INVESTIGATIVE REPORT

Low-dose Anti-thymocyte Globulin Inhibits Human B-cell Differentiation into Antibody-Secreting Cells

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Anti-thymocyte globulin (ATG) is used in the treatment of acute organ rejection. We studied *in vitro* the effect of low-dose ATG on B-cell activation and differentiation to antibody-secreting cells, as this may have an effect on B cell-driven autoimmune diseases, such as pemphigus vulgaris. Immunoglobulin production was analysed in the supernatants of peripheral blood mononuclear cells (PBMC) and CD19⁺ B cells from healthy donors and from patients with different autoimmune diseases. B-cell proliferation, viability and differentiation were analysed using flow cytometry. Differentiation of B cells to immunoglobulin G (IgG) secreting cells was significantly reduced by ATG, but not by control unspecific IgG from non-immunized rabbits (rIgG). B-cell viability was not altered by sub-depleting concentrations of ATG. In contrast, B-cell proliferation was enhanced by ATG. When PBMC from patients with autoimmune diseases were studied, specific autoantibodies could be detected in 1 out of 10 patients. In this patient, who had pemphigus vulgaris, ATG not only decreased total IgG, but decreased also specific anti-desmoglein-3. In conclusion, these data suggest that ATG at low concentrations inhibits B-cell differentiation and function. *Key words: anti-thymocyte globulin; B cells; pemphigus vulgaris.*

Anti-thymocyte globulin (ATG) is a polyclonal antibody preparation made by immunizing rabbits, horses or goats with human thymus cells, and purifying the IgG fraction (1). The different preparations have distinct characteristics, such as antibody specificity profiles and clinical efficacy. In this study, the most common preparation, rabbit ATG Thymoglobulin® (Genzyme) was used. It is used as a T-cell-depleting agent for prophylaxis and treatment of acute rejection in organ transplantation, particularly in kidney transplantation (2). It can also be used as part of the conditioning regimen before autologous stem cell transplantation in haematological malignancies or, infrequently, in refractory cases of autoimmune diseases, such as systemic lupus erythematosus (SLE) (3). Modulation of autoimmune diseases, such as systemic sclerosis, has been reported in clinical pilot studies (4). Interestingly, *in vivo* and *in vitro* B cells and even plasma cells are also depleted by specific antibodies contained in ATG when used in high concentrations (3, 5). No cytotoxic effects are observed at low doses *in vitro* (5) and low-dose ATG is currently being explored for its immunomodulatory effects in renal transplantation (6, 7).

However, beyond its cytotoxic effects, little is known about the immunomodulatory impact of low-dose ATG on B-cell differentiation, e.g. with respect to B-cell dependent autoimmune diseases with pathogenic antibodies (5, 8). The aim of this investigation was to study the impact of ATG at sub-depleting concentrations on B-cell activation and differentiation to antibody-secreting cells (ASC). Inhibition of B cells by ATG and, more importantly, ATG (Fab')2 fragments, delineates the possibility that antibodies with distinct B-cell specificities are present in this preparation, which may offer novel target structures to alter B-cell function with less B-cell toxicity.

MATERIALS AND METHODS

All procedures were approved by the local ethics committee (Charité Campus Mitte) and conform to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and patients who gave written informed consent. CD19⁺ B cells were magnetically separated from PBMC (Miltenyi Biotec, mean purity > 99%, CD14⁺ or CD3⁺ cells < 1%). Cells were stimulated for 8 days with 3 µg/ml CpG2006 and 5 µg/ml rabbit ATG (Thymoglobulin®, Genzyme) or unspecific IgG from non-immunized rabbits (rIgG), as determined by titration from 0.2 to 10 µg/ml and 0.01 to 100 µg/ml, respectively (data not shown). Secreted IgG in the supernatants was analysed by enzyme-linked immunoassay (ELISA), as described previously (9).

RESULTS

Toll-like receptor 9 (TLR9) was activated by CpG2006 to induce IgG in B cells (10). Our data show strong induction of secreted IgG, from B cells activated by TLR9, in the supernatants; 10-fold to $1,035 \pm 392$ µg/dl. Upon additional ATG, IgG was strongly decreased to $59.2 \pm 5.0\%$ (rIgG: $85.1 \pm 7.0\%$, $p < 0.05$, Fig. 1a). In order to exclude that ATG acts unspecifically through
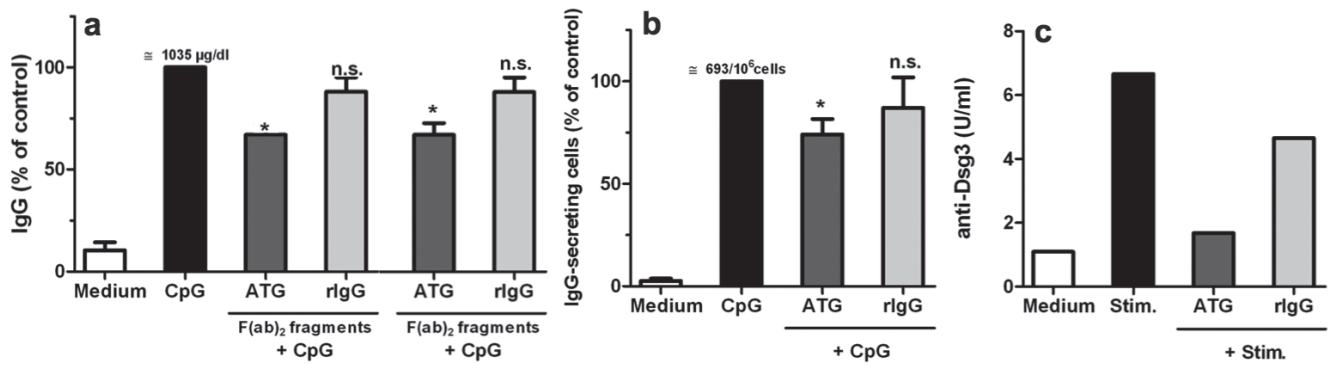
Fig. 1. Anti-thymocyte globulin (ATG) decreases immunoglobulin G (IgG) secretion in B-cell cultures by reducing the frequency of immunoglobulin G (IgG)-secreting cells. (a–b) CD19^{+} B cells were stimulated with CpG and incubated with ATG or rabbit IgG (rIgG). Unstimulated cells and cells treated with only CpG were used as controls. Results were normalized to CpG control to compensate for inter-individual variation. Data represent mean (standard error of the mean; SEM), $n = 9$. *$p < 0.05$, n.s., not significant; compared with stimulated control. (a) Secreted IgG in supernatants was measured using enzyme-linked immunoassay (ELISA). ATG decreases IgG secretion, and F(ab)2 fragments of ATG are also effective in decreasing IgG secretion. (b) IgG-secreting cells were analysed by enzyme-linked immunospot (ELISpot) assay. The frequency of IgG-secreting cells is decreased following application of ATG. (c) Peripheral blood mononuclear cells (PBMC) from a patient with pemphigus vulgaris were stimulated with CpG, anti-CD40, and anti-B-cell receptor (BCR). Cells were incubated with or without ATG or rIgG. Anti-desmoglein (Dsg) 3 autoantibodies in the supernatants were detected by ELISA. Specific autoantibodies decreased following application of ATG.

binding to Fcγ receptors, we added equimolar amounts of F(ab')2 fragments of ATG or rIgG to activated B cells. The data show, comparable to unfragmented ATG, that secreted IgG was reduced by F(ab')2-ATG fragments (Fig. 1a).

To address the question of whether antibody secretion is reduced on an individual cell basis, or if the number of ASC is reduced, we analysed IgG-secreting cells by enzyme-linked immunospot (ELISpot) assay. Paralleling the data from secreted IgG, TLR9-triggering in B cells induced the frequency of IgG-ASC 38-fold to $693 \pm 258/10^6$ cells. Upon additional ATG stimulation, only $74.1 \pm 7.5\%$ ASC were detected compared with the stimulated control (rIgG, $87.0 \pm 14.9\%$, Fig. 1b).

Finally, we analysed the impact of ATG on specific IgG expression *ex vivo* (Fig. 1c). Ten patients with high serum levels of pathognomonic antibody specificities (6 systemic lupus erythematosus patients, 3 pemphigus vulgaris patients, 1 pemphigus foliaceus patient, Table I) were analysed regarding specific IgG secretion upon stimulation of PBMC with CpG2006, anti-CD40, and anti-B-cell receptor (anti-BCR) (11). However, specific antibodies could be induced *in vitro* only in one pemphigus vulgaris patient with high disease activity and high titres of anti-desmoglein 3 (anti-Dsg3). Baseline secreted anti-Dsg3 was detectable and was enhanced 6-fold after stimulation, to 6.7 U/ml. Additional ATG, but not rIgG, resulted in a 4-fold decrease in anti-Dsg3 to 1.7 U/ml (Fig. 1c).

To explore further different cellular stages of B-cell differentiation to ASC, the cells were characterized by flow cytometry. Our data clearly show that the fre-

Table I. *Clinical characteristics of patients for specific autoantibody secretion* in vitro

ID	Age, years/ sex	Autoimmune disease	Disease duration, years	Current immunosuppressive therapy	Autoantibody examined *in vitro*	Autoantibody titre *ex vivo* (serum)	Autoantibody secretion *in vitro*
1	57/F	SLE	36	Prednisolone, Azathioprine	anti-Ro (SS-A)	Positive	No
2	43/F	SLE	23	Prednisolone	anti-Ro (SS-A)	Positive	No
3	63/F	SLE, SS	N/K	Prednisolone, Hydroxychloroquine	anti-Ro (SS-A)	Positive	No
4	42/F	SLE	20	Cyclophosphamide/dexamethasone pulse, Prednisolone, Methotrexate, Hydroxychloroquine	anti-ds-DNA	N/K	No
5	40/F	SLE	3	Methylprednisolone pulse, Hydroxychloroquine, Azathioprine	anti-ds-DNA	416 (<20) U/ml	No
6	46/M	PV	1	Prednisolone	anti-dsg-1	35 (<14) ratio	No
				Dapsone	anti-dsg-3	79 (<7) ratio	
7	45/F	PV	6	Mycophenolate	anti-dsg-1	6 (<14) ratio	No
				Methotrexate	anti-dsg-3	186 (<7) ratio	
8	23/M	PV	0	Cyclophosphamide/dexamethasone pulse (during first pulse)	anti-dsg-1	86 (<14) ratio	Yes
					anti-dsg-3	172 (<7) ratio	
9	53/M	PF	3	Cyclophosphamide/dexamethasone pulse	anti-dsg-1	171 (<14) ratio	No
					anti-dsg-3	1 (<7) ratio	
10	57/F	SLE, SS	21	Prednisolone, Hydroxychloroquine	anti-Ro (SS-A)	Positive	No

dsg: desmoglein; N/K: not known; PF: pemphigus foliaceus; PV: pemphigus vulgaris; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome.

quency of CD27^{hi}/CD38$^+$ and CD27^{hi}/CD138$^+$ plasma-blasts, and terminally differentiated CD20^{neg}CD27^{high} plasma cells is dramatically reduced in the presence of ATG (Fig. 2).

In order to investigate the impact of ATG on B-cell homeostasis, with focus on proliferation and cytotoxicity, B cells were activated *in vitro* and analysed by flow cytometry. CFSE-labelled B cells were activated by CpG2006 for 8 days and proliferation was induced in $39.1 \pm 2.6\%$ of the cells (10-fold; Fig. 3a and c). Additional ATG significantly enhanced proliferation ($61.7 \pm 5.0\%$, $p < 0.05$), in contrast to rIgG ($41.1 \pm 6.7\%$). Moreover, cell survival was not modulated by ATG (Fig. 3b and d). In detail, CpG2006 greatly improved B-cell survival compared with unstimulated cells ($16.2 \pm 2.7\%$ vs. $2.8 \pm 0.2\%$ viable cells) according to annexin V and propidium iodide staining and cells counts (Fig. 3b and d, data not shown). Viability was not affected by either low-dose ATG or rIgG ($17.0 \pm 3.4\%$ and $17.6 \pm 2.2\%$ viable cells, respectively). At a higher concentration of 100 µg/ml ATG, however, cell death in B cells was significantly elevated compared with rIgG (data not shown).

DISCUSSION

This report provides new data on the effect of low-dose ATG on B cells: we show that sub-depleting doses reduce IgG production in B cells by inhibiting the differentiation to antibody-secreting cells. Reduced secretion per cell is not likely, as ELISpot analysis shows reduced frequencies of Ig-secreting cells.
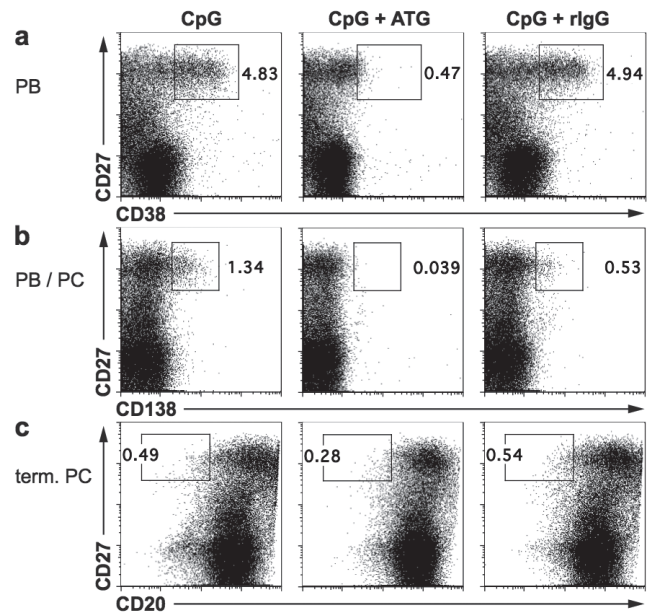

Fig. 2. Anti-thymocyte globulin (ATG) blocks plasmablast (PB) differentiation of B cells. CpG-stimulated B cells were incubated for 8 days with or without ATG or rabbit immunoglobulin G (rIgG). The expression of surface molecules was analysed by flow cytometry. The gate was on viable lymphocytes. (a) CD27^{hi}/CD38$^+$ PB, (b) CD27^{hi}/CD138$^+$ PB / plasma cells (PC), (c) CD27^{hi}/CD20^{lo} terminal PC. Results are representative of 3 independent experiments.

Low doses of ATG modulated B-cell differentiation without an impact on viability, in contrast to high concentrations (100 µg/ml) of ATG, where viability was reduced. This shows that ATG also impacts cell viability *in vitro* in a dose-dependent manner and is in line with the findings of Zand et al. (5), but that the impact on
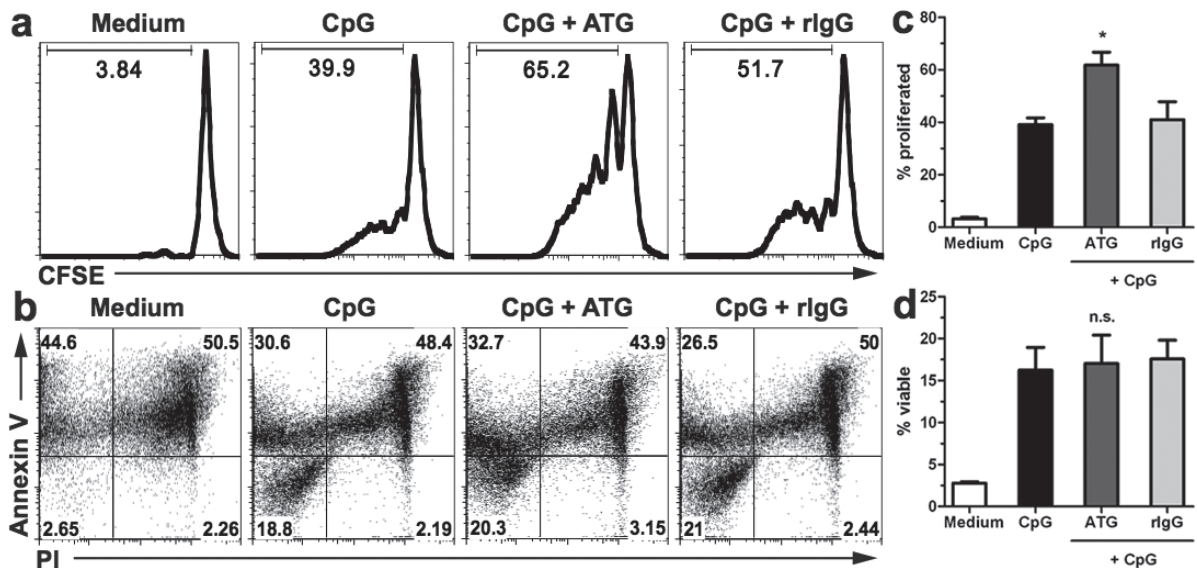

Fig. 3. Maintained B-cell proliferation and viability in the presence of anti-thymocyte globulin (ATG). (a–d) CD19$^+$ B cells were stimulated with CpG and incubated with ATG or rIgG. Unstimulated cells and cells treated with only CpG were used as controls. Data represent mean (standard error of the mean; SEM), $n = 3$. Asterisk, $p < 0.05$; compared with stimulated control. (a–b) Cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) prior to culture, and proliferation was analysed by flow cytometry on day 8. (c–d) Cells were harvested and incubated with annexin V and propidium iodide (PI) and analysed by flow cytometry. Viable cells are annexin$^-$PI$^-$, apoptotic cells annexin$^+$PI$^-$ and necrotic cells annexin$^+$PI$^+$.

cell viability was not relevant for the reduction in IgG production in our experiments. Furthermore, in patients with ultra-low-dose treatment (1.5–6 mg/kg), B-cell counts were not reduced, supporting that proliferation and survival was not compromised *in vivo* (6, 7). Modulation of B-cell differentiation is reflected by altered frequencies of B-cell subsets during cell culture in the presence of ATG. We determined that frequencies of naive B cells were stable, in contrast to reduced memory B cell and plasmablasts. This has previously been shown *in vivo*, where the overall number of B cells was not changed by ATG, whereas there were significantly less memory B cells and class switch memory B cells (12). The authors concluded that this is due to the effect of ATG on T-cell help, but our results indicate that ATG directly modulates B-cell differentiation.

Remarkably, even though B-cell differentiation was inhibited, B-cell proliferation was increased in the presence of low-dose ATG. This is most likely attributed to activating antibodies in ATG directed against BCR and CD40 and, potentially, the receptor of the cytokines interleukin (IL)-21 and IL-10. For example, synergistic effects of BCR, CD40, and TLR9, activated by CpG have been described (13). These results contrast with findings by Bonnefoy-Bérard et al. (14), who observed enhanced proliferation only in PBMC, but not in purified B cells. They concluded that B-cell activation is inhibited by ATG at concentrations that are activating for T cells. However, they did not specifically investigate B-cell activation. This discrepancy can be explained by different stimulation protocols.

This seemingly paradox finding of enhanced B-cell proliferation but disturbed B-cell differentiation can be explained by the fact that B cells are activated by low-dose ATG, but essential cytokines and co-stimulatory factors for further differentiation are missing. Such factors include IL-5, IL-6, IL-10, IL-21, tumour necrosis factor (TNF), B-cell activating factor belonging to the TNF family (BAFF) and apoptosis-inducing ligand (APRIL) (15). The modulation of B-cell function may be due to the polyclonal nature of ATG including a large variety of activating and inhibiting antibodies against B-cell surface receptors including CD40, CD54 and the B-cell receptor (5). Cross-linking of multiple cell surface receptors may also lead to a partial activation and induction of a state of anergy, in analogy to T cells (16).

Since the repeated therapeutic application of ATG in the treatment of chronic diseases, such as pemphigus vulgaris (PV) or SLE, would be limited by the induction of neutralizing anti-rabbit antibodies (17), F(ab')2 fragments may be a superior option due to reduced immunogenicity, especially if combined with cyclophosphamide and dexamethasone.

Our experiments demonstrate that F(ab')2 fragments were as potent in inhibiting antibody secretion as unfragmented ATG. Other research groups have investiga-

ted the effectiveness of F(ab')2 fragments of ATG and found them to be up to 90% as effective as the whole molecule in inducing apoptosis in PBMC, B cells and B-cell lines (5, 18).

Application of ATG not only decreased total IgG, but also specific IgG, and therefore might have a clinical benefit for antibody-mediated diseases by interfering with B-cell differentiation to ASC. The induction of specific autoantibody secretion was possible in only 1 out of 10 patient samples. This is most likely due to the low frequency of circulating antigen-specific memory B cells, as observed previously (19). Inhibition of specific autoantibody secretion may be a result of both direct action on B cells, but also of inhibition of T-cell help, as has been hypothesized by Gurkan et al. (12). However, as we did not investigate specific autoantibody secretion by purified B cells, but only in PBMC, we cannot separate the 2 effects. We can conclude from our study using purified B cells from healthy donors that ATG inhibits antibody secretion by direct action on B cells, but we cannot definitely conclude this from our experiments using PBMC from patients.

Low-dose and ultra-low-dose ATG are currently being explored for the use in renal transplantation and seem to have a comparable efficacy while being less toxic than normal-dose ATG (6, 7). We thus conclude that low-dose ATG may complement established immunosuppressive therapy in B-cell-dependent autoimmune diseases. The mechanism of action includes specific immunomodulation due to a combination of stimulatory and inhibitory antibodies and inhibition of terminal differentiation of B cells to antibody-secreting plasmablasts without affecting proliferation.

ACKNOWLEDGEMENT

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