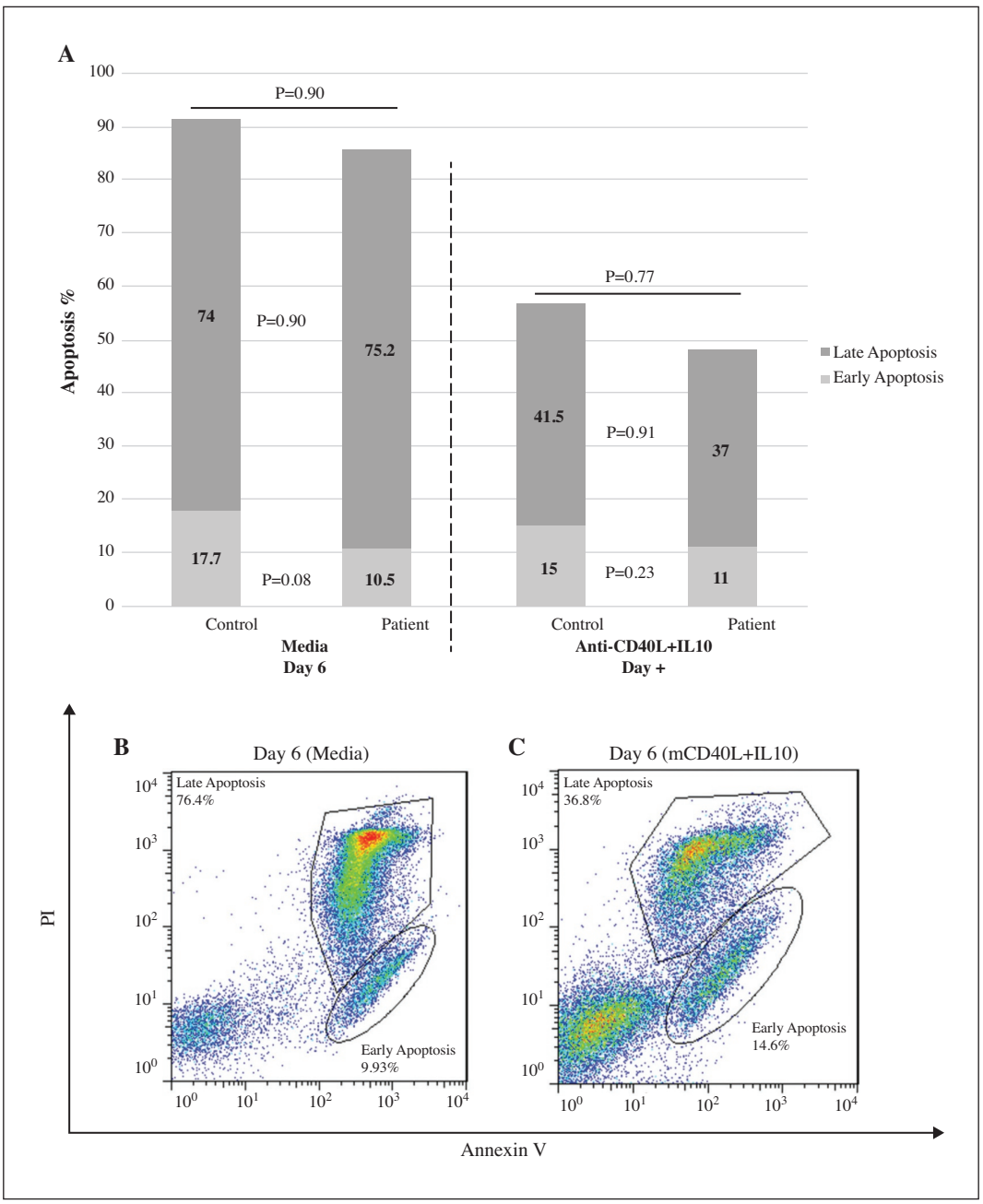

Figure 6
(A) The percentage of apoptosis in B cells isolated with MACS and stimulated with mCD40 + IL-10 in controls and patients after 6 days. (B) Representative flow cytometric pattern for apoptosis in unstimulated B cells and (C) stimulated B cells.

presence of little amounts of IgA in supernatants of cells stimulated with anti-CD40 and IL-10 [24, 27], it is postulated that the combination of these two signals is necessary to overcome the arrest of differentiation in class-switched IgA^+ B cells. Heine *et al.* [19] hypothesized another theory that IL-10 may be responsible for differentiation of lymphocytes that are committed for secretion of IgM and IgG, but it has an important role in the secretion, and not the differentiation, of IgA. Considerably, more investigations are required to determine the exact role of IL-10 in IgA CSR. In this study, we demonstrated that IL-10 significantly increases secretory IgA in both patients and healthy controls. However, in patients and all modes of treatments IL-10 secretion was significantly lower than the control group, which might be linked to the higher presence of committed B cells to IgA in control groups. A comparison of TBRII expression after IL-10 stimulation in SIgAD patients and control groups showed that the TBRII level was significantly reduced in patients. Similarly, the expression of TBRII was diminished after mCD40L stimulation although it was not significant. IL-10 significantly intensifies TGF-β secretion in control groups, but no significant increase was observed in patients. Therefore, it is possible that because of the important role of TGF-β and its receptor in class switching of IgA, attenuated responses to IL-10 are related to the regulatory effect of this interleukin on TGF-β and its receptor. The differential expression of cytokine receptors and

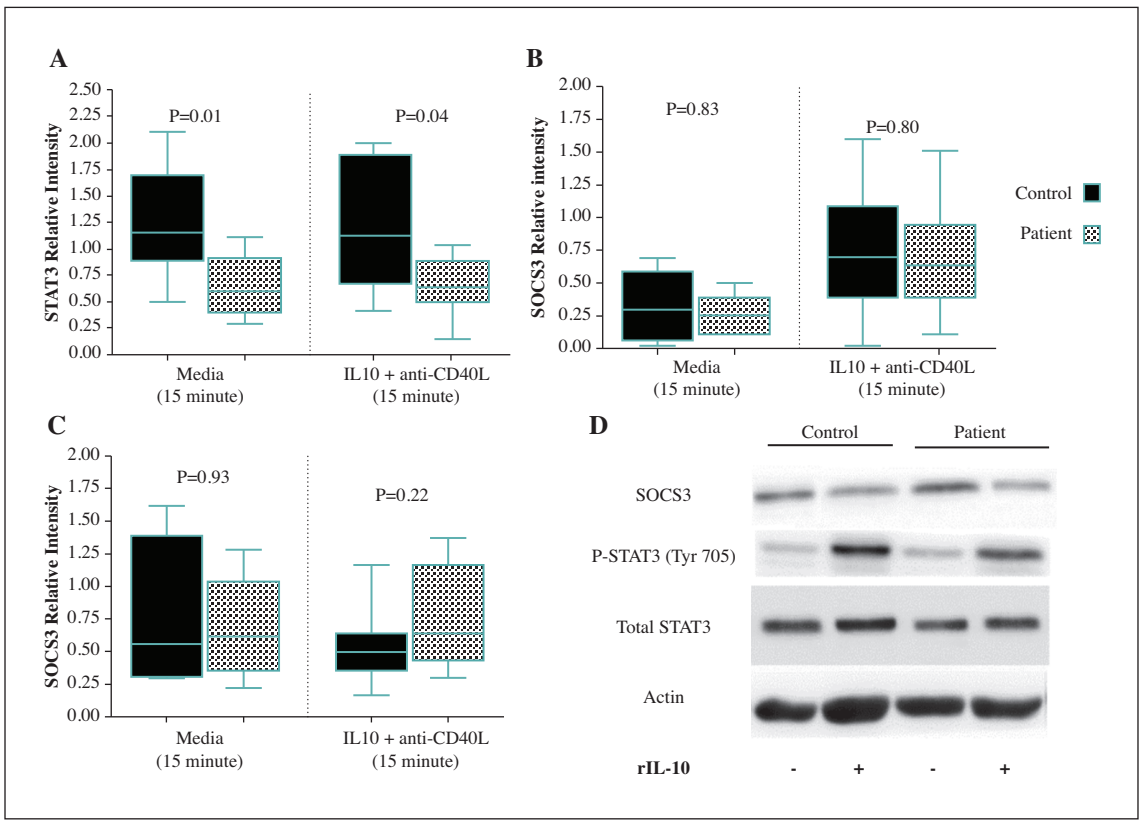

Figure 7
Comparison of (A) STAT, (B) p-STAT3 and (C) SOCS3 protein expression patients and controls in B cells isolated with MACS and treated with mCD40 + IL-10 in controls and SIgAD patients. Phosphorylation of STAT3 protein and expression of SOCS3 did not show a significant difference in patients compared with controls. (D) Representative blots from one experiment. STAT3 molecular weight is 85 KD and SOCS3 molecular weight is 25 KD.

also the regulation of their secretion is one of the immune regulatory mechanisms. Given the role of TGF-β in regulating immune responses in both physiologic and pathologic conditions such as cancer and autoimmunity, further studies could investigate the effect of IL-10 on regulatory responses of TGF-β in immune cells and its correlation with regulating immune responses. It should be noted that TGF-β is secreted by various cell types, including macrophages and T cells that exist in IgA CSR microenvironment [28, 29]. Therefore, these cell types should be considered as the source of TGF-β, a key molecule in IgA CSR process.

STAT3 could be activated by a wide range of ligands, cytokines, growth factors or G-protein-coupled receptors. Followed by the stimulation, STAT3 is phosphorylated in tyrosine 705, forms a homodimer or a heterodimer and enters the nucleus. Subsequently, it leads to the transcription of various regulators of cellular processes. Although in the present study we did not observe a defect in the STAT3 activation of B cells in SIgAD patients, the total expression of STAT3 was significantly decreased in these patients. IL-21 and IL-10 induce STAT3 phosphorylation and are involved in IgA CSR. Previous studies suggested the therapeutic effect of these two cytokines, especially IL-21, which is the most promising modality for SIgAD treatment [30]. In line with these studies, Lemarquis *et al*. [31] investigated IL-2, IL-4, IL-10, IL-21 and CpG oligodeoxynucleotides stimulation response in SIgAD patients and reported a defect in the STAT3 phosphorylation after IL-21 stimulation. Furthermore, recently a defect in STAT3 signaling followed by TLR9 stimulation in common variable immunodeficiency (CVID) with complete CSR defect was reported [32]. Therefore, several studies have suggested a defect in STAT3 signaling. Although data from Western blotting analysis is semi-quantitative, the lack of STAT3 expression might affect the content of phosphorylation and subsequently impair IgA class switching. Further studies on different levels of this pathway on more SIgAD patients are required to verify these findings as we did not observe any clinical phenotype associated with the molecular pattern.

The combination of IL-21, IL-4 and IL-10 cytokines could have an essential role in inhibiting apoptosis and compensating aberrant responses in T and B cells in CVID and SIgAD patients. Defect in IgA production in SIgAD patients could be due to the apoptosis of IgA committed B cells that express elevated levels of Caspase-1. Microarray analysis of genes related to apoptosis has demonstrated increased expression of survival genes in B cells of SIgAD patients after stimulation with IL-10 and other stimulants. Moreover IL-10 and CD40L, by inhibition of Caspase-1 transcription, may provide a condition for survival and differentiation of IgA producing B cells [24]. Despite these findings, in our study, the effect of IL-10 on the reduction of apoptosis of B cells in SIgAD

patients was almost similar to that of the control group. It should be considered that the amount of apoptosis varies among different subsets of lymphocytes [30, 33] and it is possible that increased apoptosis is limited to specific subsets of B cells in SIgAD patients and it might be better to evaluate apoptosis in different subsets.

CONCLUSION

The defect in IgA production in SIgAD patients could have different reasons. Responses of defective B cells to IL-10 stimulation in these patients may originate from defective regulation of TGF-β responses by this cytokine. Furthermore, it is suggested that baseline deficiency of STAT3 protein could impair the signaling of cytokines such as IL-10 and IL-21. Understanding the ability or inability of IL-10 and CD40 signals in retrieving antibody synthesis could help in better recognition of the etiology of SIgAD disease and also provide a new approach for repairing antibody production impairments in these patients.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Annexe A

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1684/ecn.2021.0464.

Annexe A

Figure S1 **Supplementary data**

Comparison of IL-10 receptor I expression on B cells in patients and control.
Table S1Apoptosis in B cells isolated with MACS and stimulated with mCD40+IL-10 in controls and patients.