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DISSERTATION

Modulation of the Humoral Immune Response by Antithymocyte Globulin (ATG)

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In memory of my father.

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1 Summary

Antithymocyte globulin (ATG) is a polyclonal antibody preparation made by immunising rabbits with human thymus cells and purifying the IgG fraction. ATG is widely used in the treatment of acute organ rejection. The aim of this work was to study *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.

Peripheral blood mononuclear cells (PBMC) and $CD19^+$ B cells were isolated and cultivated with ATG and control rabbit IgG (rIgG). Supernatants were analysed for the presence of immunoglobulins by ELISA. Furthermore, proliferation and cytotoxicity were measured. B-cell differentiation surface markers and expression of developmental transcription factors were analysed using flow cytometry and polymerase chain reaction (PCR), respectively.

The secretion of IgG, IgM and IgA in PBMC as well as in B cells was significantly reduced by treatment with ATG, but not control rIgG. This demonstrates that ATG is also effective in the absence of T cells and monocytes, and that the effects are not only mediated by e.g. inhibition of T-cell help. $F(ab')_2$ fragments of ATG proved to be nearly as potent in reducing the production of antibodies by B cells as unfragmented ATG, suggesting that the reduction of IgG is mediated by at least some specific effects of ATG, and not solely by unspecific binding to Fcγ receptors.

Cell death of B cells was not elevated in the presence of low concentrations of ATG. On the contrary, ATG induced strong proliferation of B cells, perhaps due to synergistic effects of ATG and CpG, since TLR9, as well as CD40 and the B-cell receptor induce MAP kinase pathways and NFκB. Flow cytometry data suggest that ATG impairs the development of B cells towards antibody-secreting cells as there was a slight reduction in memory B cells and a significant reduction in plasmablasts, while the number of naive B cells was not affected.

ATG not only decreased total IgG but also specific autoreactive IgG: Anti-Dsg3 in supernatants of PBMC from a patient with pemphigus vulgaris was decreased in the presence of ATG. This result indicates a possible clinical benefit of ATG for antibody-mediated diseases.

Summing up, the results suggest that ATG at low concentrations inhibits B-cell differentiation and function and thereby modulates the humoral immune response without exerting significant cytotoxic effects.

2 Summary (German)

Antithymozyten-Globulin (ATG) ist ein polyklonales Antiserum, das durch die Aufreinigung der IgG-Fraktion von Kaninchen gewonnen wird, die mit menschlichen Thymus-Zellen immunisiert wurden. ATG wird u. a. zur Behandlung von akuten Abstoßungsreaktionen nach Organtransplantation verwendet. Ziel dieser Arbeit war es, den Einfluss von niedrig-dosiertem ATG *in vitro* auf die Aktivierung von B-Zellen und Differenzierung zu Antikörper-sezernierenden Zellen zu untersuchen, da dies einen Effekt auf B-Zell-vermittelte Autoimmunerkrankungen wie Pemphigus vulgaris haben könnte.

Mononukleäre Zellen aus peripherem Blut (PBMC) und $CD19^+$ B-Zellen wurden isoliert und mit ATG und Kaninchen-IgG (rIgG) als Kontrolle kultiviert. In den Überständen wurden dann mittels ELISA die Immunglobuline gemessen. Darüber hinaus wurden die Proliferation bzw. Zytotoxizität gemessen. B-Zell-Differenzierung und Transkriptionsfaktor-Expression wurden mittels Durchflusszytometrie bzw. PCR analysiert.

Die Sekretion von IgG, IgM und IgA wurde durch die Behandlung mit ATG, aber nicht der Kontrolle rIgG sowohl bei PBMC als auch bei B-Zellen signifikant reduziert. Das zeigt, dass ATG auch in Abwesenheit von T-Zellen und Monozyten wirksam ist und dass die Effekte nicht durch z. B. die Hemmung der T-Zell-Hilfe vermittelt werden. $F(ab')_2$-Fragmente von ATG waren nahezu genauso potent bezüglich der Reduktion der Antikörper-Produktion von B-Zellen wie unfragmentiertes ATG, was darauf hinweist, dass die Verminderung von IgG zumindest teilweise durch spezifische Effekte des ATG vermittelt wird, und nicht nur durch unspezifische Bindung an Fcγ-Rezeptoren.

Die Zellsterblichkeit wurde unter dem Einfluss niedriger Konzentrationen von ATG nicht erhöht. Somit ist zu vermuten, dass die inhibitorischen Effekte primär durch eine Immunmodulation und nicht durch Zytotoxizität vermittelt werden. ATG induzierte bei den B-Zellen eine starke Proliferation, was auf synergistische Wirkungen von ATG und CpG zurückzuführen sein könnte, da sowohl TLR9 als auch CD40 und der B-Zell-Rezeptor mitogenaktivierte Proteinkinasen und NFκB induzieren. Die Ergebnisse der Durchflusszytometrie deuten darauf hin, dass ATG die Differenzierung von B-Zellen zu Antikörper-sezernierenden Zellen hemmt, da hier eine diskrete Reduktion der Gedächtnis-B-Zellen und eine deutliche Reduktion der Plasmablasten nachgewiesen wurde, während die Anzahl der naiven B-Zellen nicht vermindert war.

ATG verminderte nicht nur das Gesamt-IgG, sondern auch spezifisches autoreaktives IgG: Anti-Dsg3 in Überständen von PBMC eines Patienten mit Pemphigus vulgaris wurde in der Gegenwart von ATG reduziert. Dieses Ergebnis deutet auf einen möglichen klinischen Nutzen von ATG bei antikörpervermittelten Erkrankungen hin.

Zusammenfassend weisen die Ergebnisse darauf hin, dass niedrig-dosiertes ATG die B-Zell-Differenzierung und Funktion hemmt und somit die humorale Immunantwort moduliert ohne signifikante zytotoxische Effekte auszuüben.

3 Introduction

Antithymocyte globulins (ATGs) such as Thymoglobulin® play a vital role as immunosuppressants in transplantation, e.g. kidney and stem cell transplantation [93]. While the main mechanism of action is thought to be the depletion of T cells, recent investigations have determined that B cells are strongly affected by ATG as well [118]. However, neither the exact targets of ATG on B cells nor the mechanism and how ATG exactly exerts its effects on B cells are completely known.

3.1 Antithymocyte Globulin

3.1.1 Preparation

Several polyclonal antilymphocyte sera are available and have been successfully used in transplantation medicine for decades [7]. Rabbits, horses or goats are immunised with human lymphocytes, thymocytes or T cell lines. Rabbit antithymocyte globulin (rATG, Thymoglobulin®, Genzyme) is raised by immunising rabbits with human thymocytes, and in this manner it is provided with polyclonal antibodies against several surface antigens found on human leukocytes. A comprehensive list of target antigens for ATG is shown in Table 1.

Thymus fragments originate from children undergoing cardiac surgery, as they need to be removed to obtain access to the heart. Consecutively, the fragments are screened for viral infections, and further processing includes macerating the samples, digesting with collagenase and Ficoll density gradient isolation of thymocytes [68, 118]. Cells present in the thymus are mostly T cells, but also B cells, plasma cells, antigen-presenting cells and stromal cells [80]. Pathogen-free New Zealand rabbits are then immunised three times at intervals of two weeks by subcutaneous application of an aliquot of the thymus cell suspension. Following successful immunisation, 50 ml of blood is taken twice a month during the course of several years, usually by puncture of the ear vein or the ear artery. The blood is centrifuged and the immunoglobulin G (IgG) fraction purified from the serum [90]. The antiserum is pasteurised and lyophilised, and comes as a sterile powder to be reconstituted in sterile aqua ad injectionem [1].

Table 1 – List of published target antigens for ATG [6, 14, 15, 56, 66, 68, 71, 83, 84, 85, 107, 119].
For abbreviations, see appendix A.2 List of Abbreviations.

Antigen	Alternative Name	Cellular Expression	Function
CD1a		Cortical thymocytes, Langerhans cells, DC	Role in presentation of lipid antigens
CD2	T11, LFA-2	T cells, thymocytes, NK cells	Adhesion molecule, binds CD58
CD3		Thymocytes, T cells	Associated with the T cell receptor
CD4		Thymocyte subsets, T helper cells, monocytes, macrophages	Co-receptor for MHC class II molecules
CD5		Thymocytes, T cells, subset of B cells	Unknown
CD6		Thymocytes, T cells, B cells in chronic lymphatic leukaemia	Binds CD166
CD7		Pluripotent hematopoietic cells, thymocytes, T cells	Unknown
CD8		Thymocyte subsets, cytotoxic T cells	Co-receptor for MHC class I molecules
CD11a/CD18	LFA-1	Lymphocytes, granulocytes, monocytes, macrophages	Integrin, binds CD54, CD50, and CD102
CD11b	Mac-1	Myeloid and NK cells	Subunit of integrin CR3, binds CD54, complement iC3b, and extracellular matrix proteins
CD16	FcγRIII	Neutrophils, NK cells, macrophages	Mediates phagocytosis and antibody-dependent cell-mediated cytotoxicity
CD19		B cells	Forms complex with CD21 and CD81, co-receptor for B cells
CD20		B cells	Possible role in regulating B cell activation
CD25		Activated T cells, B cells, monocytes	IL-2 receptor α chain
CD28		T cell subsets, activated B cells	Activation of naive T cells, receptor for co-stimulatory signal, binds CD80 and CD86
CD29		Leukocytes	Integrin β1 subunit
CD30	Ki-1	Activated T, B, and NK cells, monocytes	Binds CD53
CD32	FcγRII	Monocytes, granulocytes, B cells	Low affinity Fc receptor for immune complexes
CD38		Early B and T cells, activated T cells, germinal center B cells, plasma cells	NAD glycohydrolase, augments B cell proliferation
CD40		B cells, macrophages, DC	Binds CD154, receptor for co-stimulatory signal for B cells
CD44	Hermes antigen	Leukocytes, erythrocytes	Binds hyaluronic acid, mediates adhesion of leukocytes
CD45	LCA	Hematopoietic cells	Tyrosine phosphatase, augments signalling through antigen receptor of B and T cells
CD49	VLA1-6	Leukocytes	Integrin, associates with CD29
CD50	ICAM-3	Thymocytes, T cells, B cells, monocytes, granulocytes	Binds integrin CD11a/CD18
CD51/CD61	Vitronectin receptor		Integrin, binds vitronectin, von Willebrand factor, fibrinogen, and thrombospondin
CD54	ICAM-1	Hematopoietic & non-hematopoietic cells	Intercellular adhesion molecule
CD56		NK cells	Adhesion molecule
CD58	LFA-3	Hematopoietic & non-hematopoietic cells	Adhesion molecule, binds CD2
CD80	B7.1	B cell subset	Co-stimulator, ligand for CD28 and CTLA-4
CD81	TAPA-1	Lymphocytes	Forms B cell co-receptor with CD19 and CD21
CD82	R2	Leukocytes	Unknown
CD86	B7.2	Monocytes, activated B cells, DC	Ligand for CD28 and CTLA-4
CD95	Apo-1, Fas	Uncertain, possibly all nucleated cells	Binds Fas ligand (CD178), induces apoptosis
CD98		Hematopoietic cells	Possibly amino acid transporter
CD99		Lymphocytes, thymocytes	Unknown
CD102	ICAM-2	Resting lymphocytes, monocytes, vascular endothelial cells	Binds CD11a/CD18
CD126	IL-6Rα	Activated B cells, plasma cells	IL-6 receptor α subunit
CD138	Syndecan-1	Plasma cells	Heparan sulphate proteoglycan, binds collagen type I
CD147		Hematopoietic cells, endothelial cells	Potential adhesion molecule
CD152	CTLA-4	Activated T cells	Receptor for CD80 and CD86, negative regulator of T cell activation
CD184		Hematopoietic stem cells	Binds SDF-1
CD195	CCR5	Promyelocytic cells	Possible role in granulocytic lineage proliferation and differentiation
CD197	CCR7	Activated T and B cells	Receptor for MIP-3β chemokine
HLA class I		All nucleated cells	Present antigenic peptides to $CD8^+$ T cells
HLA cl. II		Antigen-presenting cells	Present antigenic peptides to $CD4^+$ T cells
LPAM-1	$\alpha 4\beta 7$	Leukocytes	Integrin

3.1.2 Mechanism of Action

Effect on B cells While research initially focused on the depletion of T cells using ATG, recent investigations revealed a strong impact on B cells [118]. This is not surprising, as many of the antibodies contained in ATG preparations are specific for B-cell epitopes such as CD19 and CD20. The clinical relevance of this finding is currently in the focus of research.

In vivo, the frequency of B cells is significantly lowered after administration of ATG [33, 73]. In the cynomolgus monkey model, B-cell depletion was achieved due to both complement-mediated cell lysis and induction of apoptosis [84]. Furthermore, by depleting T cells, B cells are deprived of T-cell help, and necessary signals and factors provided by helper T cells are missing. Cell activation is therefore disturbed.

In vitro, apoptosis is induced by ATG in naive and activated human B cells, in memory B cells, plasma cells, primary myeloma cells and B-cell lines [8, 11, 13, 119, 120]. Cell killing occurs by complement-mediated cell lysis, antibody-dependent cell-mediated cytotoxicity and different apoptotic pathways.

The complement system is activated by immune complexes of ATG bound to the target cell. Complement factor C1q binds to the Fc region of rabbit immunoglobulin and forms a pentameric complex with the $C1r_2C1s_2$ tetramer leading to the initiation of a biochemical cascade. Eventually, the so-called membrane attack complex is formed, which inserts a pore into the cell membrane resulting ultimately in lysis of the cell [60].

Antibody-dependent cell-mediated cytotoxicity can occur at lower concentrations of ATG [12]. The Fc region of ATG binds to Fcγ receptors on natural killer cells (NK cells). Subsequently, the NK cell releases two mediators: Perforin forms a pore into the cell membrane of the marked cell, and granzyme B enters the cell through the pore inducing the apoptotic cascade [103, 115].

The apoptotic pathways involved are via caspase activation by cross-linking of e.g. CD38 and CD95, cathepsin B dependent apoptosis and direct loss of mitochondrial membrane potential by cross-linking of HLA-DR [119, 118]. Caspases are cleaving enzymes that degrade key intracellular substrates such as cellular and nuclear structural proteins and repair enzymes, causing the cell to perish [116].

Another mechanism of depletion appears probable but has so far not been proven, and therefore remains hypothetical: coating of cells with ATG opsonises them and makes them a target for tissue-resident macrophages in liver, spleen and lung. These recognise the Fc region of rATG with their Fcγ receptor, and phagocytise the cell [22, 84].

Mitogenic effects have not been observed at concentrations known to be mitogenic in T cells. On the contrary, ATG has been shown to inhibit both B-cell proliferation and differentiation of B cells into antibody-secreting cells (ASC) [11].

Effect on T cells, dendritic cells and natural killer cells Treatment with ATG at therapeutic doses leads to massive depletion of peripheral T cells. This is thought to be the

major mechanism by which ATG induces immunosuppression. Mechanisms of cell killing are complement-dependent cell lysis, opsonisation and subsequent phagocytosis by phagocytes and induction of apoptosis [12, 36]. ATG can furthermore induce a state of anergy in T cells. Simultaneous cross-linking of cell surface receptors and co-stimulatory molecules leads to partial activation and the induction of anergy [65].

ATG also binds to human dendritic cells (DC) and induces complement-mediated cell lysis or apoptosis [29, 71]. Furthermore, maturation of monocyte-derived DC is inhibited and polarised towards a tolerogenic phenotype [37]. The role of ATG in DC function (e.g. endocytic antigen uptake) has not yet been conclusively investigated [75]. Dendritic cells play a major role in antigen presentation and are therefore of great importance for transplant survival [104].

Natural killer cells (NK cells) play a protective role in transplantation. Relative sparing of NK cells was observed after treatment with total lymphoid irradiation and rabbit anti-mouse thymocyte globulin (mATG) in a murine model of allogeneic stem cell transplantation. This was associated with the prevention of acute graft-versus-host disease [53]. These findings were later confirmed in a small clinical trial [59]. Whereas reconstitution of T and B cells *in vivo* is delayed after HSCT conditioning with Thymoglobulin®, reconstitution of NK cells is rather quick [26, 81]. NK cells furthermore up-regulate the expression of cell surface markers associated with activation and degranulation. Data on induction of apoptosis *in vitro* remains controversial [82, 23].

Non-depleting Properties ATG not only depletes major lymphocyte populations but it also modulates lymphocyte function without exerting cytotoxic effects. This is thought to be due to antibody specificities against integrins, leukocyte adhesion receptors and chemokine receptors. Binding of antibody leads to the internalisation of the complex, thereby blocking the related pathway. This mechanism is thought to occur predominantly at low, subtherapeutic concentrations of ATG [68]. One example is the $\beta 2$ integrin leukocyte function-associated antigen 1 (LFA-1, CD11a/ CD18). It is vital for leukocyte adhesion and migration [27]. Michallet et al. demonstrated the dose-dependent down-modulation of LFA-1 by rATG [66].

Several research groups have demonstrated the potential of Thymoglobulin® to induce regulatory T cells in human and murine cell cultures [30, 58, 92]. Expansion of Treg occurs predominantly by conversion of $CD4^+CD25^-$ to $CD4^+CD25^+FOXP3^+$ cells rather than proliferation of naturally occurring Treg. This is thought to be due to the mitogenic properties of rATG leading to activation of $CD4^+$ T cells and subsequent up-regulation of co-stimulatory molecules and creation of a TH2-like cytokine milieu. Treg are essential for the maintenance of self-tolerance and the limitation of inflammation. They are characterised by the phenotype of $CD4^+CD25^+FOXP3^+$. Their function is to suppress effector T cells, dendritic cells, and B cells [57, 112].

Table 2 – Clinical application of ATG [1, 2, 68].

Application according to marketing authorisation	
Germany	Prophylaxis of transplant rejection after transplantation of kidney, heart, liver and pancreas as part of induction regimes, combined with e.g. corticosteroids, cyclosporine, tacrolimus, azathioprine or mycophenolate mofetil Treatment of acute rejection episodes after transplantation of kidney, heart and liver Second-line therapy of aplastic anaemia
USA	Treatment of acute renal transplant rejection
Application beyond marketing authorisation (off-label use)	
	Prophylaxis and treatment of transplant rejection for various other solid organs Part of conditioning regimens for bone marrow ablation before autologous and allogeneic stem cell transplantation

3.1.3 Clinical Application

In Germany, Thymoglobulin® is registered for the prophylaxis of transplant rejection after transplantation of kidney, heart, liver and pancreas as part of induction regimens, combined with other immunosuppressive medication such as corticosteroids, cyclosporine, tacrolimus, azathioprine or mycophenolate mofetil. It is furthermore used for the treatment of acute rejection episodes after transplantation of kidney, heart and liver. The third indication is as second-line therapy of aplastic anaemia [2].

In the United States, Thymoglobulin® is only licensed for the treatment of acute renal transplant rejection [1]. However, analyses have shown that rATG is also the most commonly used agent for the induction therapy in kidney transplantation (off-label) [34].

Beyond the official approval, ATG is used off-label for the prophylaxis and treatment of transplant rejection for various other solid organs [68]. It is also used as part of conditioning regimens for bone marrow ablation before autologous and allogeneic stem cell transplantation [68]. See table 2 for an overview of the clinical application of ATG.

A potentially advantageous role in umbilical cord blood transplantation is currently being discussed. Furthermore, graft-versus-host disease is commonly treated by rATG [69].

The inclusion of ATG into the induction therapy regimen e.g. in kidney transplantation offers several advantages. It not only lowers the incidence of early acute rejection episodes, but it also delays the need to introduce calcineurin inhibitors such as cyclosporine and tacrolimus. These powerful immunosuppressants are strongly nephrotoxic harming the newly implanted kidney. The use of ATG also allows for earlier reduction of steroids [34].

3.1.4 Use of ATG in Autoimmune Diseases

Autoimmune diseases are characterised by a pathological reaction of the immune system attacking the autoantigen, a self-antigen. Typical examples are systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) or progressive systemic sclerosis (PSS). Aim of therapy is to control the hyperreactivity of the immune system to the own body and to limit in-

flammation. Many therapeutics have therefore been derived from therapy regimens used in transplantation because these aim at suppressing the immune system. The potent immunosuppression achieved by ATG has prompted its therapeutic application in autoimmune diseases.

Simon et al. tested the ability of mATG to attenuate the development of type 1 diabetes mellitus (T1DM) in a murine model using non-obese diabetic (NOD) mice. Diabetes was delayed or reversed, but the timing of administration was crucial [94]. Thymoglobulin® in combination with a fusion molecule involving cytotoxic T-lymphocyte antigen 4 (CTLA4), CTLA4-Ig, was also tested in NOD mice. In newly diabetic animals, this treatment was able to reverse diabetes and normoglycemia was restored [111]. A randomised placebo-controlled trial with newly diagnosed T1DM patients demonstrated that treatment with ATG lead to elevation of endogenous insulin production, and in two cases complete remission was achieved [89].

Conversely, in a pilot study with ten PSS patients receiving ATGAM® (equine ATG, eATG) as single treatment modality, only two patients showed improvement of disease. Two patients remained stable and five became worse. The authors concluded that ATG was not sufficient for the treatment of PSS [63]. Later reports were more positive. In a clinical study by Stratton et al., patients with PSS received Fresenius ATG® (ATG from rabbits immunised with Jurkat cells, i.e. a T cell line) plus mycophenolate mofetil. The disease was stabilised, and skin sclerosis improved [98]. A recent case report of three patients receiving rATG and cyclophosphamide described improvement of skin sclerosis and overall disability [40].

Positive outcomes have also been observed in murine models or small clinical trials of SLE, multiple sclerosis (MS) and Wegener's granulomatosis [19, 48, 91]. Beyond that, ATG is used as induction therapy before stem cell transplantation, possibly allowing the cure to autoimmune diseases such as SLE or PSS.

3.2 B Cells

The adaptive immune system is indispensable in protecting the body from infections. It includes two lymphocyte lineages: T cells account for cellular immunity. B cells are classically considered to be the effectors of humoral immunity by generating antibody-secreting plasmablasts and plasma cells. However, by interaction with T cells, they also act as antigen-presenting cells (APC) and secrete important cytokines, having both activating as well as regulatory properties. Fig. 1 summarises their development and differentiation.

3.2.1 B-Cell Development in the Bone Marrow

B cells originate from hematopoietic stem cells and develop in the bone marrow. Upon the influence of specific adhesive contacts and growth factors provided by stromal cells,

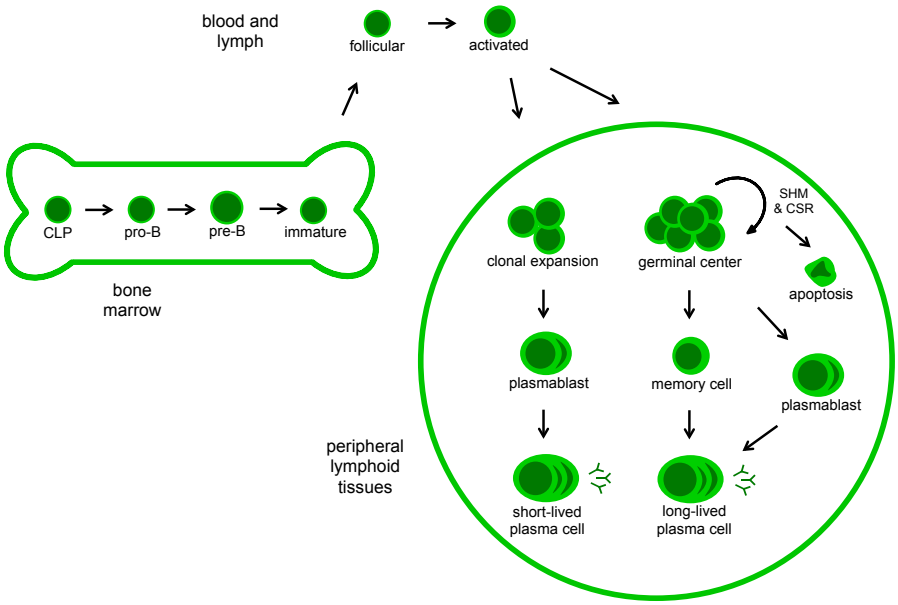

Figure 1 – Development of B cells in the bone marrow and terminal differentiation towards plasma cells.
B cells develop in the bone marrow via several precursors. Mature naive follicular B cells leave the bone marrow and recirculate between blood, lymph and peripheral lymphoid tissues until they encounter a specific antigen and become activated. They then quickly proliferate and differentiate into short-lived plasma cells, or they enter a germinal center reaction. Here they undergo somatic hypermutation and class switch recombination to produce more efficient antibodies, and differentiate into memory B cells and long-lived plasma cells.
CLP, common lymphoid progenitor; pro-B, pro-B cell; pre-B, pre-B cell; SHM, somatic hypermutation; CSR, class switch recombination.
Modified from LeBien et al. 2008 [55].

such as stem cell factor (SCF), stromal cell-derived factor 1 (SDF-1) and interleukin (IL)-7, they develop through several stages: common lymphoid progenitor, early and late pro-B cell, large and small pre-B cell and immature B cell. Several transcription factors are important in promoting the commitment to the B-cell lineage. Perhaps the most important transcription factor is Pax5 (paired box protein 5). Pax5 is expressed on all B cells but is down-regulated in plasmablasts and plasma cells [20, 70].

Fig. 1 summarises B-cell development and differentiation. The early phase of B-cell development is characterised by the assembly of the B-cell receptor (BCR). It is made up of immunoglobulin expressed on the cell-surface and is necessary for the specific recognition of antigen. Its diversity and thus the broad primary antibody repertoire is ensured by a stepwise rearrangement of its V, D and J segments. First, the heavy chain locus is rearranged. Together with surrogate light chains, this leads to transient expression of the pre-B cell receptor. In a second step, the segments of the light chain locus are rearranged [122]. Only those cells expressing a functional antigen receptor receive signals to further proliferate and differentiate (positive selection). They also undergo negative selection to maintain self-

tolerance, meaning they are eliminated by apoptosis if they recognise self-antigen. The immature B cell now expresses surface IgM representing a functional BCR. CD19 and CD45R appear as the earliest B-lineage specific markers. [4].

3.2.2 Naive B-Cell Subsets

Immature B cells then leave the bone marrow. They are short-lived with a half-life of three days, and are therefore known as transitional type 1 (T1) B cells. They migrate to lymph follicles in the spleen where they differentiate into non-circulatory T2 B cells. They receive signals necessary for their survival and further differentiation, e.g. BAFF (B-cell activating factor belonging to the TNF family) and APRIL (a proliferation-inducing ligand) [18]. Cells then proceed to become mature B cells. Both IgM and IgD are expressed on mature B cells due to alternative splicing. Via another transitional stage called follicular type II cells, the majority of cells end up as follicular type I cells while a different subset becomes marginal zone B cells [110].

Follicular B Cells The mature naive follicular B lymphocyte leaves the spleen and recirculates via lymph and blood. It then continues to reenter secondary lymphoid tissues, i.e. spleen, lymph nodes and mucosa associated lymphoid tissues (MALT), until it encounters antigen or dies. When it encounters its specific antigen, it stops recirculating and starts to proliferate and differentiate.

Upon binding of antigen to the BCR, the complex is internalised and processed. Peptides are bound to major histocompatibility complex class II (MHC class II) and presented to helper T cells (T_H cells). Typically, B cells recognise polysaccharides, whereas T cells recognise peptides. B cell and specific T_H cell recognise the same antigen, but not necessarily the same epitope (linked recognition). The two cells adhere via LFA-1 and ICAM-1 (intercellular adhesion molecule 1), and the T cell provides CD40L (CD154) and IL-4, which are vital co-stimulatory signals for the B cell. In the medullary cords of lymph nodes, they form a primary focus and proliferate vigorously. They either differentiate directly into short-lived antibody-secreting plasma cells or enter germinal center reactions [55].

Some of the activated B cells migrate to a primary lymph follicle. The proliferating cells form a germinal center within the follicle (secondary lymph follicle). Here the cells go through three processes to assure a more effective later phase of the immune response: By somatic hypermutation, those cells producing antibodies with high specificity for the antigen are selected. Affinity maturation ensures the production of antibodies with high affinity for their epitope. Furthermore, an antibody class switch occurs. These well-adapted cells differentiate into antibody-secreting plasmablasts, and further into short-lived and long-lived plasma cells, or they differentiate into memory B cells [64, 74].

Marginal Zone B Cells This type of naive B cells resides in the marginal sinus of the spleen. CD1d is expressed as a characteristic surface marker. They are able to respond directly to specific antigen in the absence of T-cell help and differentiate within a few days into antibody-secreting cells [110].

B1 B Cells While follicular and marginal zone B cells are known as B2 B cells, there exists another subset called B1 B cells. These cells have so far only been found in mice and it remains disputable whether they also exist in humans. They are assumed to stem from progenitor cells leaving the B-cell developmental path at the level of the pre-pro-B cell. They reside within the peritoneal cavity [25].

B1a B cells are $CD5^+$. They are characterised by their ability to differentiate into plasma cells in the absence of antigen, and produce so-called natural antibody (low-affinity polyvalent IgM). Therefore they are considered as part of the innate immune system. B1b B cells are $CD5^-$. Similar to marginal zone B cells, they differentiate into ASC in the presence of T-cell independent antigens [55, 110].

B10 B Cells Recent studies have unravelled yet another B cell subset, the B10 B cell or regulatory B cell. Similar to B1 B cells, their presence has only been proven in mice, but they are assumed to also exist in humans. They originate from an unknown progenitor cell. Both CD1d and CD5 are expressed, combining features of marginal zone B cells and B1 B cells. Their characteristic is strong secretion of immunomodulatory cytokines, especially IL-10, through which they are able to limit inflammation and enhance the activity of Treg cells [24, 113].

3.2.3 Plasma Cells

Some of the proliferating follicular B cells in the germinal center differentiate into antibody-secreting cells (ASC, plasmablasts). They are able to further divide and interact with T cells. After a few days, they follow one of two fates: they either stop dividing and die, or they differentiate further into plasma cells. Under the influence of PRDM1 (PR domain zinc finger protein 1), they end proliferation, affinity maturation and class switch, and specialise in the production of large amounts of antibodies. They show all characteristics of cells with high protein synthesis such as loose chromatin and abundant rough endoplasmic reticulum. Certain surface markers, e.g. CD20 and human leukocyte antigen (HLA), are down-regulated, while other typical markers such as CD38 and CD138 appear. Some plasma cells remain in the peripheral lymphoid organs and are short-lived, whereas the majority migrate to the bone marrow. These are long-lived and can maintain antibody synthesis for years, possibly for a lifetime [28, 101].

3.2.4 Memory B Cells

Other activated germinal center B cells differentiate into memory B cells. Those express high levels of MHC II molecules enabling them to recognise and present antigen. Furthermore, they are characterised by increased levels of co-stimulatory surface molecules. This enables the interaction with T_H cells at lower concentrations of antigen than necessary for naive B cells. Therefore, if during a secondary immune response, they encounter their antigen, they can differentiate more quickly into plasma cells. Class-switched, affinity-maturated immunoglobulins (Ig, mostly IgG and IgA) can then immediately be synthesised. Memory B cells are $IgD^{-}CD27^{+}$ [100, 107].

3.3 B-Cell Stimulation Using CpG Oligodinucleotides

CpG 2006 was used in this study to stimulate B cells to differentiate into antibody-secreting cells. CpG oligodeoxynucleotides (ODN) were discovered in 1995 by Krieg et al. and found to be highly immunostimulatory [50]. They are synthetically produced short sequences of unmethylated DNA containing numerous repetitions of a cytosine-guanine motif. The p indicates the presence of a phosphorothioate backbone instead of a phosphodiester backbone to prevent degradation by nucleases. Many classes and types of CpG ODN have been discovered and synthesised since 1995. CpG 2006 has the sequence 5'-TCGTCGTTTTGTCGTTTTG-TCGTT-3'. It belongs to the CpG class B that acts especially on B cells [39, 51, 31].

Unmethylated CpG ODN act as pathogen-associated molecular patterns (PAMP) because they are frequently found in bacterial DNA but are uncommon in the genome of vertebrates. PAMP (other PAMP include lipopolysaccharides, lipoproteins and peptidoglycans) are recognised by pattern recognition receptors (PRR). A major subset of PRR are the so-called Toll-like receptors (TLR). CpG is recognised by TLR9 [49].

TLR9 is expressed on B cells and dendritic cells. Ligation leads to activation of transcription factors such as nuclear factor-κB (NF-κB) and thereby promotes activation, proliferation and differentiation of B cells to ASC [117]. CpG-activated B cells up-regulate co-stimulatory molecules such as CD40, CD54, CD58, CD80, CD86, MHC class II and Fcγ receptors. Furthermore, they secrete IL-6 and IL-10, which are essential interleukins for induction of antibody production [35].

3.4 Pemphigus vulgaris

Pemphigus vulgaris is an autoimmune disease causing blistering of the skin and mucous membranes. Typically, lesions first appear in the oral mucosa [46]. Then they spread to the trunk, scalp and flexures. Blisters are fragile, they rupture easily and leave extended erosions. Often crusted erosions are the only efflorescences to be observed. In the pre-steroid era, pemphigus had a fatal prognosis with patients dying from uncontrolled fluid and protein

loss, bacterial infection and sepsis [9]. Fig. 2 shows an exemplary photograph of the clinical manifestation.

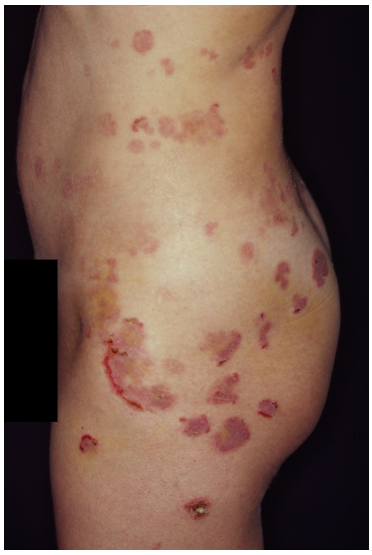

Figure 2 – Pemphigus vulgaris.
Picture of a patient with pemphigus vulgaris. Note erosive lesions with crusting and bleeding. Photo courtesy of Department of Dermatology, Charité — Universitätsmedizin Berlin.

The histology from affected skin samples shows suprabasal intraepidermal cleft formation due to loss of cell adhesion of keratinocytes (acantholysis). Intraepidermal deposits of IgG can be detected by direct immunofluorescence [121]. The hallmark of disease are pathogenic autoantibodies directed against desmoglein 1 and 3. Desmogleins are structural proteins and are, as part of the desmosomes, responsible for cell–cell adhesion [44]. The pathogenic character of these autoantibodies has been demonstrated by transfer of purified anti-Dsg3-antibodies from patient sera to neonatal mice [5].

Modern therapy is based upon suppression of the autoaggressive immune process. Immunosuppressants include high-dose corticosteroids, cyclophosphamide, azathioprine, intravenous immunoglobulin (IVIg) and rituximab [3, 88, 106]. Hematopoietic stem cell transplantation involving conditioning regimens including ATG has also been used successfully to treat drug-resistant pemphigus [109].

4 Aims and Objectives

Thymoglobulin® has been in clinical use for decades and still plays an important role as part of immunosuppressive regimens in solid organ and stem cell transplantation. The mechanism of action, however, is still not fully understood. One aspect seems to be the antibody-mediated depletion of T cells when administered in high concentrations. But other aspects have been discovered too, for example the modulation of cell surface receptors or the induction of Treg cells at low concentrations of ATG [68]. Recently, there has been a focus on induction of apoptosis by ATG in B cells and plasma cells. Little is known about the impact of ATG on the humoral immune system at sub-depleting doses.

The aim of this work was to investigate the impact of ATG on B-cell activation at sub-depleting concentrations and to focus on its immunomodulatory properties. Special consideration was given to the analysis of immunoglobulin synthesis, B-cell homeostasis and survival. Furthermore, the aim was to gain insight into the potential use of ATG in B-cell mediated diseases, such as the autoimmune blistering skin disease pemphigus vulgaris.

5 Material and Methods

5.1 Donors

Peripheral blood mononuclear cells (PBMC) and B cells were isolated from leukocyte filters that were obtained from the blood bank of the Institute of Transfusion Medicine, Campus Mitte, Charité — Universitätsmedizin Berlin. Full blood from healthy donors was centrifuged and thereby separated into a layer of plasma, a layer of erythrocytes and an intermediate layer containing thrombocytes and leukocytes, the so-called buffy coat. By filtering, the buffy coat is depleted of leukocytes to obtain thrombocyte concentrates [61]. These filters were used as sources for PBMC and B cells.

Peripheral venous blood was obtained from a 23-year old male patient recently diagnosed with pemphigus vulgaris. He was an inpatient in the Department of Dermatology, Campus Mitte, Charité — Universitätsmedizin Berlin, awaiting the first cycle of dexamethasone/cyclophosphamide pulse therapy. Further blood samples were obtained from other donors with different autoimmune diseases. The patients were thoroughly informed about the study and gave written consent.

All procedures were approved by the local ethics committee (Charité — Universitätsmedizin Berlin, Campus Mitte) and conform to the Declaration of Helsinki.

5.2 Materials

5.2.1 Thymoglobulin

Rabbit Antithymocyte Globulin (rATG, Thymoglobulin®) was kindly provided by Genzyme. Sterile, lyophilised Thymoglobulin® was reconstituted in 5 ml sterile aqua ad injectionem according to the manufacturer's instructions. The reconstituted solution then contains 5 mg/ml antithymocyte globulin (> 90% rabbit IgG), 10 mg/ml glycine, 10 mg/ml mannitol and 2 mg/ml sodium chloride. It was aliquoted and stored at −80 °C before further use. Rabbit IgG (rIgG) from non-immunised rabbits was used as control.

$F(ab')_2$ fragments of ATG were prepared by Pineda Antikörper-Service. Briefly, ATG was digested by pepsin, which cleaves immunoglobulin molecules into one $F(ab')_2$ fragment and many small pieces of the Fc fragment, and passed over a protein A column to remove Fc fragments. $F(ab')_2$ fragments of rabbit IgG (Jackson ImmunoResearch) were used as controls. Photometric analysis of protein concentration at 280 nm revealed that protein concentration in native ATG was equal to fragmented ATG. Because a given quantity of $F(ab')_2$ fragments contains twice as many reactive molecules as the whole IgG, they were used at half the respective concentration.

5.2.2 Other Material

All materials such as chemicals, buffers, antibodies, primers, and consumables, technical equipment and software, along with manufacturer's addresses, are listed in the respective tables in section A.1 List of Materials.

5.3 Methods

5.3.1 PBMC and B Cell Isolation

Leukocyte filters from the blood bank were back-flushed with sterile phosphate buffered saline (PBS) to elute the leukocytes. PBMC were isolated by standard Ficoll-Hypaque density gradient centrifugation. The method takes advantage of the differences in density of the various components of peripheral blood. The density of Ficoll, a highly branched polysaccharide, is similar to that of mononuclear cells, so that they form a phase on top of the Ficoll layer that can be easily removed with a pipette [32]. Cells were then depleted of thrombocytes by centrifugation at $200 \times g$, and rinsed repeatedly with PBS followed by centrifugation steps at $300 \times g$ (Fig. 3). Cells were counted using CASY®, an electronic cell counter, and resuspended in culture medium as described below.

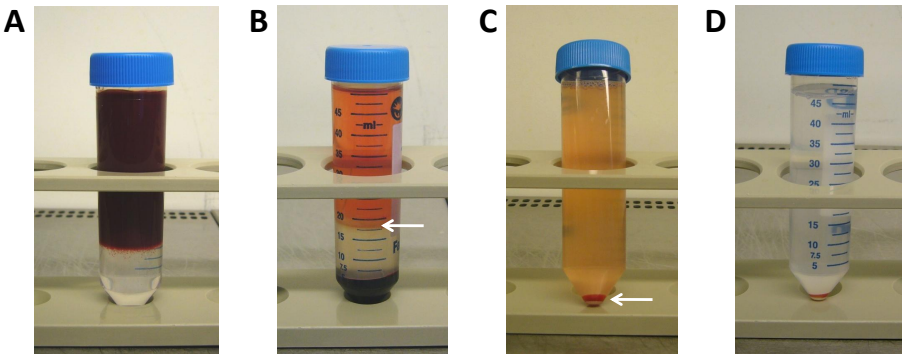

Figure 3 – Isolation of PBMC using density gradient centrifugation.
(A) Heparinised whole blood is diluted with PBS and gently layered over 15 ml Ficoll-Hypaque. (B) After centrifuging for 20 min at $450 \times g$ at room temperature, the erythrocytes and granulocytes are found at the bottom of the tube. Mononuclear cells float on top of the Ficoll layer (arrow). The plasma forms the uppermost phase. (C) After the first washing step at $200 \times g$ for 10 min at 4 °C, the thrombocytes, cell debris and Ficoll are separated from the PBMC, which form a pellet at the bottom of the tube (arrow). (D) After further washing, cells are cleared from all debris, and can be resuspended in culture medium.

B cells were positively selected by immunomagnetic separation. Cells are labelled with antibodies that are conjugated with paramagnetic beads and that recognise CD19, a B cell-specific antigen. To avoid unspecific binding, cells are stained in a buffer containing autologous serum. Cells are passed over a separation column in a magnetic field. Labelled cells stay within the magnetic field whereas unlabelled cells flow through and are collected as the negative fraction. The positive fraction is collected by removing the column from

the magnetic field and flushing with buffer to elute the cells. To yield higher purity, the positive fraction is passed over a second column. Purified cells are counted, rinsed with PBS and resuspended in culture medium. A more detailed protocol can be found elsewhere [95]. Purity was assessed by flow cytometry and was found to be greater than 99% (Fig. 4).

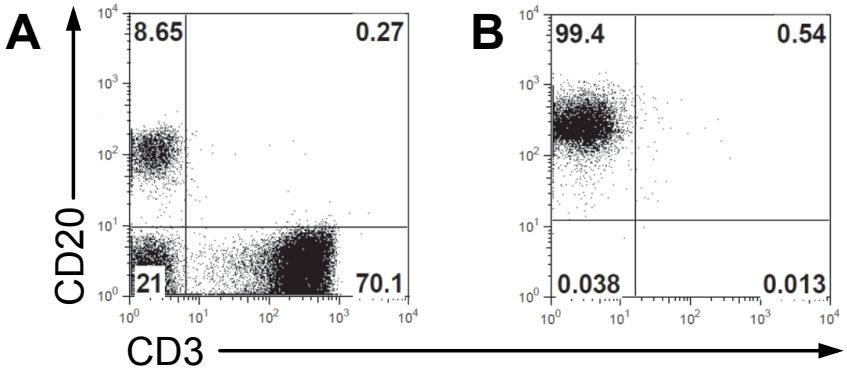

Figure 4 – Assessment of purity after MACS isolation.
(A) Freshly isolated PBMC. The majority consists of T cells ($CD3^+$); B cells ($CD20^+$) make up only a small fraction of PBMC. Other cells include monocytes and NK cells. (B) After immunomagnetic separation, B cells are enriched to a purity of over 99%.

5.3.2 Cell Culture

After counting, PBMC and B cells were centrifuged and resuspended in culture medium at a density of 10^6 cells/ml. Culture medium RPMI 1640 (Roswell Park Memorial Institute) was supplemented with 10% heat-inactivated fetal calf serum (FCS) to supply necessary proteins and growth factors, and 100 U/ml penicillin and 100 μg/ml streptomycin were added to prevent bacterial contamination. Cells were stimulated with CpG 2006 at the optimal concentration of 3 μg/ml. The cell suspension was seeded on flat-bottomed 24-well suspension culture plates, 1 ml per well. They were kept in an incubator for 8 days at 37 °C in a water-saturated atmosphere containing 5% CO_2. At the end of that period, cells and medium were aspirated off the plate and centrifuged. Cells were harvested for flow cytometry, and supernatants were collected and stored at −80 °C for further analysis.

Patients' PBMC for analysis of specific antibody production were cultivated using a protocol of triple stimulation with 3 μg/ml CpG 2006, 1 μg/ml anti-CD40 monoclonal antibody (MoAb) and 2 μg/ml anti-B cell receptor ($F(ab')_2$ fragments of anti-human IgA/IgG/IgM). 3 ml of cell suspension were seeded on 6-well suspension culture plates at 5×10^6 cells/ml and cultivated for 9 days. Supernatants were collected for detection of specific antibodies and stored at −80 °C.

5.3.3 Proliferation Assay

Cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) prior to culture, and proliferation was analysed on day 8 by flow cytometry. CFSE is a green fluorescent dye that is stably incorporated into lymphocytes by binding to intracellular proteins. With each cell division, the dye is equally distributed on the daughter cells, thereby dividing fluorescence in half, which can then be assessed by flow cytometry. In this way, up to eight divisions can be registered before intensity of fluorescence fades into the autofluorescence of unlabelled cells [72].

Freshly isolated PBMC or B cells are resuspended in 1 ml PBS. 1 μl CFSE is added to give a final concentration of 10 nM. Cells are incubated at room temperature for 1 min. The reaction is stopped by adding RPMI medium containing 10% FCS. Cells are centrifuged and resuspended in culture medium.

5.3.4 Assessment of Cell Viability

Nonviable cells were detected based on the dye exclusion technique using trypan blue (Fig. 5). Viable cells have an intact cell membrane that is impermeable for trypan blue. In apoptotic or necrotic cells, the integrity of the cell membrane is compromised, and the dye diffuses into the cell. Nonviable cells appear dark blue under a light microscope.

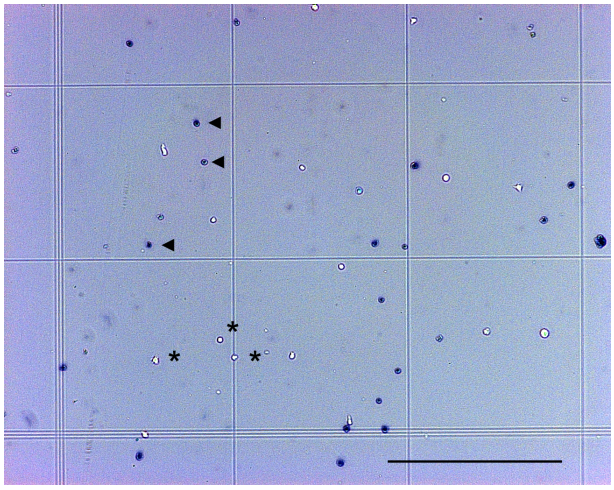

Figure 5 – B cells after 8 days of culture, stained with 0.2% trypan blue.
Viable cells (asterisks) exclude the dye, whereas nonviable cells (arrowheads) are stained dark blue. Scale bar, 25 μm.

An aliquot of the cell suspension was mixed with an equal volume of 0.4% trypan blue in PBS, and both viable and nonviable cells were counted in a hemocytometer with improved Neubauer ruling after 3 min of staining. Cells were counted in four squares at a magnification of 100×, and the average was calculated. The number of cells per microliter was calculated using the following formula:

$$\text{Cells}/\mu\text{l} = \frac{\text{average cell number} \times \text{dilution factor}}{\text{chamber volume}}$$

$$= \frac{\text{average cell number} \times 2}{0.1\mu\text{l}}$$

Nonviable cells were also detected by flow cytometric analysis of phosphatidylserine expression. Cells going into apoptosis change the constitution of their cell membrane. This results in the exposure of phosphatidylserine on the outer membrane surface. Annexin V is a ubiquitous protein binding phosphatidylserine in the presence of calcium. Annexin V conjugated to the green fluorescent dye fluorescein isothiocyanate (FITC) allows the detection of apoptotic cells with a flow cytometer [67]. Propidium iodide (PI) is a red fluorochrome binding to DNA. Analogously to the dye exclusion technique using trypan blue, viable cells are impermeable to PI, whereas nonviable cells permit the dye to pass through the membrane and can be visualised by flow cytometric analysis [21].

After the appropriate culture period, freshly harvested cells were washed with PBS and resuspended in 100 μl of a special calcium-containing binding buffer. They were incubated with FITC-conjugated annexin V. After that the cells were not washed, but 400 μl of binding buffer was added to stop the reaction. Propidium iodide (PI) was added immediately before analysis. Viable cells appear on the flow cytometer as FITC^-PI^- events, apoptotic cells are FITC^+PI^-, and necrotic cells are FITC^+PI^+.

5.3.5 Analysis of Cell Surface Molecule Expression by Flow Cytometry

The flow cytometer is a versatile instrument for characterising immune cells. A stream of fluid carrying single cells passes one or several lasers. The scattered light is caught by detectors providing information about size and granularity of the cell. Cells can be labeled with antibodies specific for certain antigens on the cell surface and conjugated with fluorochromes. The laser will then excite the fluorochrome to emit light at a characteristic wavelength. This light is registered by the detectors. A computer integrates the information, and the pattern of fluorescent signals for a given cell allows conclusions to be drawn about the type of cell and its expression of surface molecules [41].

Harvested cells at the end of the culture period were washed twice with PBS at 4 °C and resuspended in FACS buffer. They were then incubated with the respective FACS antibodies for 10 min at room temperature. Beriglobin®, a polyclonal antibody against the hepatitis A virus, was added to stabilise binding of FACS antibodies and to reduce unspecific binding. Cells were washed with PBS, resuspended in FACS buffer and kept at 4 °C protected from light until flow cytometric analysis. If appropriate, PI was added immediately before analysis

to exclude nonviable cells. All flow cytometric analyses were performed on a FACSCalibur® dual laser cytometer using CellQuest acquisition and FlowJo analysis software.

FACSort was performed on a FACSAria® under sterile conditions.

5.3.6 Assays for Immunoglobulin Production

ELISA Supernatants of PBMC and B-cell culture were analysed for the presence of IgM, IgA and IgG by enzyme-linked immunosorbent assay (ELISA, sandwich-ELISA). This assay involves antibodies specific for the antigen to be detected. These are conjugated with an enzyme that catalyses a reaction producing a dye. The amount of this dye is proportional to the amount of antigen present (Fig. 6).

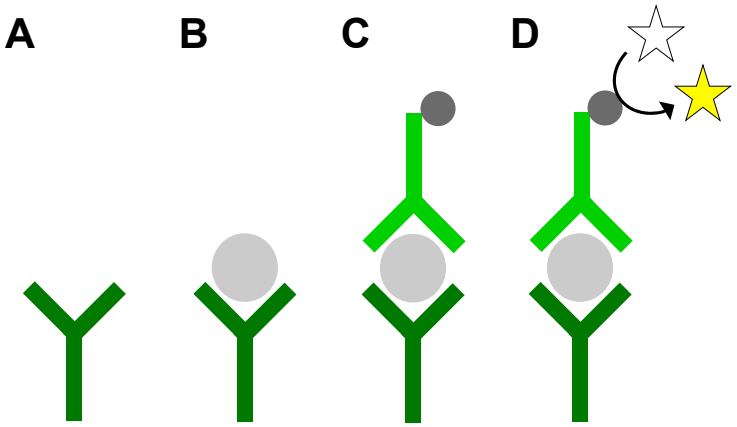

Figure 6 – The principle of enzyme-linked immunosorbent assay (ELISA).
(A) 96-well plates are coated with antibodies recognising the analyte. (B) Plates are incubated with the culture supernatants, and only the analyte binds to the coating antibody. (C) The secondary antibody is also specific for the analyte and binds to it. (D) An enzyme that is conjugated to the secondary antibody catalyses the reaction of a colourless chromogenic substance to a yellow product.

MaxiSorb® ELISA plates were coated and kept overnight at 4 °C with the appropriate primary antibody (goat anti-human IgA, IgG, or IgM) in 0.1 M bicarbonate buffer. Unspecific binding sites were blocked using 2% bovine serum albumin (BSA) in Tris buffered saline (TBS) for 1 h. After washing with TBS containing 0.05% Tween 20 (a detergent), supernatants and internal standards were incubated in duplicate for 2 h at room temperature. Plates were washed again, and incubated with secondary antibodies (alkaline phosphatase-conjugated goat anti-human IgA, IgG, or IgM) in 0.2% BSA/TBS for 2 h at room temperature. Alkaline phosphatase catalyses the reaction of *para*-nitrophenylphosphate, a colourless chromogenic substance, to *para*-nitrophenol, a yellow pigment. Following a final washing step and the reaction with phosphatase substrate, optical density was measured in a micro plate ELISA reader at 405 nm, and the amount of the respective Ig was calculated according to the standard curve.

Specific antibodies (anti-Dsg1, anti-Dsg3, anti-Ro, anti-ds-DNA) were detected using commercially available ELISA kits (MESACUP Desmoglein TEST "Dsg1", "Dsg3"), with minor modifications.

ELISpot Assay The enzyme-linked immunosorbent spot assay (ELISpot assay) allows for the enumeration of antibody-secreting cells. After 6 days of culture, B cells were transferred onto 96-well MultiScreen filter plates. Plates had been prepared by equilibrating with 35% ethanol, coated and kept overnight at 4 °C with goat anti-human IgG in bicarbonate buffer and blocked for 1 h with 3% BSA/PBS. Cells were applied in serial dilution of 1 : 2. Cells were incubated for 6 h at 37 °C. Plates were washed with PBS/Tween 0.05% and incubated with the secondary antibody (biotinylated anti-IgG in 0.3% BSA/PBS) overnight at 4 °C. Plates were washed and incubated with streptavidin-horseradish peroxidase for 45 min. Streptavidin binds covalently to biotin. Horseradish peroxidase catalyses the oxidation of a colourless chromogenic substance, in this case 3-amino-9-ethyl-carbazole, in the presence of hydrogen peroxide as the oxidising agent to a red product. After washing and developing with 3-amino-9-ethyl-carbazole in dimethylormamide, spots were counted with the ELISpot plate reader.

5.3.7 Real-Time Reverse-Transcription PCR

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) was used to analyse gene expression. In a first step, all ribonucleic acid (RNA), including messenger RNA (mRNA), is extracted from cells using a silica membrane. RNA is then transcribed into complementary deoxyribonucleic acid (cDNA) with reverse transcriptase. In a third step, cDNA is selectively amplified by means of PCR, involving specific primers for the desired gene. In order to quantify the amplified product, the absorption of a fluorescent dye that intercalates with double-strand DNA is detected. A reference gene, the so-called housekeeping gene, is used to normalise the expression of the target gene.

RNA Isolation RNA was purified from cultured cells using the commercially available kit NucleoSpin® RNA II by MACHEREY-NAGEL. At the end of culture, cells were lysed by adding the lysis buffer RA1 and β-mercaptoethanol. The lysates were stored at -80 °C until further processing. On the day of analysis, samples were filtered and 70% ethanol was added. They were passed onto a silica membrane where both DNA and RNA bind. DNA was digested by adding DNase. After several washing steps, RNA could be eluted in RNase-free H_2O.

cDNA Synthesis RNA was transcribed to cDNA with the TaqMan® Reverse Transcription kit by Applied Biosystems. For each sample, 7.7 μl of RNA was added to 12.3 μl of master mix containing TaqMan RT buffer, $MgCl_2$, deoxyNTPs, random hexamer primer,

oligo $d(T)_{16}$ primer, RNase inhibitor and MultiScribe reverse transcriptase. Reverse transcription was performed in a thermal cycler under the following conditions: 10 min at 25 °C (primer incubation), 40 min at 48 °C (reverse transcription) and 5 min at 95 °C (reverse transcriptase inactivation).

Real-Time PCR qPCR was performed using the LightCycler® system by Roche. cDNA was used at a dilution of 1 : 4 in H_2O. 2 μl of cDNA was added to 3 μl of master mix in a glass capillary. The master mix consisted of LightCycler® reaction mix, $MgCl_2$, the primer pair and H_2O. SYBR® Green I was used as the fluorescent dye. PCR was performed in a LightCycler® 1.5. Conditions for PCR are summarised in Table 3.

Table 3 – PCR Conditions

Mode	Cycles	Segment	Temperature	Hold Time
Pre-Incubation	1		95 °C	10 min
Quantification	40	Denaturation Annealing Extension	95 °C 58–65 °C 72 °C	10 s 8–10 s 10 s
Melting Curve	1	Denaturation Annealing Melting	95 °C 65 °C 0.1 °C/s	0 s 60 s
Cooling	1		40 °C	30 s

Quantification Hypoxanthine-guanine-phosphoribosyltransferase (HPRT), a ubiquitous enzyme from purine metabolism, was chosen as reference gene. Relative expression levels (R) were calculated according to the following formula, where E is qPCR efficiency of gene transcript, CP is the crossing point of the melting curve, target is the target gene, and ref is the reference gene:

$$R = \frac{(E_{target})^{CP_{target}}}{(E_{ref})^{CP_{ref}}}$$

5.4 Statistical Analysis

Statistical analysis was performed after consultation with Dipl. math. Christine Gericke, Institute of Biometry and Clinical Epidemiology, Campus Mitte, Charité — Universitätsmedizin Berlin in accordance with the canon of the institute.

The two-sided Mann-Whitney *U*-test was applied for comparison of experimental groups. This is a non-parametrical test, which is necessary because of the small sample size where Gaussian distribution cannot be assumed. The test is unpaired—even though cells in the respective experimental groups originate from the same donor, they are not the same cells.

Data on antibody secretion was expressed in the figures as percentage of CpG control group. Cells in this group were only stimulated with CpG, and not treated with either ATG or rIgG. They were normalised to 100% and Ig levels of other groups were compared to that. This was done in order to improve clarity of data because of wide inter-donor variance of immunoglobulin production. In this case, the one-sided t-test was applied to compare the sample mean to the mean of the CpG group (100%).

Statistical analysis was performed using GraphPad Prism 5. Differences were considered to be statistically significant at $P < 0.05$.

6 Results

6.1 Immunoglobulin Secretion in ATG-treated PBMC Cultures

To investigate the impact of ATG on the humoral immune response, PBMC from healthy volunteers were incubated with ATG at different concentrations. Immunoglobulin production was induced by stimulation with CpG, and the supernatants were analysed by ELISA at the end of culture. Unspecific IgG from non-immunised rabbits (rIgG) was used as a control. Data was expressed as percentage of CpG which was normalised to 100%. Normalisation was performed to compensate inter-individual variation.

Unstimulated cells produced very little IgG (1.9%). CpG induced IgG to 762 μg/dl. At a concentration of ATG of 5 μg/ml, secretion of IgG was reduced to 26.5% of CpG control ($P < 0.05$). Even with 0.1 μg/ml of ATG, secretion of IgG was reduced to 45.9%. With the lowest concentration of 0.01 μg/ml, IgG secretion was strongly elevated to 284.2% of CpG control. It is of note that control rIgG also led to a reduction of antibody secretion (57.7% at 5 μg/ml of rIgG, statistically not significant). At 0.1 and 0.01 μg/ml of rIgG IgG levels were 78.3 and 81.5% (Fig. 7 A).

Fig. 7 B shows levels of IgM in supernatants of PBMC cultures. Baseline secretion of IgM of unstimulated PBMC was 1.6%. Under CpG stimulation, 788 μg/dl was detected. IgM secretion was lowered to 63.1% of CpG control at 5 μg/ml of ATG. With 0.1 μg/ml of ATG, IgM levels were 70.0%, and with 0.01 μg/ml 98.6%. 5 μg/ml of rIgG led to a decrease of IgM secretion to 87.7%. At 0.1 μg/ml, IgM was 93.1%, at 0.01 μg/ml 72.5%.

Also the secretion of IgA was lower in the presence of ATG (Fig. 7 C). With 5 μg/ml ATG, IgA levels were 67.3% of CpG control. At 0.1 μg/ml ATG 67.8%, and at 0.01 μg/ml 108.7% of IgA was determined in comparison to the control. In the presence of 5 μg/ml of rIgG, the IgA concentration was 105.4% of CpG, at 0.1 μg/ml 99.2%, and at 0.01 μg/ml 67.3%. The baseline level of IgA in supernatants of unstimulated cells was 7.8% of CpG-stimulated cells (82 μg/dl).

IgE was not detectable in cell culture supernatants (data not shown).

Taken together, the results show a dose-dependent decrease of immunoglobulin production in the presence of ATG that was observed when PBMC were stimulated with CpG. This finding was determined for different isotypes including IgA, IgM, and IgG.

6.2 Immunoglobulin Secretion in ATG-treated B Cells

The next step was to determine whether the reduction of Ig levels in PBMC cultures shown above was dependent on T cells or monocytes, or if it was related to direct action of ATG on B cells. $CD19^+$ B cells were positively selected by immunomagnetic cell sorting and cultured for eight days. Again, immunoglobulin production was induced by CpG. Total IgG in the supernatants was measured by ELISA. In this set of experiments, only IgG was determined

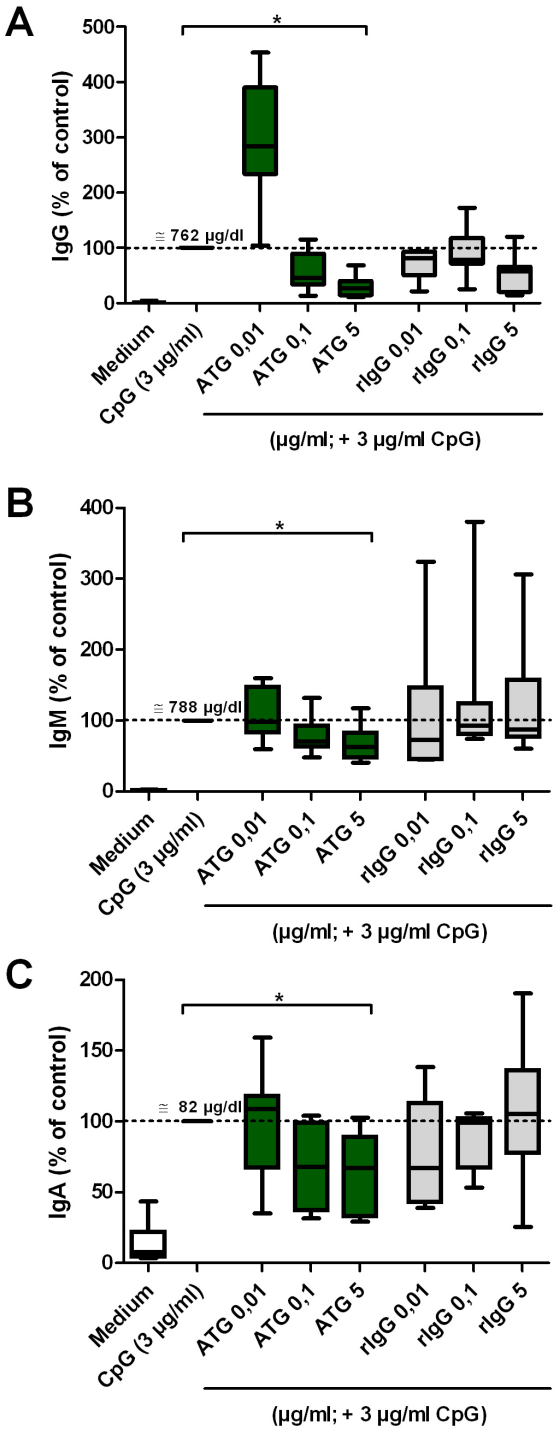

Figure 7 – Decreased immunoglobulin secretion in ATG-treated PBMC cultures.
PBMC were stimulated with 3 μg/ml CpG and incubated for 8 days with ATG or rIgG at concentrations of 0.01–5 μg/ml. Unstimulated cells and cells treated only with CpG were used as controls. Secreted immunoglobulin in supernatants was measured by ELISA. (A) IgG, (C) IgM, (E) IgA. Results are normalised to CpG control and depicted as box-and-whisker-plots: minimum, 25th percentile, median, 75th percentile, maximum; n = 9. Asterisk, $P < 0.05$.

because the data from PBMC had shown the most pronounced results on IgG production.

Baseline secretion of IgG by unstimulated B cells was 8.3% of control B cells stimulated with CpG (466 μg/dl). B cells treated with 5 μg/ml ATG secreted 61.6% of CpG control. At 0.1 μg/ml, IgG levels were 89.6%, and at 0.01 μg/ml 93.3% of CpG control. Under the influence of 5 μg/ml rIgG, IgG secretion was also slightly decreased, but to a lesser extent, to 88.2%. With 0.1 μg/ml rIgG, it was 82.4%, and with 0.01 μg/ml 88.3% (Fig. 8).

Cells that were not stimulated with CpG, but only treated with 5 μg/ml ATG produced only little IgG (10% of CpG control, data not shown).

These data suggest that the modulation of immunoglobulin production depends to a large extent on a direct action of ATG on B cells.

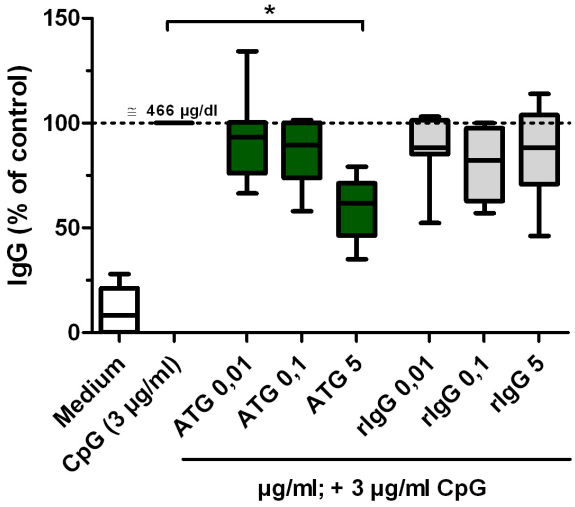

Figure 8 – Decreased IgG secretion in ATG-treated B-cell cultures.
$CD19^+$ B cells were stimulated with 3 μg/ml CpG and incubated for 8 days with ATG or rIgG at concentrations of 0.01–5 μg/ml. Unstimulated cells and cells treated only with CpG were used as controls. Secreted IgG in supernatants was measured by ELISA. Results are normalised to CpG control and depicted as box-and-whisker-plots: minimum, 25th percentile, median, 75th percentile, maximum; n = 9. Asterisk, $P < 0.05$.

6.3 Frequency of IgG-Secreting Cells

To address the question whether antibody secretion is reduced on an individual cell basis, or if the number of antibody-secreting cells (plasmablasts) is reduced, IgG ELISpot assay was performed. This method allows the detection of single IgG-secreting cells. B cells were stimulated with CpG and treated with 5 μg/ml ATG or rIgG. After 6 days of incubation, cells were transferred onto a MultiScreen filter plate coated with goat anti-human IgG and incubated for a further 24 hours. Then the cell spots were developed and counted.

Fig. 9 A shows a representative example of a developed ELISpot plate. Fig. 9 B shows the corresponding graph. In unstimulated B cell cultures, 1.3% antibody-secreting cells (ASC)

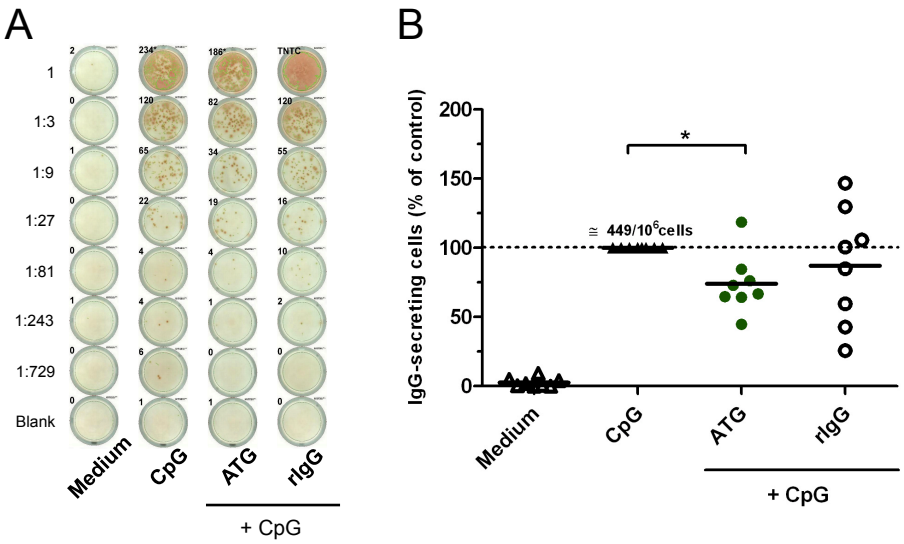

Figure 9 – ATG reduces the frequency of IgG-secreting cells.
B cells were stimulated with 3 μg/ml CpG and incubated for 6 days with 5 μg/ml ATG or rIgG. Unstimulated cells and cells treated only with CpG were used as controls. Cells were then transferred onto a MultiScreen filter plate coated with goat anti-human IgG and incubated for further 24 hours. Spots were developed with a peroxidase reaction, and counted with the ImmunoSpot Analyser. (A) Representative photograph of ELISpot plate. (B) Graphical depiction. Results are normalised to CpG control. Line indicates median, n = 8. Asterisk, $P < 0.05$

were detected. Upon CpG stimulation, there was an absolute number of $449/10^6$ cells. In ATG-treated cultures, only 69.9% ASC were detected, compared to CpG control. With control rIgG, 92.8% ASC were detectable.

6.4 F(ab')$_2$ Fragments of ATG

In order to investigate whether the modulation of ATG on Ig-secreting B cells is mediated by specific antibodies or unspecific binding of the Fc part of ATG to Fcγ receptors expressed on B cells, primary B cells were incubated with F(ab')$_2$ fragments of ATG. F(ab')$_2$ fragments are the specific antigen-binding part of immunoglobulin. They were prepared by pepsin digestion of ATG. F(ab')$_2$ fragments of rIgG were used as controls. Because a given quantity of F(ab')$_2$ fragments contains twice as many reactive molecules as the whole IgG, they were used at half of the respective concentration.

Fig. 10 shows the concentration of IgG in supernatants of B cells treated with F(ab')$_2$ fragments of ATG and rIgG as percent of CpG control (466 μg/dl, unstimulated cells 8.3%). With 2.5 μg/ml ATG F(ab')$_2$, IgG was 67.7%. With 0.05 μg/ml, it was 84.9%. Under the influence of 2.5 μg/ml rIgG F(ab')$_2$, the amount of IgG in the supernatant was 86.9%, and with 0.05 μg/ml 90.6%.

These results indicate that F(ab')$_2$ fragments of ATG are comparably effective in reducing

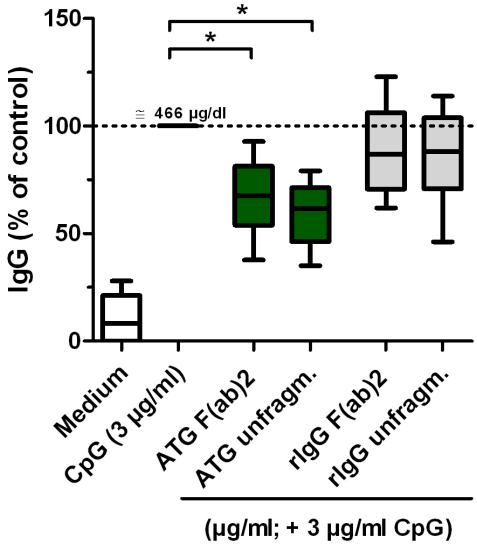

Figure 10 – F(ab')$_2$ fragments of ATG are comparably effective in reducing IgG secretion.
B cells were stimulated with 3 μg/ml CpG and incubated for 8 days with F(ab')$_2$ fragments of ATG or rIgG at concentrations of 0.05–2.5 μg/ml. Unstimulated cells and cells treated only with CpG were used as controls. Secreted IgG in supernatants was measured by ELISA. Results are normalised to CpG control and depicted as box-and-whisker-plots: minimum, 25th percentile, median, 75th percentile, maximum; n = 9. Asterisk, $P < 0.05$

IgG secretion, and these effects are not mediated by unspecific binding of the Fc part.

6.5 Proliferation in B Cells

As the previous findings demonstrate that the secretion of immunoglobulin is decreased under the influence of ATG, B-cell proliferation was assessed next by measuring CFSE-labelled B cells by flow cytometry after eight days (gated on viable cells).

Cultures of unstimulated cells displayed hardly any cells in the FACS gate of viable cells ($<$ 1% of total). This indicates poor survival without stimulation, making assessment of proliferation futile (data not shown). A higher percentage of cells survived in the presence of CpG stimulation, which caused a moderate proliferative response (39.1% proliferating cells, Fig. 11). When 5 μg/ml ATG was added, even more cells proliferated (61.7% proliferating cells). 44.2 % of cells treated with 5 μg/ml rIgG proliferated.

F(ab')$_2$ fragments of ATG were likewise able to induce an increased proliferation in B cells (data not shown).

6.6 B-Cell Survival

After finding out that the proliferation of B cells was elevated in the presence of ATG, it was of interest to explore the viability of the cells next. B cells were stimulated with CpG

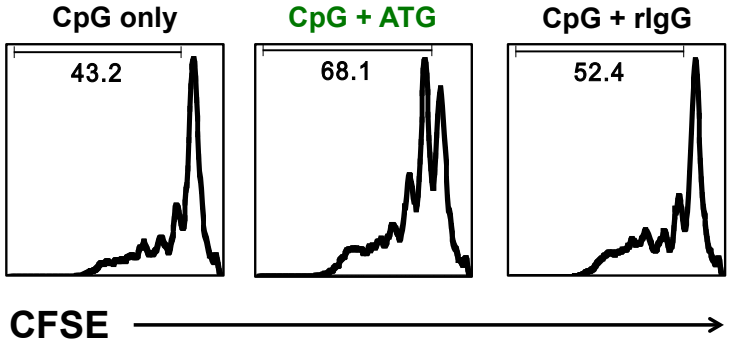

Figure 11 – ATG enhances rate of proliferation in B cells.
B cells were stimulated with 3 μg/ml CpG and incubated with 5 μg/ml ATG or rIgG. Cells treated only with CpG were used as control. Cells were labelled with CFSE prior to culture, and proliferation was analysed on day 8 by flow cytometry. Results are representative of three independent experiments.

and cultured for eight days as described above. Viability was determined by trypan blue staining and flow cytometric analysis of phosphatidylserine expression.

Trypan blue staining revealed no difference in cell death in B-cell cultures treated with low concentrations (5 μg/ml) of ATG (37.1% non-viable cells) compared to CpG control (42.7% non-viable cells) or rIgG control (35.8%, Fig. 12 A). Cell death in unstimulated B cells was 52.2%. At high concentrations (100 μg/ml) of ATG, however, the number of non-viable cells increased to 57.2% (statistically significant, $P < 0.05$). By contrast, 100 μg/ml of rIgG did not elevate the cell death rate (38.9%).

Flow cytometric analysis of phosphatidylserine expression confirmed that cell death was not elevated at 5 μg/ml ATG (76% annexin V^+ (non-viable cells), Fig. 12 B). CpG control was 79%, and rIgG control 77%. At 100 μg/ml of ATG, but not rIgG, elevated cell death was observed (95.1% vs. 82% annexin V^+).

6.7 Frequency of Memory B Cells and Plasmablasts

After determining that IgG secretion is decreased under the influence of ATG, which is related to a lower frequency of IgG-secreting cells without elevated cytotoxicity, further insight into the mechanism of action was to be obtained. Therefore, different stages of B-cell differentiation (memory B cells and plasmablasts) were analysed *in vitro* under the influence of ATG by performing flow cytometry. Memory B cells were defined as $CD20^+CD27^{lo}$, plasmablasts as $CD27^{hi}CD38^+$ and $CD27^{hi}CD138^+$. CpG-stimulated B cells were incubated for 8 days with 5 μg/ml ATG or rIgG and consecutively analysed by FACS. Cells were analysed in a gate of viable B cells.

As previous results had demonstrated that survival of unstimulated B cells is very poor, they were not analysed for this experiment. Survival in CpG-stimulated cells was moderate.

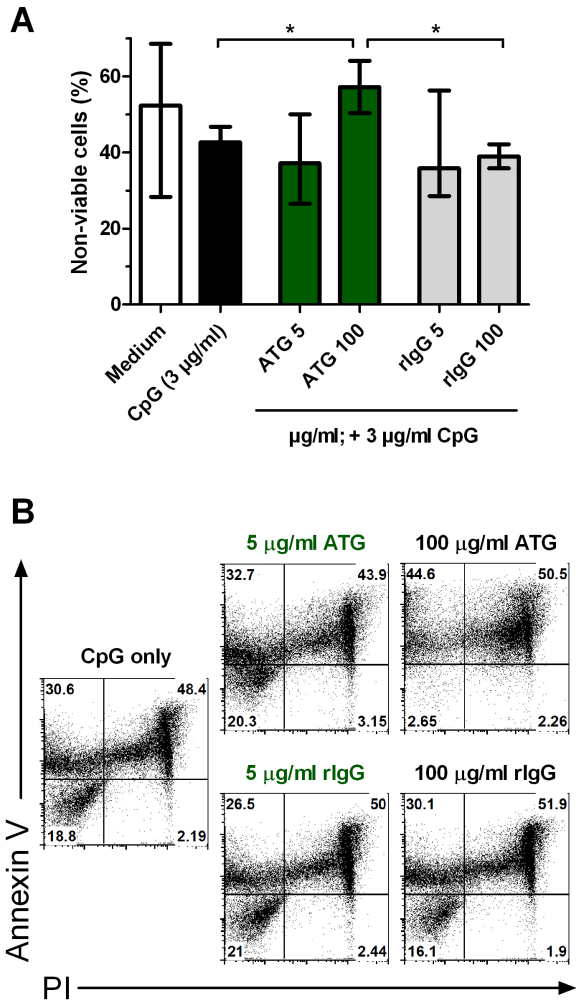

Figure 12 – No additional cell death at low concentrations of ATG.
B cells were stimulated with 3 μg/ml CpG and incubated for 8 days with ATG or rIgG at concentrations of 5–100 μg/ml. Unstimulated cells (data not shown) and cells treated only with CpG were used as controls. (A) Non-viable cells were stained with trypan blue and counted in a hemocytometer. Data represent median (range), n = 7. Asterisk, $P < 0.05$. (B) Cells were incubated with annexin V and PI and analysed by flow cytometry. Viable cells are annexin$^-$PI$^-$, apoptotic cells annexin$^+$PI$^-$ and necrotic cells annexin$^+$PI$^+$. Results are representative of three independent experiments.

CD20$^+$CD27^{lo} memory B cells were detected at a frequency of 28% in CpG-stimulated cells. Their frequency was 22% under the influence of ATG and 29% with rIgG (Fig. 13 A).

CpG as a stimulant was not able to induce the maturation of plasmablasts in all B cell cultures. CD27^{hi}CD38$^+$ plasmablasts were found in cultures of five out of nine donors. Their frequency was reduced from 7% (CpG stimulation alone) to 1% with ATG treatment. With rIgG, it was 5% (Fig. 13 B).

CD27^{hi}CD138$^+$ plasmablasts were detectable in three out of nine cultures. Their frequency with CpG stimulation alone was 6%. It was reduced to 1% under the influence of ATG. With control rIgG, their frequency was 7% (Fig. 13 C).

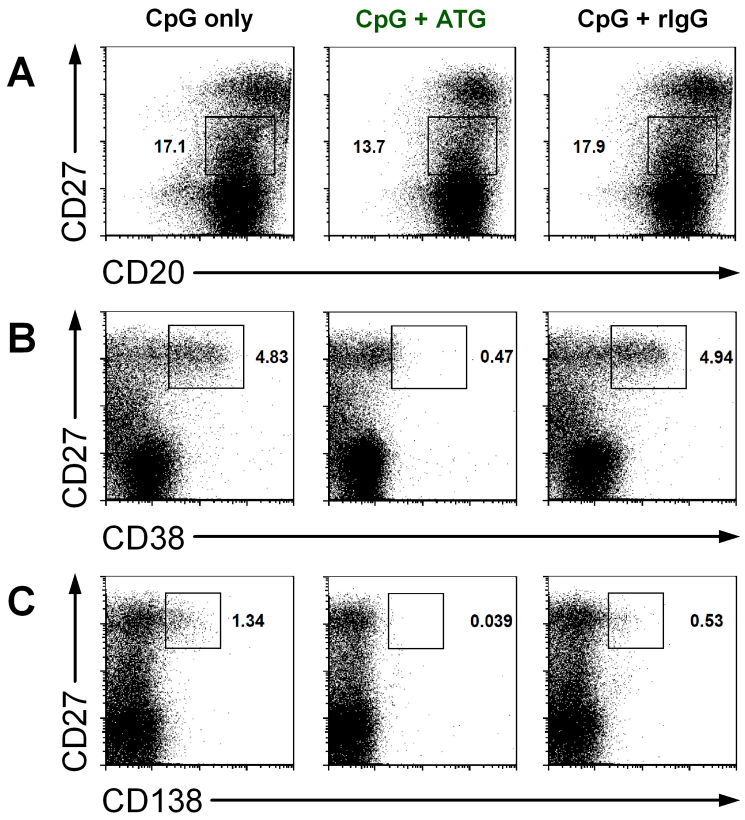

Figure 13 – ATG slightly reduces the frequency of memory B cells and strongly reduces the frequency of plasmablasts.

CpG-stimulated B cells were incubated for 8 days with or without 5 μg/ml ATG, or rIgG. Expression of surface molecules was analysed by flow cytometry. The gate was on viable lymphocytes. (A) $CD20^{+}/CD27^{lo}$ memory B cells, (B) $CD27^{hi}/CD38^{+}$ plasmablasts, (C) $CD27^{hi}/CD138^{+}$ plasmablasts. Results are representative of three to nine independent experiments.

6.8 Gene Expression of B-Cell Differentiation Markers

As the phenotypical analysis of B-cell subsets showed reduced frequency of memory B cells and particularly plasmablasts, disturbed differentiation of B cells under the influence of ATG was presumed. To further analyse these findings at the level of gene expression, quantitative real-time polymerase chain reaction (qRT-PCR) of differentiation markers was performed. To yield highest purity of the cell population of interest, $CD27^{+}$ memory B cells were sorted via flow cytometry. Afterwards, the memory B cells were stimulated with CpG and incubated with either 5 μg/ml ATG or rIgG. On days 4 and 8, qRT-PCR of different genes, which are important for B-cell differentiation and immunoglobulin synthesis including Pax5, IRF-4, PRDM1 and AID was performed. Due to limited cell numbers, there was no control group of cells that were only stimulated with CpG and not incubated with ATG or rIgG.

Pax5 is B cell specific and expressed in all cells belonging to the B-lymphocyte lineage, but is down-regulated during terminal differentiation towards plasmablasts and plasma cells (compare section 3.2.1 B Cell Development, Activation and Differentiation). IRF4 and

PRDM1 are up-regulated in plasmablasts. AID is expressed to promote class switch recombination. The chronological sequence of these differentiation markers is depicted in Fig. 14 A.

The relative expression of Pax5 mRNA on day 4 was 0.017 in ATG-treated cells, and 0.016 in rIgG-treated cells. On day 8, it was 0.008 with ATG, and 0.007 with rIgG (Fig. 14).

Relative expression of IRF-4 was 0.039 under the influence of ATG on day 4, and it was 0.043 with rIgG treatment. On day 8 it was 0.021 with ATG, and 0.022 with rIgG.

PRDM1 expression on day 4 was 0.18 with ATG and 0.15 with rIgG. On day 8, cells treated with ATG showed a relative expression of PRDM1 of 0.1, cells treated with rIgG showed relative expression of 0.12.

AID mRNA level was 0.36 on day 4 for ATG and 0.35 for rIgG. On day 8, AID mRNA was 0.29 for ATG and 0.32 for rIgG.

Taken together, these results suggest that the impact of ATG on B-cell differentiation via comparison to rIgG is not mirrored in the expression of the specific genes that were analysed in this experiment.

6.9 Secretion of Specific Autoantibody

The experiments so far demonstrate that the production of antibodies is decreased in B cells from healthy subjects. The question of interest, however, was whether this is also valid and relevant in a clinical setting, e.g. with patients suffering from an autoimmune disease who would possibly benefit from the treatment with ATG.

The generation of specific antibodies *in vitro* proved to be demanding. Ten patients from the outpatient clinic for autoimmune diseases of the Department for Dermatology and Allergy, Charité, Berlin, Germany, with high serum levels of pathognomonic antibody specificities gave a blood sample. Among these were six patients with systemic lupus erythematosus (four with anti-Ro antibodies, two with anti-ds-DNA antibodies), three patients with pemphigus vulgaris (anti-Dsg3) and one patient with pemphigus foliaceus (anti-Dsg1) (Tab. 4).

Specific antibodies could be measured after *in vitro* culture only in one out these ten patients. This young male patient with pemphigus vulgaris had been recently diagnosed with the disease and had high clinical disease activity with high titres of anti-Dsg3 (160 U/ml). He had not previously received any systemic immunosuppressive medication.

Because B cells specific for a certain antigen are found in peripheral blood only at low frequencies, patients' PBMC were cultivated at a high density of 1.5×10^7 cells per well. Using a protocol of triple stimulation involving CpG, anti-CD40 and anti-BCR, antibody production in PBMC *in vitro* was maximised to detect specific autoantibodies. Supernatants were analysed for production of anti-Dsg3 by ELISA.

Anti-Dsg3 autoantibodies were detectable after stimulation (6.7 U/ml, unstimulated 1.1 U/ml). Cells treated with ATG produced only 1.7 U/ml anti-Dsg3. In supernatants

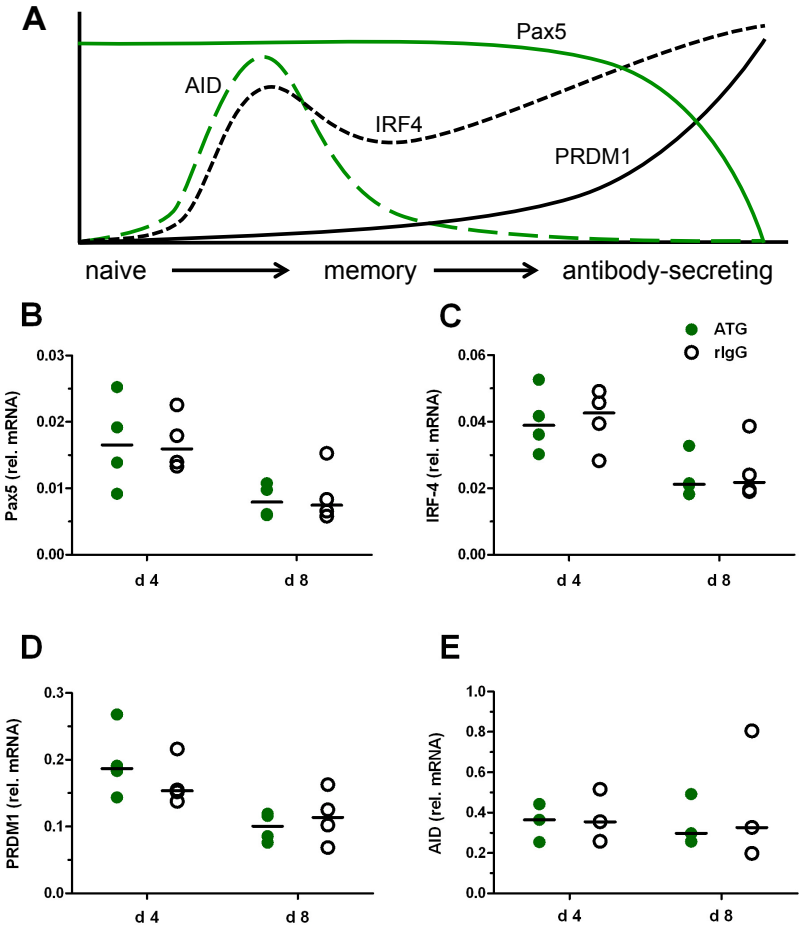

Figure 14 – Gene expression of B-cell differentiation markers upon ATG.
(A) Relative expression of differentiation factors during B-cell differentiation. (B–E) FACSorted CD27$^+$ memory B cells were stimulated with CpG and incubated with 5 μg/ml ATG or rIgG. On days 4 and 8, qRT-PCR of differentiation markers was performed. (B) Pax5, (C) IRF-4, (D) PRDM1, (E) AID. Line indicates median, n = 4.

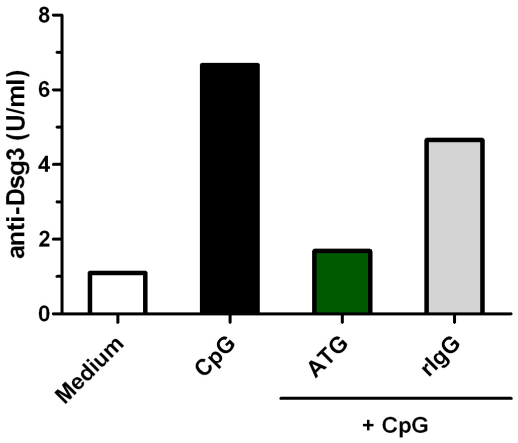

Figure 15 – ATG lowers secretion of specific autoantibody production *in vitro*.
PBMC from a patient with pemphigus vulgaris were stimulated with CpG, anti-CD40 and anti-BCR. Cells were incubated for 9 days with or without 5 μg/ml ATG or rIgG. Anti-Dsg3 autoantibodies in the supernatants were detected by ELISA.

of cells treated with control rIgG 4.7 U/ml were detected (Fig. 15).

Table 4 – Clinical characteristics of patients for detection of specific autoantibodies *in vitro*.
dsg, desmoglein; f, female; m, male; N/K, not known; PF, pemphigus foliaceus; PV, pemphigus vulgaris; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; yrs, years.

ID	age (yrs)	sex	auto-immune disease	disease duration (yrs)	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, dapsone	anti-dsg-1, anti-dsg-3	35 (< 14) ratio, 79 (< 7) ratio	no
7	45	f	PV	6	mycophenolate, methotrexate	anti-dsg-1, anti-dsg-3	6 (< 14) ratio, 186 (< 7) ratio	no
8	23	m	PV	0	cyclophosphamide/ dexamethasone pulse (during first pulse)	anti-dsg-1, anti-dsg-3	86 (< 14) ratio, 172 (< 7) ratio	yes
9	53	m	PF	3	cyclophosphamide/ dexamethasone pulse	anti-dsg-1, anti-dsg-3	171 (< 14) ratio, 1 (< 7) ratio	no
10	57	f	SLE, SS	21	prednisolone, hydroxychloroquine	anti-Ro (SS-A)	positive	no

7 Discussion

The data of this study provide evidence that ATG at low, non-toxic concentrations modulates B-cell differentiation and immunoglobulin production *in vitro*. For the first time, it is demonstrated that immunoglobulin secretion by B cells is reduced by treatment with Thymoglobulin® in a T cell independent manner. The modulation occurs by impairing the differentiation of B cells to immunoglobulin-secreting cells. See Fig. 16 for a synopsis of the results of this study.

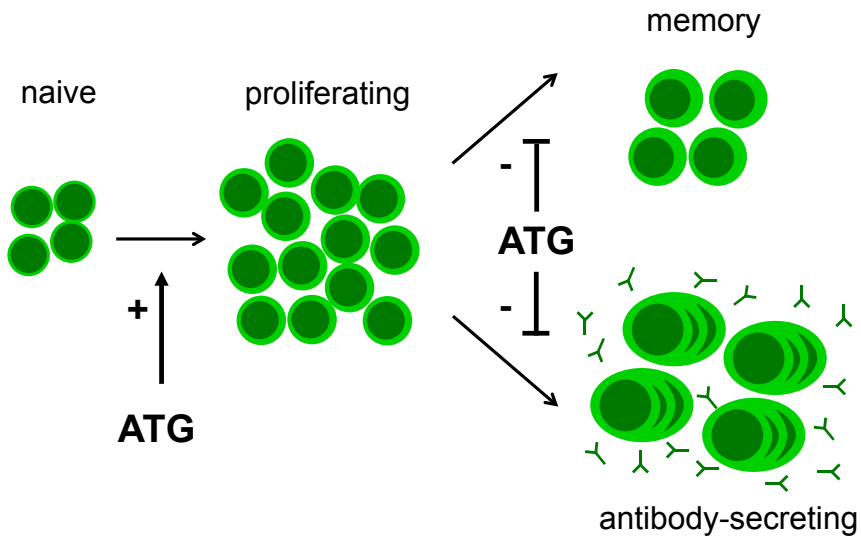

Figure 16 – Synopsis of the effects of ATG on B cells at sub-depleting concentrations. Naive B cells from peripheral blood in this culture model proliferate and differentiate into memory B cells and antibody-secreting cells. ATG amplifies proliferation but inhibits differentiation.

7.1 Antibody Secretion by PBMC

In order to investigate the influence of ATG on PBMC, cells were incubated with different stimulations and different concentrations of ATG and control rIgG.

Cells that were incubated without any stimulation showed a very low baseline secretion of immunoglobulins. This indicates that stimulation, e.g. with CpG, is necessary to induce Ig secretion in PBMC. When cells were incubated without CpG, but with ATG only, there was almost no secretion of immunoglobulins, either. Adding to the fact that CpG is necessary, this indicates that incubation with ATG alone is not sufficient to induce antibody secretion, in spite of the well-known stimulatory properties of ATG [11].

When CpG was added to PBMC cultures, IgG, IgM and IgA, but not IgE, were detected in high amounts. This is not surprising as CpG is known as a potent inducer of IgG, IgM and IgA in B cells. CpG typically does not induce IgE positive memory cells, only in people

with allergy, it induces low numbers of IgE positive memory B cells [10]. To induce IgE secretion, e.g. anti-CD40 and IL-4 can be used as stimulant [45].

Upon the addition of ATG to CpG-stimulated PBMC, the secretion of IgG, IgM and IgA was decreased in a dose-dependent manner. Because all three isotypes were affected, it appears that these effects are not specific for one subset. Interestingly, the secretion of IgG at the lowest concentration of ATG (0.01 μg/ml) was elevated to levels three times as high as the reference group (CpG alone). At very low concentrations, the activation through ATG seems to predominate over its inhibitory effects. Here, indirect inducing effects, e.g. on T cells or monocytes may be relevant.

The data obtained by the experiments in PBMC raised the question whether the observed effects were induced directly on B cells or indirectly e.g. via T cells. In 1992, Bonnefoy-Bérard et al. investigated the effect of different antilymphocyte sera on antibody production in PBMC. They demonstrated a decreased secretion of immunoglobulins and attributed this to a direct interaction of ATG with B cells. The authors postulated that ATG inhibited B-cell activation. They made no suggestions as to how B-cell activation is inhibited [11].

7.2 Direct Impact of ATG on B cells

Helper T cells and monocytes provide cytokines and co-stimulatory molecules for B cells to produce antibodies. Therefore, they needed to be removed from the culture system to determine whether ATG affects B cells directly. Purified primary B cells were cultivated with CpG at different concentrations of ATG and rIgG. In these experiments, only IgG was measured because ATG had the most pronounced influence on IgG when PBMC were used.

As in PBMC, purified B cells did not produce significant amounts of IgG in the absence of CpG, but when stimulated with CpG, they secreted high amounts of IgG. When CpG-stimulated B cells were treated with ATG, a dose-dependent reduction of IgG levels was observed. This demonstrates that ATG is also effective in the absence of T cells and monocytes, and that its effects do not depend on other cells. Assuming that this is not due to cytotoxic but rather immunomodulatory effects, this may be advantageous in the treatment of autoimmune disease by directly modulating autoreactive B cells.

Of note is that IgG levels were also decreased by control rIgG, albeit to a lesser extent. This might be mediated by the Fc part of rIgG binding to Fc receptors on B cells. One of several Fc receptors, FcγRIIB, is expressed on B cells and plasmablasts. It has inhibitory functions on B cells, leading to reduced antibody secretion [76]. The therapeutic role of FcγRIIB is implied by the fact that this receptor is assumed to be responsible for the efficacy of IVIg [97].

7.3 Efficacy of F(ab')$_2$ Fragments

F(ab')$_2$ fragments of ATG were used to determine the extent to which ATG exerts specific effects by binding to certain cell surface molecules. The fragments proved to be nearly as potent in reducing the production of antibodies by B cells as unfragmented ATG. This indicates that the reduction of IgG is most likely mediated by specific binding of ATG to B cell surface receptors and not by unspecific binding to their Fc receptors.

Several research groups investigated previously the efficacy of F(ab')$_2$ fragments of ATG as well. They found them to be up to 90% as effective as the whole molecule in inducing apoptosis in PBMC, B cells and B cell lines [13, 36, 119]. These authors also concluded that most of the effects of ATG were by specific binding to various epitopes. However, at least some might be mediated by unspecific binding to Fc receptors as well.

Zand et al. proposed that F(ab')$_2$ fragments of ATG might be advantageous in the treatment of autoimmune diseases due to reduced induction of cell necrosis, while rather exerting apoptotic and modulatory effects [119].

7.4 No Cell Death at Low Concentrations of ATG

To determine whether the reduced IgG secretion by B cells depends on a decreased number of antibody-secreting cells (ASC) or is caused by decreased IgG secretion of the individual cell, ASC were enumerated by performing ELISpot assay. Indeed, the numbers of ASC were reduced by ATG. To rule out cytotoxic effects, B cell survival was assessed.

During a time frame of 8 days, no increased cell death in B cells was observed at low concentrations of ATG (5 μg/ml). At high concentrations (100 μg/ml) of ATG, however, significant cell death occurred in B cells. This observation proves that the concentration chosen to inhibit antibody secretion in this study was not cytotoxic per se. The inhibitory effects are most likely mediated by immunomodulation and may complement traditional immunosuppressive therapy.

When ATG is administered intravenously over a course of several days, ATG accumulates *in vivo* and reaches a peak after the last dose. Therapeutic levels are around 135 μg/ml. Levels then decline, after 90 days they are around 12 μg/ml [86, 87]. So-called active ATG, which is the specific fraction of ATG able to bind to lymphocytes, is cleared from the body faster. Its half-life is only seven days whereas total ATG has a half-life of fourteen days [114].

When comparing the results of this study to other studies it becomes apparent that they are within the range of other investigations: Bonnefoy-Bérard found cell death in B cell lines to occur at concentrations above 50 μg/ml [13]. Zand observed a slight elevation of cell death in naive B cells, CD40L-stimulated B cells and plasma cells at 10 μg/ml, and significant cell death above 100 μg/ml [119]. Two other authors found cell death to occur in myeloma cell lines and primary myeloma cells above 100 or 500 μg/ml, respectively [8, 105]. The variations in the identification of cytotoxic concentrations by different authors can be

explained by the use of different ATGs, different cells and cell lines. Cell death occurs by complement-mediated cell lysis, antibody-dependent cell-mediated cytotoxicity and the induction of apoptosis.

7.5 ATG Enhances Proliferation in B Cells

ATG is known as a potent inducer of T-cell activation, but there are no reports of ATG inducing proliferation in B cells. This study provides evidence that ATG is also a potent inducer of B-cell activation. This could be attributed to activating antibodies contained in ATG, such as anti-BCR, anti-CD40, anti-IL6R, anti-IL10R and anti-IL21R. Out of these, only anti-IL6R antibodies have been identified in ATG to date [68]. Therefore, the presence of the other antibody specificities mentioned above remains hypothetical.

The findings of the present study stand in contrast to findings by Bonnefoy-Bérard et al. who observed enhanced proliferation 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 [13].

It furthermore conflicts with the observation that ATG alone (without CpG) is not sufficient to induce proliferation in B cells. Enhanced proliferation can however be explained by CpG inducing the expression of cell surface markers in B cells that serve as a target for activation by ATG. It could also be explained by synergistic effects of ATG and CpG. Recent reports confirm that TLR9 and CD40 synergise in amplifying B-cell activation [79]. This is related to the underlying molecular pathways. Both TLR9 and CD40 induce mitogen-activated protein kinase (MAPK) pathways p38 and c-Jun NH2-terminal kinase (JNK) and nuclear factor κB (NFκB) [51]. By inducing MAPK, TLR9 furthermore synergises with the BCR in B-cell activation [50, 38].

7.6 Phenotypical Analysis of Differentiating B Cells

By using flow cytometry, differentiating B cells were analysed regarding the expression of activation and differentiation markers. The frequency of naive B cells remained unchanged whereas the frequency of memory B cells and even more pronounced plasmablasts was reduced. This indicates that late terminal differentiation of B cells to plasmablasts is disturbed while activation and proliferation are unaffected. Cells seem to be arrested at the early germinal center stage: They proliferate vigorously, but they do not continue to develop into the memory or plasmablast phenotype.

Again, this stands in contrast to Bonnefoy-Bérard's assumptions who proposed that inhibition of antibody secretion was due to inhibition of B-cell activation. However, he did not investigate B-cell activation in specific B-cell subsets [11].

To further characterise the cells that actually secrete antibody, more specific markers are needed. This is critical because in this setting both memory cells ($CD27^+$) and plasmablasts

($CD38^+$, $CD138^+$) can produce antibodies. Even though surface Ig may indicate class switch and memory cells, it is not limited to ASC. A more specific B cell subset marker would allow single cell analysis and better phenotypical characterisation. Recent investigations have suggested that CD93 could be such a marker. CD93 is expressed during B-cell development in the bone marrow and down-regulated upon further maturation, but re-induced during plasma cell development [17].

7.7 Differentiation Markers at the mRNA Level

Real Time PCR revealed no difference in the expression of selected B-cell differentiation markers (PAX5, Blimp1, IRF4 and AID) on the mRNA level. Unlike flow cytometry, suitable markers for ASC are available for PCR. Nonetheless, there was no difference in the expression profile of these markers detectable at the time-points examined.

Several reasons may account for this: Only a very small percentage of B cells are ASC; FACS analysis showed a maximum of 4%. Low overall expression of the housekeeping gene HPRT indicates low cell numbers per sample, which limits the accuracy of the method. $CD27^+$ cells were sorted by FACS before culture. Although this is a homogenous population, it becomes more heterologous during time of culture. FACSort for e.g. CD38 after culture might therefore be advantageous, but it is impractical due to even lower cell numbers. Blimp1 is also found in switching cells, it is not a marker exclusive for ASC, and antibody secretion can occur even in the absence of Blimp1 [47]. The number of experiments was very small (n = 4), and for each experiment, several donors were pooled to achieve higher cell numbers, which could further conceal effects.

Chen et al. investigated the immunomodulatory effects of vitamin D on B cells and found that vitamin D decreases the frequency of plasma cells. To look into the mechanism of action, they analysed the presence of PAX5, Blimp1, AID and IRF4, but found no difference in their expression, either. However, another analysed differentiation marker, X-box binding protein 1 (XBP1), was expressed to a lesser extent in the presence of vitamin D [16]. This transcription factor has been shown to be vital for the secretion of high amounts of antibodies [42]. Based on these findings, one could speculate that XBP1 may also play a role in the mechanism of action of ATG.

7.8 Effect on Specific Autoantibodies

In order to demonstrate that the findings of this study can be relevant in a clinical setting, PBMC from patients with certain antibody-associated autoimmune diseases were incubated with ATG. Specific autoantibodies could only be detected in supernatants of one out of ten patients. In this patient with pemphigus vulgaris, levels of anti-Dsg3 were indeed found to be decreased in the presence of ATG.

It may appear critical that in this study, specific autoantibodies were found only in one

patient. Autoimmune diseases such as pemphigus vulgaris are rare diseases with a yearly incidence of one to five per million [46]. Not all patients are eligible for inclusion in the study because they are already under immunosuppressive therapy which may have a negative impact on the cultivation of their lymphocytes. Furthermore, specific antibodies cannot be detected in all patients *in vitro*. Nishifuji et al. found Dsg3-specific B cells in peripheral blood only in three patients with severe and active disease while none in seventeen patients with moderate or mild disease [77].

The generation of specific antibodies *in vitro* is not trivial. Typically, a specific antigen is needed as a stimulant. Specific stimulants (Ro, ds-DNA, Dsg1, Dsg3) were not available, however. Polyclonal antibody production was therefore maximised by CpG + anti-BCR + anti-CD40 in order to trigger specific antibody production *in vitro* [54]. Another approach might have been the transduction of specific memory B cells with Bcl-6 and Bcl-xL and culture in the presence of CD40L and IL-21 [52]. Neither this assay nor the fluorescent antigen were available during this study.

The pemphigus patient who donated PBMC for this study was a young patient who had been recently diagnosed. He had high titres of anti-Dsg3 and high clinical disease activity. Disease progressed in spite of intensive therapy regimens. These included cyclophosphamide/dexamethasone pulse therapy, interval therapy with prednisolone and azathioprine, and ultimately immunoadsorption. At the same time increasing side effects i.e. Cushing's syndrome occurred due to cortisone therapy. Anti-Dsg titres remained at constantly high levels between 2008 and 2010. Improvement was finally achieved after anti-CD20 therapy in 2010. ATG decreased anti-Dsg3 in this study *in vitro* by 50%. This indicates that ATG might be effective in terminating disease flares in pemphigus. The repeated application of ATG in clinical practice may however be limited by the development of neutralising anti-rabbit antibodies.

7.9 Modulatory Effects of ATG on B-Cell Differentiation

Due to its polyclonal nature, ATG can simultaneously exert activating and inhibitory effects on B cells. On the one hand, B cells are strongly activated and proceed to the GC stage, on the other hand, they do not further differentiate into memory cells or plasmablasts. Several factors may account for this phenomenon.

ATG can induce antagonists of B lymphocyte-induced maturation protein 1 (Blimp1). Blimp1 (corresponding gene PRDM1) is known as "master regulator" of plasma cell development [62]. One transcription factor known to repress Blimp1, and thereby repress plasma cell differentiation, is basic leucine zipper factor Bach2. Little is known how Bach2 is regulated [43, 78]. Another factor is B cell lymphoma protein 6 (Bcl-6), which promotes proliferation and GC formation, but inhibits ASC formation by repressing Blimp1 [102, 108]. The proposed relationship between Blimp1 and Bcl6 is as follows: When Bcl6 is down-regulated and

Blimp1 up-regulated, B cells differentiate to plasma cells. When Bcl6 is down-regulated, but Blimp1 remains stable, memory cells form, and when Bcl6 is up-regulated, B cells recycle through the GC [101].

Another possible explanation might be the down-regulation of B cell lymphoma 2 (Bcl-2) family members by ATG. These are known as anti-apoptotic transcription factors and can lead to enhanced ASC formation [96]. Their down-regulation might therefore inhibit ASC formation.

It is possible that after a strong activation by ATG, essential factors are missing for B cells to further differentiate to ASC. Such factors include IL-5, IL-6, IL-21, TNF, BAFF and APRIL, which might be of interest for further studies [101]. Cells may then be arrested in the cell cycle. 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 [65].

8 Outlook

The findings of this study support the concept that B cell-targeted therapies are useful in autoimmunity. ATG can be used for the development of new B-cell targets. ATG would have the advantage that it can also reach bone marrow-resident plasma cells. These are resistant to common immunosuppressive medication. Low-dosed ATG therapy should have fewer side effects than ATG administered at high doses. Trials using low-dosed ATG have already been suggested by Genestier et al. [36].

One should keep in mind that ATG is a powerful treatment and care must be taken regarding the translation of the findings of this *in vitro* study to the clinical setting. Adverse effects of ATG include the induction of the so-called cytokine storm. This became a disaster for the pharmaceutical company TeGenero who in 2006 tested a very promising new monoclonal antibody against CD28. Although *in vitro* and murine experiments had not suggested any complications, during a phase 1 clinical trial, all six participants became critically ill due to a systemic inflammatory response [99]. ATG, however, has already been in use for a long time, and the ATG-induced cytokine storm occurs very rarely.

Another limitation is that the repeated application of ATG is not possible. It ultimately leads to the formation of neutralising anti-rabbit antibodies in the patient and requires the discontinuation of treatment [86]. $F(ab')_2$ fragments might be less immunogenic, and the combined administration of ATG with cyclophosphamide and dexamethasone might inhibit the immunologic sensitisation against ATG.

The different aspects of the mechanism of action of ATG provide effective immunomodulation by targeting and impairing both autoreactive T and B cells, and even pathogenic memory B cells and plasma cells. The side effects can be within the range of other non-humanised biologicals. Using ATG at low doses might limit the extent of immunosuppression and thereby lower the risk of severe infections.

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A Appendix

A.1 List of Materials

FACS antibodies

Table 5 – FACS antibodies.

Species	Epitope	Fluorochrome	Working Dilution	Manufacturer
mouse anti-human	CD3 (clone UCHT-1)	PE	1:400	DRFZ
mouse anti-human	CD19 (clone BU12)	Cy5	1:400	DRFZ
mouse anti-human	CD20	FITC	1:20	BD
mouse anti-human	CD27	PE	1:20	BD
mouse anti-human	CD38	PerCP-Cy5.5	1:20	eBioscience
mouse anti-human	CD138	APC	1:20	BD

Antibodies and secondary reagents for ELISA and ELISpot

Table 6 – Antibodies and secondary reagents for ELISA and ELISpot.

Species	Epitope	Comment	Working concentration	Manufacturer
goat anti-human	IgG		1:5000	Jackson
goat anti-human	IgM		1:5000	Jackson
goat anti-human	IgA		1:5000	Jackson
goat anti-human	IgG	AP-conjugated	1:5000	Jackson
goat anti-human	IgM	AP-conjugated	1:5000	Jackson
goat anti-human	IgA	AP-conjugated	1:5000	Jackson
mouse anti-human	IgG	biotinylated	1:5000	BD

Primers

Table 7 – Primers.

Primer		Sequence 5' → 3'	Efficiency
AID	forward	AgAggCgTgACAgTgCTACA	1.88
	reverse	ATgTAgCggAggAAgAgCAA	
HPRT	forward		2.0
	reverse		
IRF4	forward	CCAggTgACTCTATgCTTTgg	1.9
	reverse	CTTTgCgCTCATAACgTCAg	
PAX5	forward	AAACCAAAggTCgCCACAC	1.81
	reverse	gTTgATggAACTgACgCTAg	
PRDM1	forward	gTgCTCggTTgCTTTAgACTgCT	2.0
	reverse	TAAgCCCATCCCTgCCAACCA	

Consumables

Table 8 – Consumables.

Consumable	Model	Manufacturer
Culture plates	Cellstar® Suspension (24 well, 28 well, 96 well)	Greiner Bio-One
FACS tubes	Polystyrene Round Bottom Tube 5ml	BD
ELISA plates	MaxiSorb®	NUNC
ELISpot plates	MultiScreenHTS-IP	Millipore
MACS columns	MS columns	Miltenyi
Microtubes	Safe-Lock (0.5 ml, 1 ml, 2 ml)	Eppendorf
Pipette tips	10 μl, 200 μl, 1000 μl (with filter)	SARSTEDT
Pipette tips	10 μl, 200 μl, 1000 μl	SARSTEDT
Pipettes	Serological pipet (2.5 ml, 10 ml, 25 ml)	BD
Reaction tubes	Blue Max Polypropylene Conical Tube (15 ml, 50 ml)	BD
PCR capillaries	LightCycler® Capillaries (20 μl)	Roche
Syringe filter	0.2 μml	SARSTEDT
Syringes	1 ml, 10 ml, 50 ml	BD
Transfer pipette	3.5 ml	SARSTEDT

Laboratory equipment

Table 9 – Laboratory equipment.

Equipment	Model	Manufacturer
Cell incubator	HERAcell®	Heraeus
Centrifuge	Multifuge® 4KR	Heraeus
	Megafuge® 1.0R	Heraeus
	5417R	Eppendorf
Electronic cell counter	CASY® 1, Modell TT	Schärfe
ELISA plate reader	SpectraMax® 190	Molecular Devices
ELISA plate washer	Nunc-Immuno Wash 12	NUNC
ELISpot plater reader	S5 Core Analyzer	CTL
Flow cytometer	FACSAria®	BD
	FACSCalibur®	BD
Hemocytometer	Neubauer Improved	LO — Laboroptik
Laminar flow hood	HERAsafe®	Heraeus
Microscope	Axioskop	Carl Zeiss
Microscope camera	Axioplan 2 / Axio Cam HRc	Carl Zeiss
Pipette	Research (10 μl, 10-100 μl, 20-200 μl, 100-1000 μl)	Eppendorf
	Pipettusboy	Hirschmann
Real-time PCR system	LightCycler® 1.5	Roche
Thermal cycler	Px2	Thermo Electron Corporation
Vortex mixer	REAX 2000	Heidolph
Water bath		Memmert

Software

Table 10 – Software.

Application	Software	Manufacturer
ELISA analysis	Revelation G 3.2	Dynex Technologies
ELIspot analysis	Immunospot®	CTL
FACS acquisition	CellQuest Pro®	BD
FACS analysis	FloJo	Tree Star
Illustrations	Powerpoint 2007	Microsoft
Microscopy	Axio Vision LE Application 4.5.0.0	Carl Zeiss
Picture editing	Photoshop® CS3	Adobe
qRT-PCR	LightCycler® 3	Roche
Statistics	Prism	GraphPad
Table calculation	Excel 2007	Microsoft

Chemicals and Reagents

Table 11 – Chemicals and reagents

Reagent	Specification	Manufacturer
β-Mercaptoethanol	2-Mercaptoethanol	Sigma-Aldrich
Acetic acid (CH_3COOH)		Sigma-Aldrich
AEC	3-Amino-9-Ethylcarbazole	Sigma-Aldrich
Annexin FITC		ImmunoTools
anti-BCR	AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgA + IgG + IgM (H+L)	Jackson
anti-CD40 antibodies		Santa Cruz Biotechnology
anti-Dsg3 ELISA kit	MESACUP Desmoglein TEST "Dsg3"	MBL
BSA	Albumin bovine Fraction V	Serva
$CaCl_2$		Sigma-Aldrich
CASY cleaner	CASYclean	Schärfe
CASY dilution liquid	CASYton	Schärfe
cDNA synthesis kit	TaqMan® Reverse Transcription	Applied
Cell culture medium	RPMI 1640 (with 2 g/l $NaHCO_3$ and stable glutamine	Biochrom
CFSE		
CpG 2006		TIB MOLBIOL
Diethanolamine		Sigma-Aldrich
DMF	n,n-Dimethylormamide	Sigma-Aldrich
EDTA		Serva
Ethanol (CH_3CH_2OH)		Merck
FACS block	Beriglobin®	CSL Behring
FACS cleaning solution	BD FACS Clean Solution	BD
FACS sheeth fluid	BD FACSFlow Sheath Fluid	BD
FCS	Fetal calf serum	Biochrom
Ficoll	Biocoll separating solution	Biochrom
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma-Aldrich
Hydrogen peroxide (H_2O_2)		Merck
IL-4	rhuIL-4	R&D
MACS beads	CD19 MicroBeads	Miltenyi Biotec
Magnesium chloride (MgCl)		Sigma-Aldrich
p-nitrophenylphosphate	phosphatase substrate	Sigma-Aldrich
Penicillin/ Streptomycin	10000 E/ml / 10000 μg/ml	Biochrom

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Reagent	Specification	Manufacturer
PI		
qRT-PCR kit	FastStart DNA Master SYBR Green I	Roche
rIgG		Jackson
rIgG F(ab')$_2$		Jackson
RNA purification kit	NucleoSpin® RNAII	MACHEREY
Sodium acetate (CH_3COONa)		Merck
Sodium bicarbonate ($NaHCO_3$		Merck
Sodium carbonate (Na_2CO_3)		Merck
Sodium chloride (NaCl)		Merck
Sodium azide NaN_3	Natriumazid reinst	Merck
Sterile PBS	Dulbeccońs PBS without Ca/Mg	PAA Laboratories
Streptavidin-Horseradish Peroxidase	Conjugate E2886	Sigma-Aldrich
Tris	TRIZMA® (tris(hydroxymethyl)aminomethane)	Sigma-Aldrich
Trypan blue	0,40 % in PBS w/o $Ca2^+$/ $Mg2^+$	Biochrom
Tween 20		Sigma-Aldrich

Protocols for Preparation of Buffers and Solutions

AEC in DMF	1 tablet	AEC
	2 ml	DMF
Annexin binding buffer	4.1 g	NaCl
	0.14 g	$CaCl_2$
	ad 50 ml	HEPES 0.1 M
	use at 1 : 10 dilution in Aqua bidest.	
BSA/TBS (2 %)	2 g	BSA
	100 ml	TBS
Cell culture medium	500 ml	RPMI 1640
	5 ml	Penicillin/ Streptomycin
	50 ml	FCS (heat-inactivated, sterile filtered)
Coating buffer	1.46 g	$NaHCO_3$
	4.5 g	NaCl
	0.8 g	Na_2CO_3
	500 ml	Aqua bidest.; pH 8.0
FACS buffer	500 ml	PBS
	10 g	BSA
MACS buffer	500 ml	sterile PBS
	2.5 g	BSA
	10 ml	100 mM EDTA
PBS	8 g	NaCl
	0.2 g	KCl
	1.44 g	Na_2HPO_4
	0.24g	KH_2PO_4
	1000 ml	Aqua bidest.; pH 7.4
PBS/EDTA 0.2 mM	500 ml	sterile PBS
	10 ml	100 mM EDTA

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Substrate buffer (ELISA)	48.5 g	Diethanolamine
	0.107 g	$MgCl_2$
	0.05 g	NaN_3
	500 ml	Aqua bidest.; pH 9.9
Substrate buffer (ELISpot)	7.5 ml	$C_2H_4O_2$
	10.55 ml	Ch_3COONa
	15 ml	Aqua bidest.
	1 ml	AEC in DMF (slowly)
	filtrate 22 ml, before use add 12 μl H_2O_2	
TBS	6.05 g	Tris base
	45 g	NaCl
	0.5 g	NaN_3
	5000 ml	Aqua bidest.
TBST	250 μl	Tween 20
	500 ml	TBS

Manufacturer's Addresses

Adobe Systems GmbH	München, Germany
Applied Biosystems Deutschland GmbH	Darmstadt, Germany
CSL Behring GmbH	Marburg, Germany
BD Becton Dickinson GmbH	Heidelberg, Germany
Biochrom AG	Berlin, Germany
CTL-Europe GmbH	Bonn, Germany
Dynex Technologies GmbH	Berlin, Germany
DRZF (Deutsches Rheuma-Forschungszentrum)	Berlin, Germany
eBioscience	Frankfurt, Germany
Eppendorf AG	Berlin, Germany
Genzyme GmbH	Neu-Isenburg, Germany
GraphPad Software, Inc.	San Diego, CA, USA
Greiner Bio-One GmbH	Frickenhausen, Germany
Heidolph Instruments GmbH & Co.KG	Schwabach, Germany
Heraeus Holding GmbH	Hanau, Germany
Hirschmann Laborgeräte GmbH & Co. KG	Eberstadt, Germany
ImmunoTools GmbH	Friesoythe, Germany
Jackson ImmunoResearch Europe Ltd.	Newmarket, Suffolk, UK
LO — Laboroptik GmbH	Bad Homburg, Germany
MACHEREY-NAGEL GmbH & Co. KG	Düren, Germany
MBL International	Woburn, MA, USA
Memmert GmbH & Co. KG	Schwabach, Germany
Merck KGaA	Darmstadt, Germany
Microsoft Deutschland GmbH	Unterschleißheim, Germany
Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
Millipore GmbH	Schwalbach/Ts., Germany
Molecular Devices GmbH	Ismaning, Germany
nunc (Thermo Electron LED GmbH)	Langenselbold, Germany
PAA Laboratories GmbH	Pasching, Austria
Pineda Antikörper-Service	Berlin, Germany
R&D Systems GmbH	Wiesbaden-Nordenstadt, Germany
Roche Diagnostics Deutschland GmbH	Mannheim, Germany
SARSTEDT AG & Co.	Nürnbrecht, Germany
Schärfe System GmbH	Reutlingen, Germany
SERVA Electrophoresis GmbH	Heidelberg, Germany
Sigma-Aldrich Chemie Gmbh	München, Germany
TIB MOLBIOL Syntheselabor GmbH	Berlin, Germany
Tree Star, Inc.	Ashland, OR, USA
Carl Zeiss AG	Oberkochen, Germany

A.2 List of Abbreviations

AID	activation-induced (cytidine) deaminase
ALG	anti-lymphocyte globulin
AMR	antibody-mediated rejection
anti-Ig	antibodies against human immunoglobulin
AP	alkaline phosphatase
APC	antigen-presenting cell
APRIL	a proliferation-inducing ligand
ASC	antibody-secreting cell
ATG	antithymocyte globulin
BAFF	B-cell activating factor belonging to the TNF family
BCR	B cell receptor
BSA	bovine serum albumin
C terminus	carboxy terminus
C1q	complement factor 1q
CCR	C-C chemokine receptor
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary deoxyribonucleic acid
CFSE	carboxyfluorescein succinimidyl ester
ConA	Concanavalin A
CTLA-4	cytotoxic T-lymphocyte antigen
Cy5.5	cyanine 5.5
DC	dendritic cell
DNA	deoxyribonucleic acid
DRFZ	Deutsches Rheuma-Forschungszentrum
Dsg3	Desmoglein-3
eATG	equine antithymocyte globulin
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunosorbent spot assay
et al.	et alii
FACS	fluorescence-activated cell sorting
FcϵR	receptor for the Fc domain of IgE
FcγR	receptor for the Fc domain of IgG
FCS	fetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
FOXP3	forkhead box P3
HLA	human leukocyte antigen
HPRT	hypoxanthine-guanine-phosphoribosyltransferase

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HSCT	homologous stem cell transplantation
ICAM	inter-cellular adhesion molecule
IVIg	intravenous immunoglobulin
Ig	immunoglobulin
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IGF	insulin-like growth factor
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IRF-4	interferon regulatory factor 4
L	ligand
LCA	leukocyte common antigen
LFA	lymphocyte function-associated antigen
LPAM-1	lymphocyte Peyer's patch adhesion molecule 1
MACS	magnetic activated cell sorting
MALT	mucosa associated lymphoid tissues
mATG	rabbit anti-mouse thymocyte globulin
MHC	major histocompatibility complex
MoAb	monoclonal antibody
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
N terminus	amino terminus
NAD	Nicotinamide adenine dinucleotide
NDCM	Nocardia-delipidated cell mitogen
NF-κB	nuclear factor-κB
NK cell	natural killer cell
NOD mice	non-obese diabetic mice
ODN	oligodeoxynucleotides
PAMP	pathogen-associated molecular patterns
Pax5	paired box protein 5
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	priding chlorophyll protein complex
PHA	phytohemagglutinin
PI	propidium iodide
PRDM1	PR domain zinc finger protein 1
PRR	pattern recognition receptor

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PSS	progressive systemic sclerosis
PWM	pokeweed mitogen
qRT-PCR	quantitative real-time reverse-transcription PCR
RA	rheumatoid arthritis
rATG	rabbit antithymocyte globulin
rIgG	rabbit immunoglobulin G
RNA	ribonucleic acid
SAC	formalinized Cowan I strain Staphylococcus aureus
SCF	stem cell factor
SDF-1	stromal cell-derived factor-1
SLE	systemic lupus erythematosus
T_H cell	T helper cell
T1DM	type 1 diabetes mellitus
TAPA-1	Target of anti-proliferative antibody
TBS	Tris buffered saline
TCR	T cell receptor
TGF-β1	tumour growth factor β1
TH1 cell	type 1 helper T cell
TH2 cell	type 2 helper T cell
TLR	Toll-like receptor
TNF	tumour necrosis factor
Treg	regulatory T cell
VEGF	vascular endothelial growth factor
VLA	very late antigen

A.3 Affirmation in Lieu of an Oath

Eidesstattliche Versicherung

"Ich, Pascal Klaus, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: "Modulation of the Humoral Immune Response by Antithymocyte Globulin (ATG)" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder VortrŁgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe "Uniform Requirements for Manuscripts (URM)" des ICMJE -www.icmje.org) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s. o.) und werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit der Betreuerin, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s. o.) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst."

Datum Unterschrift

Anteilserklärung an etwaigen erfolgten Publikationen

Pascal Klaus hatte folgenden Anteil an der folgenden Publikation:

P. Klaus, G. Heine, C. Rasche, M. Worm, Low-Dose Anti-Thymocyte Globulin (ATG) Inhibits Human B Cell Differentiation into Antibody-Secreting Cells, Acta Dermato-Venerologica, 2015

Beitrag im Einzelnen:

Planung der Experimente gemeinsam mit der betreuenden Hochschullehrerin, Durchführung und Auswertung der Experimente, Entwurf des Manuskripts und Fertigstellung des überarbeiteten Manuskripts.

Unterschrift, Datum und Stempel der betreuenden Hochschullehrerin

Unterschrift des Doktoranden

A.4 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

A.5 Publications

Klaus P, Heine G, Rasche C, Worm M. Low-dose Anti-thymocyte Globulin Inhibits Human B-cell Differentiation into Antibody-Secreting Cells. Acta Derm Venereol. 2015 Jan 14. doi: 10.2340/00015555-2046. [Epub ahead of print]

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