DISSERTATION

Mechanisms of action of immunomodulatory activities of oral IgG

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1. Zusammenfassung

Seit langem ist bekannt, daß die orale Verabreichung von Proteinantigenen in experimentellen Autoimmunerkrankungsmodellen Immuntoleranz induziert und die Entwicklung der Erkrankung verzögert oder sogar verhindert. In meiner Studie habe ich die immunmodulierende Kapazität von humanem polyvalentem IgG (IVIG) in einem Mausmodel untersucht, in dem ich Rheumafaktor-ähnliche Antikörper induzierte durch die Injektion von unlöslichen humanen Immunkomplexen (IC). Rheumafaktoren (RF) sind Autoantikörper, die den Fc-Teil des IgG-Moleküls erkennen und in diversen Autoimmunkrankheiten zu finden sind, wie zum Beispiel bei Rheumatoider Arthritis, Systemischem Lupus Erythematosus, und dem Sjögren Syndrome. Der Fc-Teil des IgG Moleküls repräsentiert das Antigen, das von RF-expimierenden B Zellen und RFs erkannt wird. Daher sollte IVIG die Fähigkeit haben, orale Toleranz gegen die systemische Induktion von RFs zu induzieren.

In anfänglichen Untersuchungen konnten wir zeigen, dass die präventive Behandlung mit einer einzigen Dosis an oralem IVIG in der Lage ist, primäre RF Immunantworten zu verhindern. Eine kontinuierliche Behandlung mit oralem IVIG schützt nicht nur vor primären Immunantworten, sondern sogar vor sekundären RF Immunreaktionen. Eine therapeutische Behandlung mit oralem IVIG kann zwar nicht vor einer primären RF Immunantwort schützen, aber sie ist in der Lage, sehr effektiv die sekundäre Immunantwort zu reduzieren. Diese Befunde sind sehr interessant für mögliche klinische Ansätze, da normalerweise Autoimmunerkrankungen in Patienten erst nach Etablierung der Krankheit diagnostiziert werden.

Mittels sogenannten Transferexperimten haben ich die Wirkungsmechanismen von oralem IVIG untersucht. Ich konnte zeigen, dass zelluläre Mechanismen für die immunmodulatorischen Aktivitäten von oralen IVIG verantwortlich sind.

Weiters konnte ich zeigen, dass in die immunsupprimierenden Effekte von oralem IVIG möglicherweise sogenannte regulatorische T Zellen involviert sind. Eine wichtige Subpopulation von regulatorischen T Zellen gehört zur $CD4^+CD25^+$ T Zellsubpopulation. Durch „in vivo" Abreicherung dieser T Zellsubpopulation konnte ich zeigen, dass die Abwesenheit dieser Zellen die immunmodulatorische Effekte von oralem IVIG aufhebt. Aus diesem Grund spekuliere ich, dass $CD4^+$

$CD25^+$ regulatorische T Zellen für die beobachteten Effekte von oralem IVIG verantwortlich sind. Trotzdem kann ich nicht ausschließen, dass auch andere Mechanismen an diesen Effekten beteiligt sind.

Wenn ich meine Ergebnisse zusammenfasse, kann ich schlußfolgern, dass die orale Applikation von IVIG ein interessanter neuer Therapieansatz sein könnte für die Behandlung von Autoimmunerkrankungen, die mit RF asssoziert sind.

2. Summary

Oral administration of protein antigen has been shown to be effective in the induction of immune tolerance in a number of experimental studies. In the present study we examined the immunomodulatory capacity of human polyvalent IgG (IVIG) in a mouse model were we experimentally induced rheumatoid factor (RF) like antibodies by the injection of insoluble human immune complexes (IC). RFs are autoantibodies that recognize the Fc-part of the IgG molecule. They can be detected in several autoimmune diseases, such as Rheumatoid Arthritis, Systemic Lupus Erythematosus, and Sjögren Syndrome. The Fc-part of the IgG molecule represents the antigen that is recognized by RFs and RF-expressing B cells. Therefore, IVIG might have the capacity to induce oral tolerance against the induction of systemic RF responses.

Earlier results have demonstrated that preventive treatment with a single dose of oral IVIG prevents the induction of primary RF responses. Furthermore, continuous treatment with oral IVIG prevents both primary and memory RF responses. Therapeutic treatment with oral IVIG fails to prevent primary RF responses but is effective in the down-modulation of memory RF responses. These results are very relevant for potential clinical applications of oral IVIG because autoimmune diseases in patients are usually diagnosed after the autoimmune process has established itself.

Using transfer experiments I examined the mechanisms of action of the oral IVIG. I demonstrated that cellular mechanisms are responsible for the immunomodulatory activities of oral IVIG. Furthermore, I could show that T cells are very likely to be involved in the immune suppressive effects of oral IVIG. Therefore, I assume that the induction of unresponsiveness to the induction of RFs induced by oral IVIG might involve the activation of regulatory T cells.

An important subpopulation of regulatory T cells has been shown to be part of the $CD4^+CD25^+$ T cell subpopulation. Therefore, I did in vivo depletion of this T cell subpopulation and showed that the absence of these cells prevents the immunmodulatory effects of oral IVIG. Based on these results, I speculate that $CD4^+CD25^+$ regulatory T cells are responsible for the observed immunosuppressive effects of oral IVIG. Nevertheless, I cannot exclude that other mechanisms contribute to these effects of oral IVIG, too.

Summarizing my findings, I believe that the oral application of IVIG might be a useful clinical approach for the treatment of RF associated autoimmune diseases.

3. Introduction

3.1. Autoimmune diseases

The primary function of the immune system is to distinguish components of body tissues (self) from foreign (non-self) antigens like pathogens. Contrary to foreign antigens, which normally cause an active immune response, autoantigens induce self-tolerance (unresponsiveness to self). Self-tolerance is mediated by different mechanisms like clonal deletion, clonal anergy (central tolerance) or by active suppression (peripheral tolerance) of autoreactive B or T cells (1).
But sometimes there is a failure in the maintenance of self tolerance caused by a failure in the discrimination between self and non-self antigens. This failure leads to an autoimmune response, characterized by the activation and clonal expansion of autoreactive lymphocytes and/or the production of autoantibodies targeted against autologous antigens of normal body tissue.
The initial cause for autoimmune diseases is not fully understood, risk factors could probably be genetic disposition or environment. It is also suggested that infections may trigger the disease initiation (2, 3).

3.2.Oral Tolerance

The intestinal immune system, the most complex part of the immune system is able to distinguish between potentially harmful antigens (e.g. pathogens) and potentially harmless antigens (e.g. food proteins and commensal bacteria). It reacts to harmful antigen with an active immune response whereas it reacts to potentially harmless antigens with unresponsiveness (4, 5, 6). This kind of immune tolerance is called oral tolerance. Oral tolerance is thought to be of great importance in the development of new immunotherapies for autoimmune diseases and might be also a barrier for the development of recombinant oral vaccines.

Mechanisms of action of oral tolerance are still unclear. Knowing the compartments of the intestinal immune system, it is most likely that specialized

cells as well as the uptake and presentation of antigen in the gut might contribute to the phenomenon of oral tolerance.

3.2.1 GUT-associated lymphoid tissues (GALT)

The mucosa-associated lymphoid tissues (MALT) is a set of organized lymphoid structures found in the mucous membranes that line the gastrointestinal and respiratory tracts. The gastrointestinal part includes Peyer's Patches (PP), Lamina propria lymphocytes, and intraepithelial lymphocytes, which form together the GUT-associated lymphoid tissues. In a broader sense, mesenteric lymph nodes (MLN) also belong to the GALT.

PP are lymphoid aggregates that are found in the mucosa and submucosa of the small intestine. Mature PP contain large B-cell follicles that are intermediated by T cell areas. MLN are the largest lymph nodes of the body and their development is distinct from other compartments like PP or peripheral lymph nodes (6).

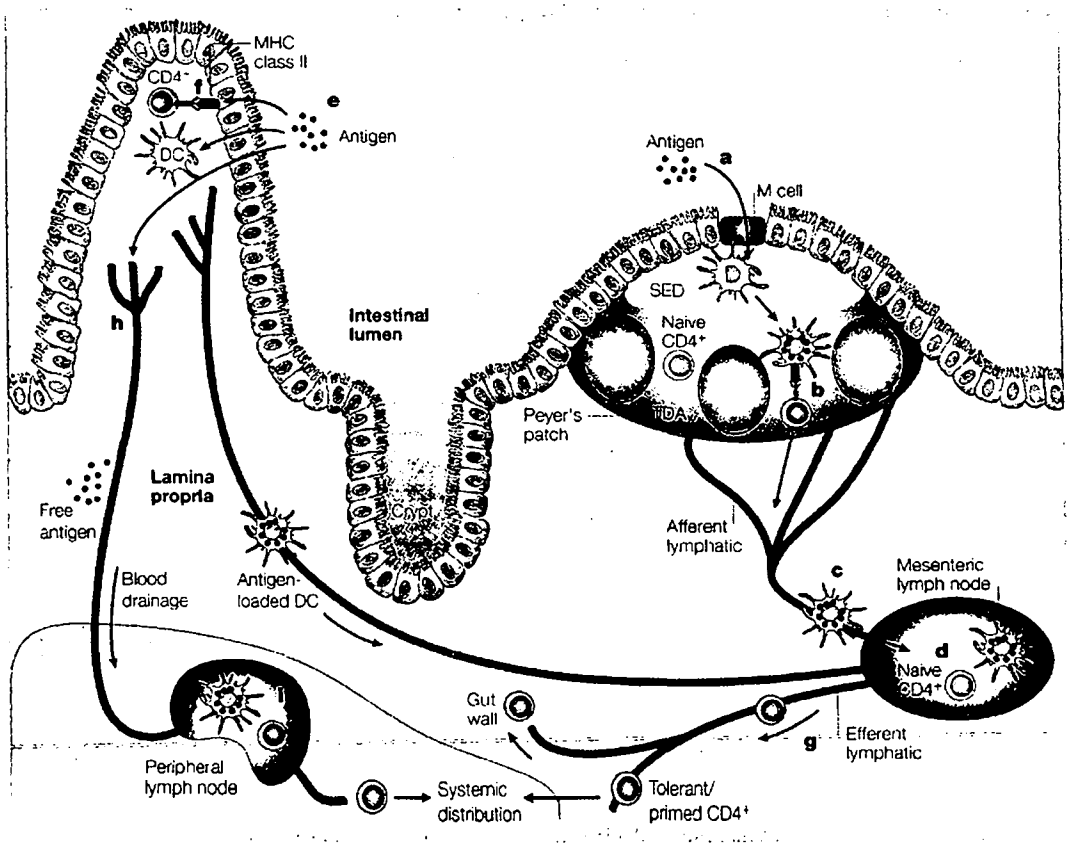
3.2.2. Antigen uptake by the GALT

Excessive entry of pathogens is prevented by the gut mainly through intestinal epithelial cells connected by tight junctions but also by factors like a low luminal pH, mucus release and the glycocalyx (5). The uptake of antigens by the intestinal immune system could be realized by several ways. The so-called M cells (membranous microfold), are specialized enterocytes that lack surface microvilli and are interspersed between the epithelial cells of PP. For many years these M cells were considered to be the main entry side for antigen from intestinal lumen (4, 5, 6). Nevertheless there seem to be alternative routes of antigen uptake from the intestinal lumen. Although it was always considered that villus epithelium is an impermeable membrane to macromolecules, it has recently been shown that proteins like ovalbumin (OVA) can be taken up and processed by enterocytes in situ (7).

3.2.3. Antigen presentation and induction of oral tolerance in the gut

A lot of work has supported the idea that antigen presentation as well as induction of regulatory cells takes place in the PP after the uptake of antigen through M cells (5, 8, 9). It was shown that the absence of PP descended T cells leads to the failure of oral tolerance induction (9, 10). However, published results of experiments examining the importance of PP as the side of antigen presentation and induction of oral tolerance have been very conflicting. Spahn et al. demonstrated that mice deficient in PP were still able to develop oral tolerance (11, 12). Reasons for this discrepancy could be that mice deficient in PP have MLN that are also potential candidates for tolerance induction. Especially the fact that mice deficient in MLN were not able to induce oral tolerance suggests that MLN might be the side of oral tolerance induction (11, 12). Recent works supported the model of antigen uptake by dendritic cells in the lamina propria and migration of these dendritic cells to the MLN to interact with naïve $CD4^+$ T cells to induce oral tolerance (4, 6, 13).

Figure1.



A McMowat: Nature Rev Immunol 2003; 3: 331-341

3.3. Mechanisms of oral tolerance

Oral tolerance is an active immunologic process that is mediated through more than one mechanism. It is thought that low doses of antigen favour the induction of active suppression mediated by some form of regulatory cells, whereas higher doses favour the induction of anergy or deletion of autoreactive cells (14). At the moment active suppression by the induction of so-called regulatory T cells enjoys great popularity and an immense number of publications have been dealing with this subject (4). Nevertheless the exact mechanisms of action of oral tolerance are still unclear and the characterizations of regulatory cells involved in oral tolerance diverge (4, 6). This might be due to the possibility that more than one regulatory cell type and more that one mechanism contribute to the initiation of oral tolerance (4, 5).

3.3.1.Deletion

Antigen-specific deletion of T cells in the spleen and GALT have been shown by experiments with T-cell receptor (TCR) transgenic mice that were fed with high doses of antigen. However, this mechanism has been observed primarily in transgenic animals and might not be in accordance with natural conditions in normal animals (5).

3.3.2. Anergy

Anergic cells are unresponsive to a stimulating signal, which means that they are unable to secrete interleukine 2 (IL-2) and to proliferate. Nevertheless, anergic cells can still be reactivated by restimulation with exogenous IL-2 (5).

3.3.3. Active suppression by regulatory T cells (Tregs)

An alternative to clonal deletion or anergy of specific T cells is the active suppression of autoreactive T cells by regulatory cells. Earlier studies suggested that $CD8^+$ "suppressor" T cells are mediators of systemic tolerance, but today it is

believed that $CD4^+$ T cells are the primary population of regulatory T cells (2). Until now several different classes of regulatory T cells have been identified. The most popular cells have been $CD4^+CD25^+$ regulatory T cells, T helper 3 (TH3) cells and T regulatory 1 (Tr1) cells. Other candidates mentioned have been $CD8^+$ T cells or $\gamma\delta$ T cells (6).

CD4+ CD25+ Treg cells:

About 10% of the peripheral $CD4^+$ T cells express CD25 (15, 16, 17, 18). Transfer of these natural occurring $CD4^+CD25^+$ cells from normal donors was able to inhibit development of autoimmune diseases in animal models (15). Further on, a number of experimental studies have shown that in vivo depletion of CD25+ T cell accelerates parthenogenesis of disease (19). This findings, led to the suggestion that $CD4^+CD25^+$ T cells must have regulatory properties towards self-antigen and therefore might maintain immunological tolerance (15, 16, 17, 18). There is evidence that potential regulatory $CD4^+CD25^+$ T cells can also arise in vivo after oral administration of antigen (20, 21). It is assumed that these antigen–specific $CD4^+CD25^+$ T cells play a major role in the induction of oral tolerance in autoimmune diseases like RA (22). This consideration is supported by the findings that adoptive transfer of antigen-specific $CD4^+CD25^+$ Tregs into recipient mice decreases the severity of disease, when recipients were immunized after the transfer (19).

On the other hand, there are indications that Tregs are also contained in the $CD4^+CD25^-$ T cell population of normal animals. In different experiments it has been shown that $CD4^+CD25^-$ T cells also bear the ability to control autoimmune diseases in animal models (16). In addition, Chung et al. found that depletion of $CD4^+CD25^+$ T cells did not reverse the immune tolerance towards the antigen in Ovabulin (OVA)-tolerized mice (23). Moreover, Bardos and his colleagues showed that neither adoptive transfer of $CD4^+CD25^+$ T cells did protect severe combined immunodeficient mice (SCID) from arthritis nor did depletion of $CD4^+CD25^+$ enhance the onset or severity of the disease (24).

In addition to these controversial findings it should be considered that CD25 is also a marker for all activated T cells, which includes effector T cells. Therefore, the discovery of a unique marker for Tregs is necessary.
The transcription factor forkhead box p3 (FoxP3) was a promising candidate for such a marker. It was demonstrated that CD25- T cells failed to express FoxP3. Morever, ex vivo retrovirally introduced expression of FoxP3 led to conversion of $CD4^+CD25^-$ T cells into the $CD4^+CD25^+$ regulatory T cells. This suggested that FoxP3 was necessary for $CD4^+CD25^+$ Treg development and that FoxP3 was exclusively expressed on $CD4^+CD25^+$ T cells (25, 26). In contrary to these findings, other groups showed that also $CD4^+CD25^-$ T cells that possess regulatory properties express FoxP3 (27). Besides D. Wraith and his colleagues found a population of $CD4^+CD25^-$ Treg cells that failed to express FoxP3 (16, 27).

Summarizing these conflicting perceptions, we have to realize that regulatory T cell could belong to the $CD25^+$ as well as the $CD25^-$ T cell subsets and FoxP3 expression is not obligatory as a marker for immunoregulatory properties of a cell. In the future the establishment of a better phenotypic profile of regulatory T cell subsets that is related to their function in vivo will be essential.

T Helper 3 cells (TH3):

Another population of regulatory T cells are TH3 CD4+ cells. These cells were described to produce transforming growth factor-β (TGF-β). They can be isolated in vitro after repeated restimulation of MLN or spleen lymphocytes from mice previously fed with low doses of antigen. Similar cells were found in vivo (4, 6).

T regulatory 1 cells (Tr1):

Tr1 CD 4+ cells were described to produce mainly interleukin-10 (IL-10). They can be isolated in vitro in the presence of antigen together with IL-10, IL-15 and/or IFN-γ. Tr1 cells could not be isolated in vivo after oral tolerance induction (4, 6).

Mechanisms of how active suppression by regulatory T cells is mediated are still under examination. It has been observed that there are differences in how regulatory T cells behave in vivo and in vitro. Active suppression can be achieved by the release of the anti-inflammatory cytokines IL-10 (e.g. Tr1, Th3, $CD4^+CD25^+$) and TGF-β (e.g. Th3, $CD4^+CD25^+$) but also by cell-cell contact. Another existing form of suppression is the so-called bystander suppression, in which regulatory T cells activated by an antigen can inhibit their surrounding cells from responding to any antigen (6).

3.3.4. Role of antigen-presenting cells in oral tolerance

The decision of the immune system whether to react with tolerance induction or with the production of an active immune response towards an antigen seems to depend essentially on the activation state of the antigen-presenting cells. It is supposed that the uptake of antigen by immature antigen-presenting cells (mainly dentritic cells) and the presentation to naïve $CD4^+$ T cells in the absence of costimulatory signals or with the involvement of inhibitory signals, might also contribute to tolerance induction in the mucosal immune system (4, 6).

3.4. Regulatory T cells in the therapy of autoimmune diseases

Induction of antigen-specific regulatory T cells by oral administration of antigen could be a promising approach for the immunotherapy of autoimmune diseases like rheumatoid arthritis. In experimental models of autoimmune or inflammatory disease, oral administration of antigen often leads to an improvement of the diseases. Clinical trials were not always successful which might arise from the circumstances that the exact mechanisms responsible for oral tolerance are still unknown (4).

3.5. Rheumatoid arthritis (RA)

Rheumatoid arthritis is a chronic inflammatory disease that causes irreversible destruction of cartilage, tendons and bone. The disease can involve all peripheral joints but the most commonly affected joints are those of the hands, feet, knees and distal interphalangial joints. RA is two to three times more frequent in women than in men. Although it can start at any age there is a high incidence between the fourth and sixth decade of life. In untreated patients RA can lead to reduction in life expectancy (28). By genetic studies it was found, that there seems to be a genetic disposition in the HLA-DR locus. But there also exists the presumption that environmental factors might play a role in developing RA (2, 3).

3.5.1.Pathogenesis

In RA the synovium of the joints is infiltrated by a large number of activated lymphocytes and macrophages. Cytokine release of activated lymphocytes induces the induction of destructive enzymes, mainly matrix metalloproteinases. These destructive enzymes are believed to be mainly responsible for the destruction of cartilage and bone (29, 30).

3.5.2. Autoantibodies in rheumatoid arthritis

Autoantibodies are characteristic for rheumatoid autoimmune diseases. Although most of them do not have a fundamental role in the disease some of them could be used for a diagnostic approach or as indicators of disease activity. Among these are the anti-DNA antibodies in systemic lupus erythematosus or anti-topoisomerase antibodies in scleroderma (31).

Rheumatoid factors (RF):

RFs were said to be the hallmark of rheumatoid arthritis, but they also appear in different additional autoimmune diseases as well as in a number of non-autoimmune conditions. Besides they only appear in about 60-80% of RA patients and are in low levels at early disease, what impedes the early identification of RA (3, 31).

RFs are produced by plasma cells in inflamed synovial tissues. They recognize the Fc-Region of human IgG that is present in the synovia of patients with RA (2, 3, 31, 32). Besides of RA and other autoimmune diseases, RFs also occur in normal elderly people or in healthy immunized individuals, as well as in people with chronic infections. The physiological role of these "natural" RFs might be the clearance of circulating immune complexes consisting of foreign antigen and bound antibodies. These physiological ("natural") RFs differ from the so-called "pathogenic" RFs in isotype, specificity, frequency of mutation, site of production or ability of complement-activation. "Pathogenic" RFs also seem to have an increased affinity to IgG-Fc when compared to "natural" RFs (32). Because RFs are often expressed during infections, pathogenic RFs may be a result of crossreactivity between microbial epitopes and IgG-Fc (32).

RF autoantibodies can appear in all immunoglobulin subclasses (IgG, IgM, IgE, IgD and IgA). Most of the RA patients have IgM RFs, while IgG RFs are less frequent. Because IgM and IgG RFs differ from the germ line sequence, it is conceivably that RF induction is an antigen driven process (31). RFs are associated with the severity of RA and after therapies RF positive patients can turn into RF negative patients. Although there is a correlation between RFs and severity of RA, the contribution of RFs to the disease itself remains unclear (31).

Other autoantibodies in rheumatoid arthritis:

Besides of RFs there are a couple of other autoantibodies associated with RA like antikeratin, antifilaggrin, anticitrulline, anti-RA33 antibodies. Antikeratin (AKA) antibodies have been shown to be very specific for rheumatoid arthritis. Other

autoantibodies like anticollagen antibodies or anticalpastatin antibodies could contribute to the pathology of the disease. Anticalpastin antibodies are directed against calpastatin, a natural inhibitor of calpains, which are calcium-ion-dependent neutral cystein proteases. Antibodies against calpastatin could increase calpain activitiy and therefore lead to an increased damage of the cartilage (3). The participation of many other autoantibodies like anticitrulline in RA parthenogenesis is still unclear.

3.5.3. The role of B and T lymphocytes in RA

Autoantibodies are produced by terminally differentiated B cells, so-called plasma cells. Although B cells are responsible for the production of autoantibodies it is still not clear if they are involved in mechanisms that lead to the onset of inflammation in autoimmune diseases like rheumatoid arthritis. As shown in different studies, B-cell depletion disrupts the production of inflammatory cytokines. Furthermore it has been shown that B cells are able to bind and present antigens and it is therefore possible that they act as antigen presenting cells that stimulate autoreactive T lymphocytes (33). B-cells seem to have an important role in the activation of $CD4^+$ T cells in the synovial tissue (34).

The autoreactive T cells are activated by antigenic peptides presented by major histocompatibility complex (MHC)-class II, but the antigen involved in this activation is still unidentified. Antigen-activated CD4+ T cells stimulate other cells like monocytes, macrophages and synovial fibroblasts to produce inflammatory cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor-α (TNF-α). These cytokines are the key players in inflammation in rheumatoid arthritis.
In vitro experiments using blocking antibodies against cytokines demonstrated the existence of a cascade of cytokines involved in inflammation in rheumatoid arthritis. TNF-α seems to play a central role in this cascade. It has been shown that the blockade of TNF-α also interferes the action of other pro-inflammatory mediators, suggesting that TNF-α is at the top of the cascade (30).

3.6. Therapeutic strategies for Rheumatoid arthritis

Today RA therapy is either based on treatment with non-steroidal anti-inflammatory drugs (NSAIDs) or on treatment with disease modifying antirheumatic drugs (DMARDS). Most of the NSAIDs inhibit cyclooxygenase 1 and cyclooxygenase 2 isoenzymes. These cyclooxygenases catalyze the formation of prostaglandin and thromboxane from arachidonic acid. Prostaglandines are mediators of inflammation (28).

DMARDs impede the inflammatory and the destructive process of the disease. DMARDS can be divided in two groups, small molecule DMARDS and biological agents. One of the best known examples for small-molecule DMARDS is Methotrexate (MTX). Unfortunately small-molecule DMARDS have limited efficacy and often also toxicity problems (28, 35).
Biological DMARDS target pro-inflammatory cytokines by different strategies. Blockade of pro-inflammatory cytokines by monoclonal antibodies, soluble receptor or antagonist is the most commonly used one (28). In RA therapy antibodies against TNF-α and IL-1prevent the molecules from binding to their receptors (28, 35).

Despite the success of anti-TNF therapies, it should be kept in mind that at least 30% of RA patients do not respond to any of these therapies. Furthermore, it should be mentioned that these drugs induce serious side effects in patients (28, 35).

Administration of intravenous immunoglobulins in RA:

Polyclonal intravenous immunoglobulins (IVIG) consists of human IgG preparations that are purified from large plasma pools obtained from thousands of healthy donors. IVIG exhibits a number of immunomodulatory activities that are mediated via the Fc-part of IgG and by the spectrum of variable (V) regions contained in the immunoglobuline preparation. Mechanisms of action of the immunomodulatory effects of IVIG are very complex. The most important mechanisms are the functional blockade of Fc receptors, the interference with

complement activation and the cytokine network, the modulation of the activation, differentiation and effector functions of B and T cells. Besides, circulating autoantibodies are neutralized by anti-idiotypic antibodies that are contained in IVIG (37, 38, 39). Therefore, IVIG is indicated as the treatment of choice in primary immune deficiencies and in a number of inflammatory and autoimmune diseases (37, 38, 39).

3.7. Objectives of my project

At the beginning of our study we asked the question whether oral application of IVIG can inhibit the induction of systemic responses of RF-like antibodies, since the Fc-part of the IgG molecule should provide the antigen that is required for the induction of oral tolerance. In preliminary experiments we could show that oral administration of IVIG prevented the systemic induction of rheumatoid factors in mice. My part of the project was to characterize the mechanisms of action of the immunomodulatory capacity of oral IVIG. In particular I tried to establish whether the immunomodulatory activity of oral IVIG might be due to the induction of regulatory T cells.

4. Results

4.1. Quality of spleen cells is determined by preparation procedure and influences the outcome of transfer experiments

4.1.1. Abstract

Oral administration of protein antigen has been shown to be effective in a number of experiments studies. In the present study we showed that oral treatment with IVIG for a short-term period of 45 days can induce systemic tolerance in a mouse model were we experimentally induced rheumatoid factors (RFs) by immunization with insoluble human immune complexes (ICs). Next, we asked the question whether these immunomodulatory effects are mediated by cellular mechanisms. Therefore we used adoptive transfer experiments where we transferred spleen cells derived from naïve mice and spleen cells derived from mice that received oral IVIG into naïve recipients. Afterwards we induced RF responses by the immunization with human ICs. To optimize the isolation procedure for the preparation of spleen cells to be transferred, we compared cells isolated using hemolysis with cells isolated by density gradient centrifugation. Our results demonstrate that only cells prepared by density gradient centrifugation contained a sufficient proportion of viable cells. When we transferred these cells into naïve mice, they did not show any prevention of primary RF responses, but a significant down-modulation of RF memory responses after booster injection. When we transferred cells obtained by hemolysis, on the other hand, cells did not express any immunosuppressive effect in the naïve recipient but rather amplified RF immune responses. The amplification of immune responses might be due to adjuvants effects induced by apoptotic cells present in the cell preparation. We conclude that the quality of cells to be transferred significantly influences the outcome of any transfer experiment.

4.1.2. Introduction

Intravenous immunoglobulins (IVIG) are polyvalent human IgG preparations that are purified from large plasma pools obtained from thousands of healthy donors. They are indicated as the treatment of choice in primary immune deficiencies and in a number of inflammatory and autoimmune diseases (1, 2, 3). Based on the fact that the Fc-part of the IgG molecule is the autoantigen that is recognized by B cells that express RFs. We asked the question whether IVIG might provide a suitable antigen for the induction of oral tolerance in several autoimmune diseases that are associated with the development of RFs.

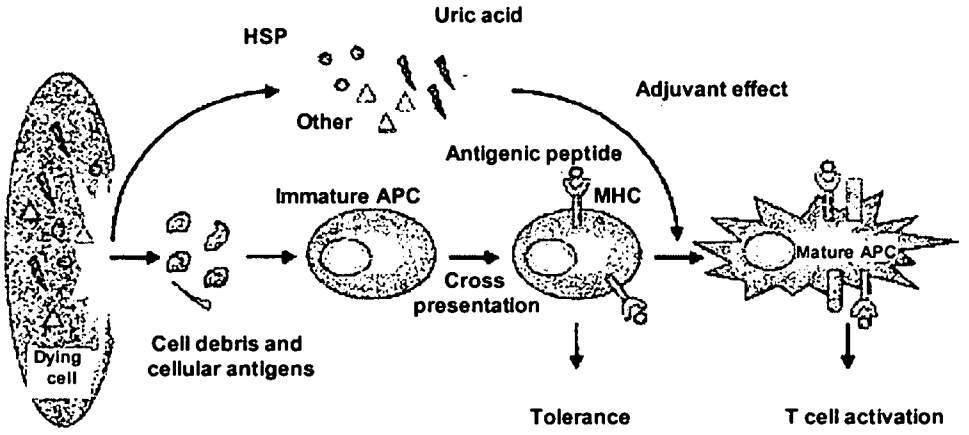
To examine the immunomodulatory potential of oral IVIG, we used a murine model first published by Abedi-Valugerdi et al. (4, 5) were we experimentally induced human rheumatoid factors. In this model, a single intravenous injection of insoluble ICs containing the antigen human IgG, induces a sustained systemic IgG response with RF like properties. Using this mouse model we could show previously that oral application of IVIG in drinking water prevents the systemic induction of RFs both in primary and secondary immune responses (see 4.2).

Based on these earlier results, we wanted to characterize the mechanisms of action of the immunomodulatory capacity of oral IVIG. In particular, we tried to ascertain whether these immunomodulatory activities might be due to the induction of regulatory T cells. To approach this question we performed adoptive cell transfer experiments, which are considered to be the method of choice for examining mechanisms of tolerance induction (6).

Initially, we compared different procedures for cell isolation to obtain cells of sufficient quality for transfer. As cell preparation procedures represent a stressful manipulation of cells, these procedures induce apoptosis in a considerable number of cells. Different authors presented evidence that stressed apoptotic cells are immunogenic and might release natural endogenous adjuvants that amplify immune responses to self and foreign proteins (7, 8). Fig. 1 shows a model for apoptotic cells releasing endogenous adjuvant molecules such as heat shock

protein (HSP) or uric acid, that stimulate the maturation of antigen presenting cells (APCs) to become immunostimulatory cells (8).

Figure 1.



KL Rock et all., Springer Semin Immunopathol. 2005 26:231-46

Previous studies showed that the injection of tumor cells bearing a foreign antigen induced an immune response against the tumor cells. Furthermore these immune responses could also be directed against intracellular antigens presented during cell injury. Many of these injuries are caused by experimental manipulations and by injection of cells into an ectopic side. As a result, many of these cells die (8). Immunization of mice with killed syngenic cells that were mixed with antigen lead to an immune response whereas immunization with the antigen alone showed no effect. Moreover, it was shown that killed syngenic cells injected with an antigen even increased the immune responses against the antigen when they were physically separated from the antigen. In addition, previous studies showed an about 10-fold increase in adjuvant effects when cells were stressed or injured for several hours before dying (8).

This led us to the presumption that apoptotic cells occurring during conventional cell preparation (9) might induce an immune response in the recipient mice against the immune complexes. Therefore, we examined if our conventional cell

preparation method results in a higher number of apoptotic cells and whether these cells influence the outcome of the transfer experiment when compared with cell preparations were apoptotic cells were removed by density gradient centrifugation.

After methodical establishment of the transfer experiments, we transferred spleen cells obtained from either naïve mice or from mice that received oral IVIG in drinking water for a certain time period. The spleen cells were injected intravenously into naïve donors. After cell transfer, donors were immunized with ICs to induce systemic RF responses (Fig. 2).

4.1.3. Material and methods

Animals:

Female Balb/c mice were obtained from Harlan Winkelmann GmbH (Borchen, Germany) and maintained in our own animal facility (Dep. Of Exp. Pharmacology and Transgenic Animals, Vienna, Austria). All animals were aged 10-12 weeks at the beginning of the experiments. All studies were carried out in accordance with Austrian federal Law (Act BG 501/1989) regulating animal experimentation.

Preparation of insoluble human immune complexes:

The preparation of insoluble human immune complexes was according to a protocol described by Abedi-Valugerdi (4, 5). Human IgG (Pierce, USA) and mouse anti-human IgG (Jackson Immuno Research Lab., USA) were mixed in a ratio 1:6 and incubated over night at 4°C. Thereafter, the antibody mixture was spun down at 17400 x g for 15 min. The supernatant was removed and the remaining pellet that contained the insoluble human immune complex was resuspended in 0,9% sodium chloride (Mayrhofer Pharmaceutics, Austria).

Induction of human rheumatoid factors:

Human rheumatoid factors (mouse antibodies specific for the Fc-part of human IgG) were induced in Balb/b mice by i.v. treatment with 100 µg of insoluble immune complexes. At several time points after the injection of IC, blood samples were taken by puncture of the retro-orbital vein. Serum was prepared and stored at -20°C until further analyses. Titers of human RF were measured by ELISA as described below. At several time points after primary immunization, mice received i.v booster injections with 100 µg IC.

Oral treatment with human IgG:

Balb/c mouse received human IVIG (Endobulin S/D, Baxter AG, Vienna, Austria) in drinking water for 45 days. The daily water consumption per cage was measured to estimate the water uptake per mouse per day. The amount of IVIG in the drinking water was adjusted for a daily uptake of 10 mg IVIG per mouse. Drinking water was replaced every day.

Isolation of spleen cells:

Spleens obtained from mice were minced and passed through an 70-µM nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ.) The cells were then washed in RPMI 1640 (Life Technologies, Paisley, Scotland) containing 5% fetal calf serum (Hyclone, Logan, UT).

Cell isolation by hemolysis:

Red blood cells were removed by hemolysis using a lysis buffer (pH 7.2) containing 0.15M ammonium chloride, 10M potassium bicarbonate (both from Merck, Darmstadt, Germany) and 0.1M ethylene-diaminetetraacetic acid (Life Technologies, Paisley, Scotland).

After hemolysis cells were washed in RPMI 1640 supplemented with 5% fetal calf serum.

Cell isolation by density gradient centrifugation (Lympholyte-M):

After single cell preparation, cells were purified from erythrocytes, apoptotic cells and debris by density gradient centrifugation using Lympholyte-M (Cedarlane, Ontario, Canada). For density gradient centrifugation, cells were resuspended in RPMI-1640 with 2% FCS.

After centrifugation, cells were washed in RPMI 1640 supplemented with 5% fetal calf serum.

Adoptive cell transfer:

$2x10^7$ spleen cells in 300µl Dulbecco's PBS (DPBS, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were i.v. injected into recipient mice on day 0. Control groups received DPBS only. One day after cell transfer (day 1), mice were immunized with insoluble human ICs. When RF titers were declining, mice received booster injections.

Detection of human rheumatoid factors by ELISA:

Polysorb microplates (Nunc, Roskilde, Denmark) were coated with 1µg/ml Fc fragments of human IgG (Jackson Lab, Maine, USA) in carbonate buffer, pH 9.6 and incubated overnight at 4°C. Plates were washed and unbound sites were blocked with 1% bovine serum albumin (BSA, Aurion, Vienna, Austria) in Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Paisley, Scotland). Serum samples obtained from mice were added at serial dilutions for 2 hours at room temperature (RT). Human RFs (mouse IgG specific for the Fc-part of human IgG) bound to the plates were incubated with horseradish peroxidase (HRP) labelled rat anti-mouse IgG1 (Pharmingen Int. Heidelberg, Germany) or HRP-labelled goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA). Substrate development was done using O-phenylene diamine dihydrochloride (Sigma-Aldrich, Steinheim, Germany) in citrate puffer (pH 5.0). The reaction was stopped with 2 N H_2SO_4 (Merck, Darmstadt Germany) after 30 min and analyzed using an ELISA reader at 492 nm (620 nm reference wave-length). Titers of RF are expressed as the highest sample dilution showing a positive result (optical density > 0.2) in the ELISA.

Preparation of total-RNA and Reverse Transcription:

Total RNA of spleen cells was isolated using TRIzol reagent (Invitrogen Life Technologies, Lofer, Austria) following the manufacturers protocol. The amount of total RNA was measured using a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Inc, Montchanin, USA).
Reverse transcription was carried out with 1µg total RNA using Hexamer primers (MBI Fermentas GMBH, St.Leon-Rot, Germany). 5x reaction buffer, 10mM dNTP mix Ribonulease inhibitor and RevertAid^{TM} M-MuLV were obtained from MBI Fermentas.

Real Time PCR:

Real Time PCR analysis was performed using TaqMan real-time reverse transcriptase–polymerase chain reaction (RT-PCR) assay on the AB Prism 7000 (Applera Austria Handels GMBH, Brunn am Gebirge) sequence detection system.
Gene expression levels were measured using pre-developed assays ("Assay-on-demand^{TM}" Gene expression products) for forkhead box P3 (FoxP3), CD3 and the TATA-box binding protein (TBP) as described by the manufacturer (Applera Austria Handels GMBH, Brunn am Gebirge). TBP, a housekeeping gene, was used for internal control. Calculation was done using the ^^CT-calculation-method and Microsoft Exel software.

Statisical analyses:

Results for antibody titers are expressed as means +/- standard deviation (SD). Statistical analysis was performed based on calculations of areas under the curve (AUC). The AUC data were assessed for normal distribution using the Anderson-Darling method. Means, SD, minimum, maximum, least square means (LSMEANS) and 95% confidence limits (95% CI) were calculated for each group using the program SAS. Comparisons between the treated groups and the control groups were performed by variance analyses using the program SAS.

4.1.4. Results

The quality of spleen cell preparation depends on the procedure used for isolation of cells

For optimization of transfer experiments different procedures for cell isolation were compared to obtain cells of sufficient quality for transfer. I investigated whether our conventional preparation using hemolsyis to remove erythrocytes (9) results in a high number of apoptotic cells when compared, with spleen cells prepared by density gradient centrifugation. The amount of apoptotic cells was studied by Forward-scatter (FSC) and Side-scatter (SSC) analysis using a FACS-Calibur and cell quest pro software (BD Becton Dickinson Bioscience, Heidelberg, Germany). Results of FACS-analysis demonstrate that only cells prepared by density gradient centrifugation contained a sufficient proportion of viable cells (Fig. 3b), whereas the preparation using hemolysis resulted in a high number of apoptotic cells (Fig. 3a).

Induction of systemic rheumatoid factor (RF) responses

Intravenous injection with a single dose of insoluble human immune complexes into Balb/c mice induced a sustained RF antibody response against the Fc-part of human IgG (Fig. 4-7). These results confirm previous findings published by Abedi-Valugerdi et al. (4, 5). After decline of antibody titers, mice were again i.v. treated with IC to booster the titers of RF (Fig. 4-7).

Prevention of rheumatoid factor responses after oral IgG

IVIG is a human polyvalent IgG preparation and therefore contains the antigen recognized by RF-expressing B cells. I asked the question, whether oral treatment with IVIG prevents the induction of RF in mice. Here, I show that oral treatment with IVIG in drinking water for 45 days prevents the induction of RFs in naïve mice (Fig. 4-7). Next, I was interested to know whether oral treatment with IVIG also has

a positive influence on memory responses after booster injections. In my experiments I could show that preventive treatment with oral IVIG for 45 days did not only prevent primary RF responses (Fig. 4-7) but did also prevent booster effects of RF responses (Fig. 4-7).

Adoptive transfer of spleen cells prepared by hemolysis does not prevent RF responses

To address the question whether regulatory T cells are involved in the immunomodulatory activities of oral IVIG, we transferred spleen cells from either naïve mice or mice treated with oral IVIG for 45 days into naïve recipients. The spleen cells were injected intravenously into naïve donors, in which we subsequently induced RF responses by immunizing with human IC. Control groups received PBS instead of cell transfer (Fig. 2). Transfer experiments showed that transfer of spleen cells prepared by hemolysis does not prevent the induction of RF responses in recipient mice. Cells obtained from naïve mice even amplified the RF response (Fig. 4a, 4b). These results confirm data obtained from FACS-analysis (Fig. 3), which showed that cell preparation using hemolysis results in a high amount of apoptotic cells. We would therefore suggest that the amplification of the immune response might be due to a stimulation of the immune system by the apoptotic cells.

Adoptive transfer of spleen cells prepared by density gradient centrifugation prevent RF responses

Based on the results from transfer experiments with spleen cells obtained by cell preparations using hemolysis, we decided to use spleen cells obtained by cell preparations using density gradient centrifugation for further experiments. In these experiments we could show that transfer of spleen cells prepared by density gradient centrifugation does not inhibit the induction of primary RF responses (Fig. 5-6) but significantly down-modulates the immune response after booster injections (Fig. 5, 7).

Cells obtained from mice treated with oral IVIG for 45 days showed a tendency to prevent primary immune responses when compared to cells obtained from naïve mice (Fig. 6a, 6b). There is no significant difference in the suppression of RF memory responses after transfer of cell obtained from naïve mice compared with cells obtained from mice treated with oral IVIG for 45 days (Figs. 5a, 5b, 7a, 7b). This might be due to natural regulatory cells in naïve mice.

FoxP3 expression of spleen cells from naïve or IVIG treated mice did not show any differences

Another way to address the question whether the immunmodulatory activities of oral IVIG are due to regulatory T cells is to investigate the expression of FoxP3 as a potential marker specific for regulatory T cells. Using real time PCR, we examined the expression of FoxP3 in spleen cells from naïve mice as well as from mice that received oral IVIG for 45 days in drinking water. Results from real time PCR showed that the expression level of FoxP3 in spleen cells from naïve mice or from IVIG pre-treated mice did not show significant differences (Fig. 8a). Even when we adjusted the expression of FoxP3 to the amount of T cells in the cell populations, we did not see any significant difference (Fig. 8b).

4.1.5. Discussion

Our studies investigated the immunomodulatory activities of oral IVIG. For our studies we used a mouse model in which we induced an antibody response against the Fc-part of human IgG by a single dose injection of insoluble human immune complexes. Since IVIG is a human polyvalent IVIG preparation and should provide the antigen that is required for the induction of oral tolerance, we asked the question, whether oral treatment with IVIG prevents the induction of RF in mice. In different experiments we could show that administration of IVIG in drinking water for a time period of 45 days prevents the induction of RFs and RF memory responses in naïve mice.

Further on, we tried to characterize the mechanisms of action that are responsible for the immunomodulatory activities of oral IVIG. For this purpose we used adoptive transfer experiments, were we transferred spleen cells from naïve mice or cells obtained from mice that received IVIG in drinking water for 45 days. We found that the quality of the preparation method has a significant influence on the outcome of the transfer experiments. Spleen cell preparation prepared using hemolysis of the red blood cells resulted in a high amount of apoptotic cells, whereas preparations using density gradient centrifugation resulted in a sufficient amount of viable cells. Transfer of spleen cells prepared using hemolysis resulted in an increase of the RF-titers rather than a down-modulation. Those results are probably due to the high amount of apoptotic cells that resulted from the preparation. In several publications it has already been shown that apoptotic cells are immunogenic (7, 8). Some papers suggest that the injection of damaged cells in combination with an antigen leads to an immune response against the antigen, even when cells and antigen are physically separated (8). These results suggest that the high amount of apoptotic cells caused by the preparation method might lead to an immune response against the IC in our model. This is confirmed by the finding that spleen cell preparations obtained by using density gradient centrifugation contained much less apoptotic cells and did not amplify the immune response in recipient mice after transfer.

Transfer of spleen cells prepared by density gradient centrifugation significantly down-modulated RF memory responses after booster injections, although it did not

prevent primary responses in naïve recipient. We also saw a suppression of immune responses after transfer of cells obtained from naïve mice. This indicates the presence of natural regulatory cells, e.g. natural regulatory T cells in naïve mice. There is evidence that these natural regulatory T cells are $CD4^+CD25^+$ $FoxP3^+$ cells. In several studies it has been shown that depletion of these cells leads to autoimmune diseases (10, 11, 12). In contrast to our expectations, there was no significant difference in the suppression of immune responses after transfer of cell obtained from naïve mice compared with cells obtained from mice treated with oral IVIG for 45 days. These results might have different causes. One reason might be that the transfer of total spleen cells obtained from IVIG treated mice is not sufficient to transfer the immunomodulatory activities of oral IVIG. The concentration of regulatory cells activated by oral IVIG might be too low in the preparation of total spleen cells. Therefore, transfer experiments with enriched T cell subpopulations might be more appropriate.

Another possibility might be that the time period of IVIG administration is not sufficient to recruit enough regulatory T cells to the spleen to prevent the induction of RF responses after transfer. Maybe a longer time period of oral administration of IVIG administration would be necessary. Furthermore, it is not clear whether spleen cells are the ideal cell population to mediate tolerogenic effects in transfer experiments. Both localization and exact mechanisms of the induction of oral tolerance are still unclear (13). Transfer of cells from Peyer's patches, mesenteric lymph nodes or lamina propria might be more suitable to transfer the immunomodualtory effects of oral IVIG (14, 15, 16, 17, 18).

Another very interesting point is that we saw no difference in the expression of FoxP3, a potential marker for regulatory T cells (19, 20) in spleen cells from naïve mice compared with spleen cells obtained from mice that received oral IVIG for 45 days. The findings of FoxP3 as a marker for regulatory T cells have been very conflicting. There are publications that described regulatory T cells that do not express FoxP3 (21). Therefore it is possible that regulatory T cells involved in the immunmodulatory activities of oral IVIG do not express FoxP3.

Further investigations regarding the mechanisms of action of oral IVIG will be necessary. These investigations should involve transfer experiments with enriched T cell subsets and experiments in which regulatory T cells are in vivo depleted.

4.1.6. Figures

Figure 2.

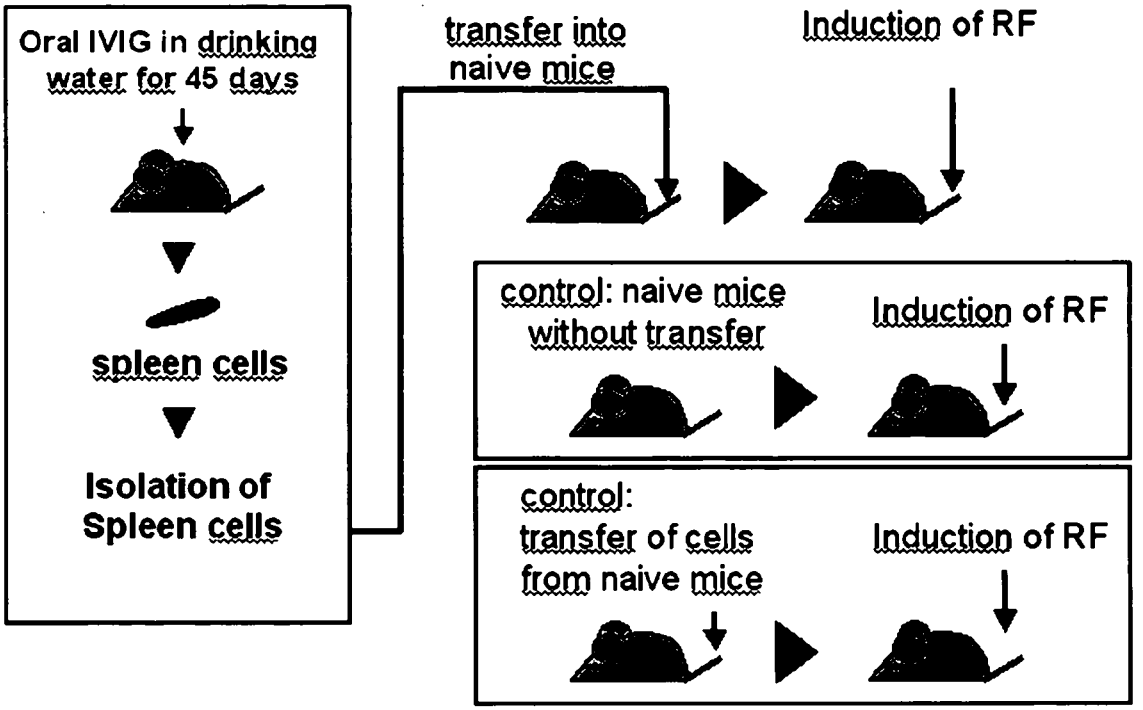


Figure 2. Scheme of transfer experiments

Mice received IVIG in drinking water for 45 days. Afterwards spleens were removed and spleen cells isolated. $2x10^7$ cells were transferred into naïve recipient mice. One day after cell transfer, recipient mice were immunized with insoluble human IC to induce the development of human rheumatoid factors.
Control groups received spleen cells from naïve mice and were also immunized with insoluble human immune complexes one day after transfer. Controls without transfer received i.v. DPBS instead, but were also immunized with IC following the same scheme as in the transfer groups.

Figure 3.

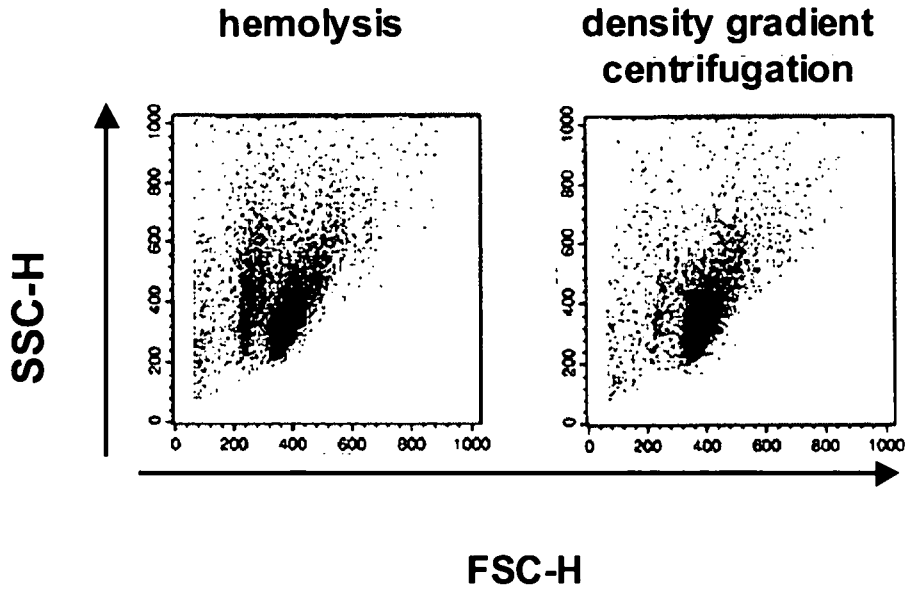


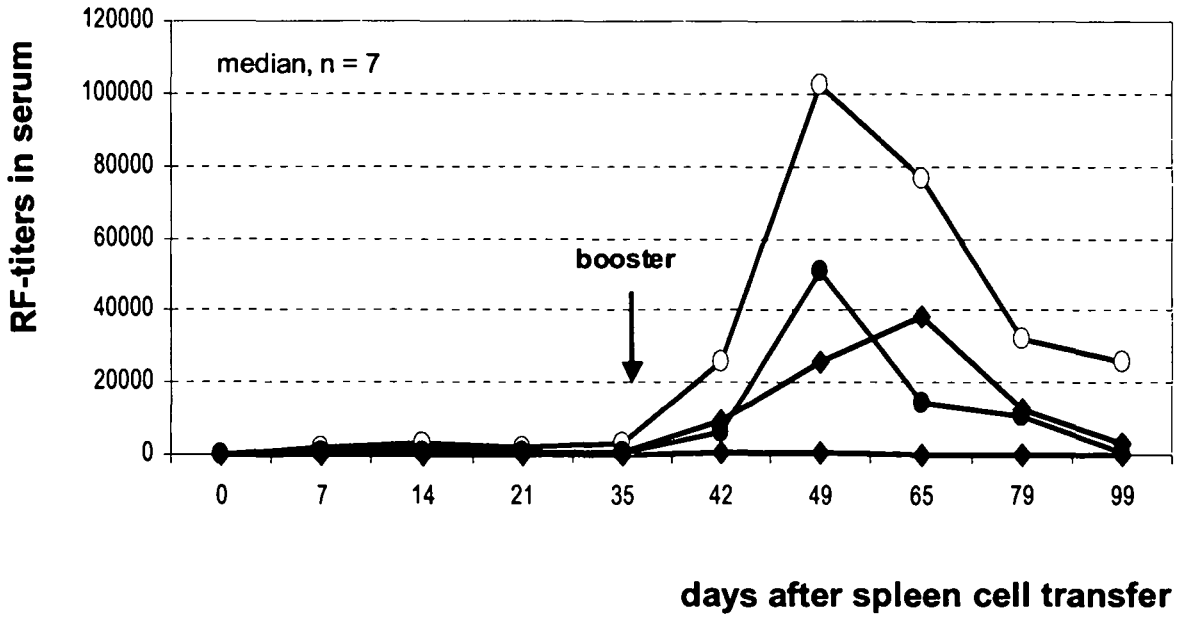
Figure 3. FACS analysis of spleen cells from different preparation methods

Forward scatter analysis (FSC-H) is representative for the cell size. Side scatter analysis (SSC-H) is determined by the granularity of the cell. The cell population at the left shows a lower cell size and less granularity. These cells are apoptotic cells.

The left picture shows the FSC-H/SSC-H analysis of spleen cells prepared using hemolysis. The right picture shows the FSC-H/SSC-H analysis of spleen cells prepared using density gradient centrifugation.

Figure 4.

4a



4b

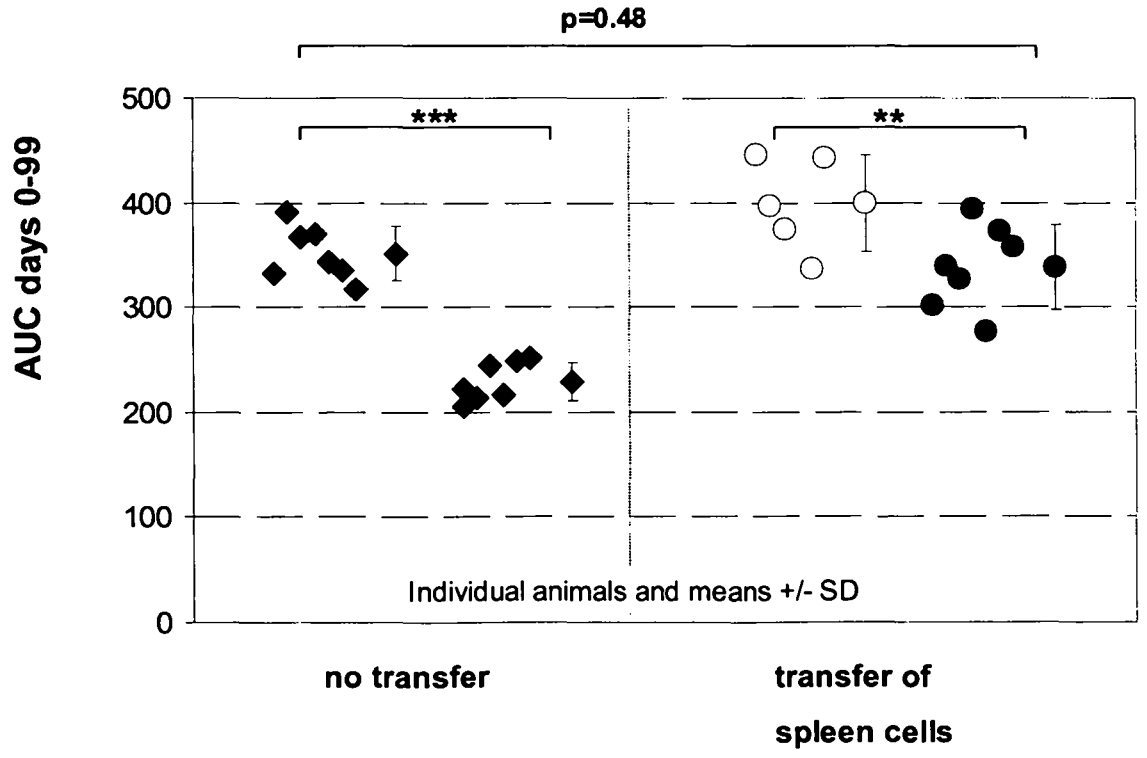
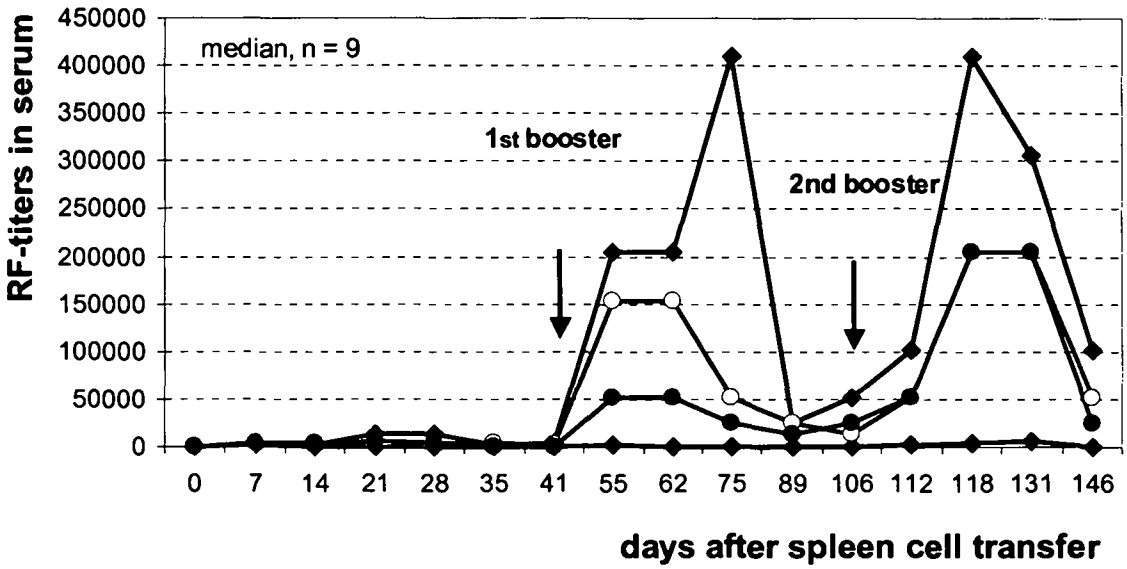


Figure 4. Transfer of Spleen cells prepared using hemolysis

Spleen cells (O) from naïve mice or from mice that had received oral IVIG for 45 days in drinking water (●) were transferred on day 0. Control mice received DPBS buffer instead of cells and either no further treatment (◆) or IVIG in drinking water for 45 days (◆). All groups were first immunized with IC on day 1 and received booster injections on day 36. RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (4a) or the areas under the curve (AUC) for the development of ELISA titers from days 0-99 (4b). AUCs were calculated for each individual animal. Means and standard deviations for AUCs were calculated for each group. **p<0.01; ***p<0.001

Figure 5.

5a



5b

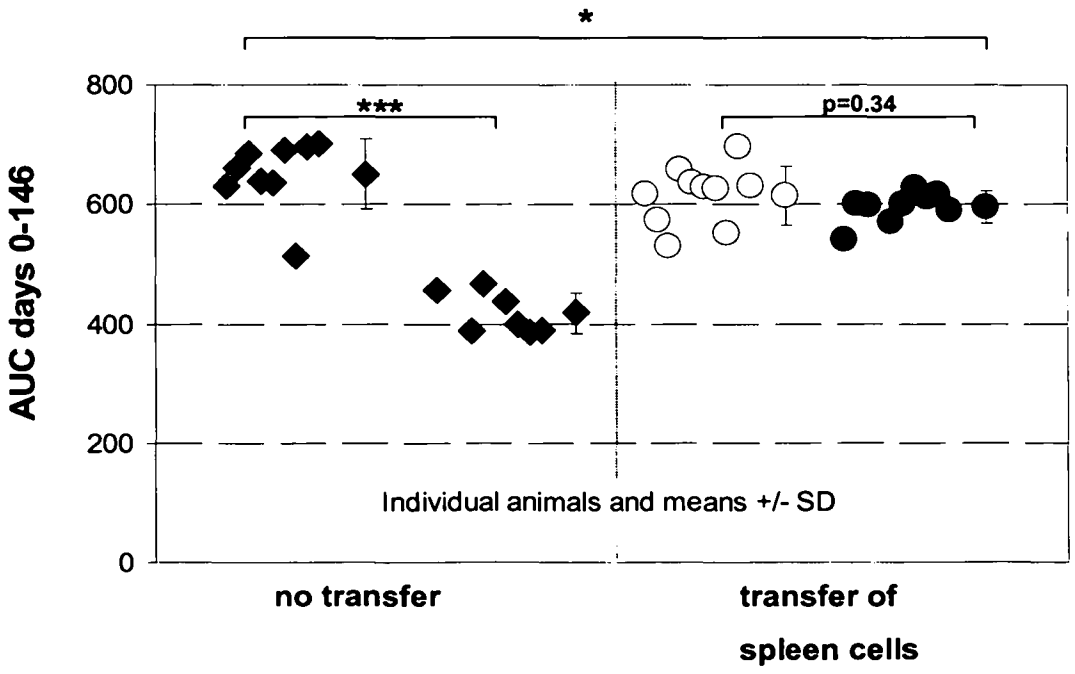
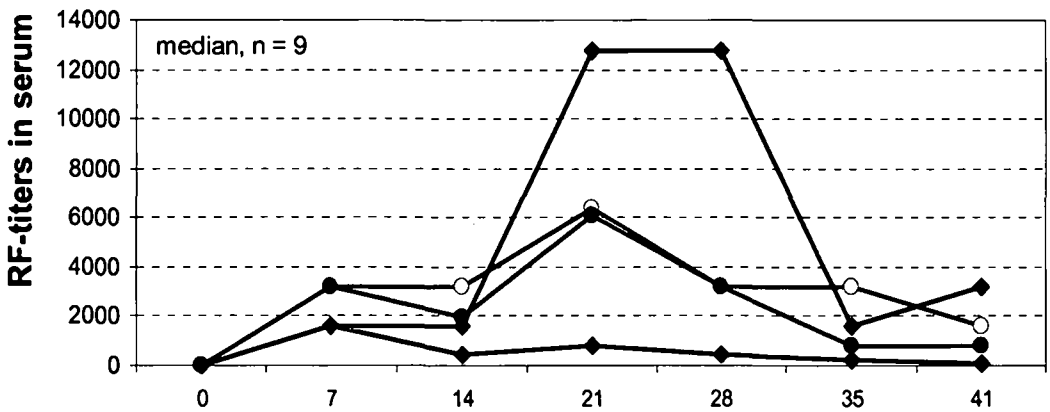


Figure 5. Transfer of spleen cells prepared using density gradient centrifugation

Spleen cells (O) from naïve mice or from mice that had received oral IVIG for 45 days in drinking water (●) were transferred on day 0. Control mice received DPBS buffer instead of cells and either no further treatment (◆) or IVIG in drinking water for 45 days (◆). All groups were first immunized with IC on day 1 and received booster injections on day 42 and 106. RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (5a) or the areas under the curve (AUC) for the development of ELISA titers from days 0-146 (5b). AUCs were calculated for each individual animal. Means and standard deviations for AUCs were calculated for each group. *$p<0.05$; ***$p<0.001$

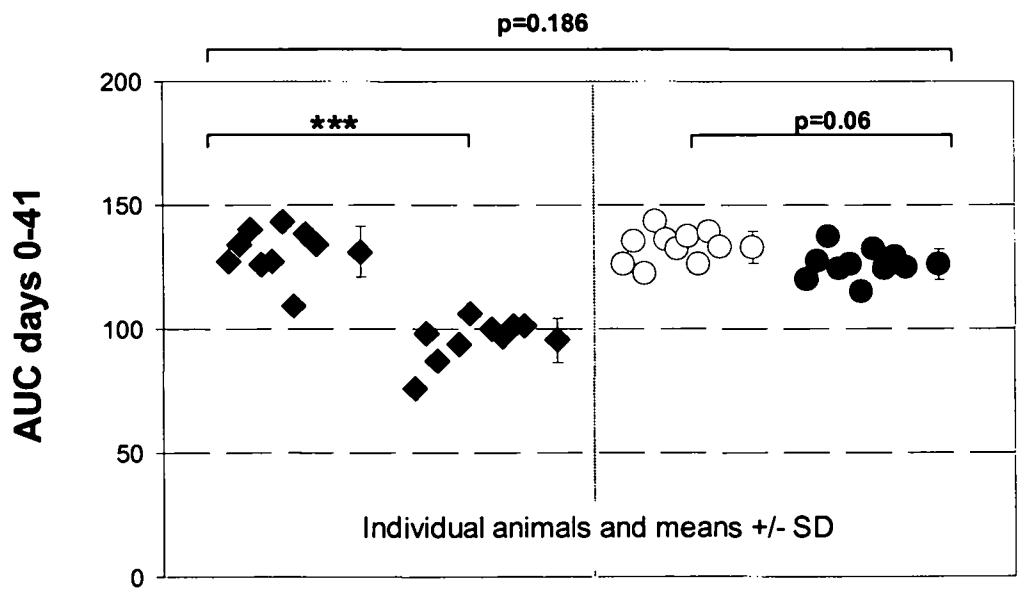
Figure 6.

6a



days after spleen cell transfer

6b



no transfer

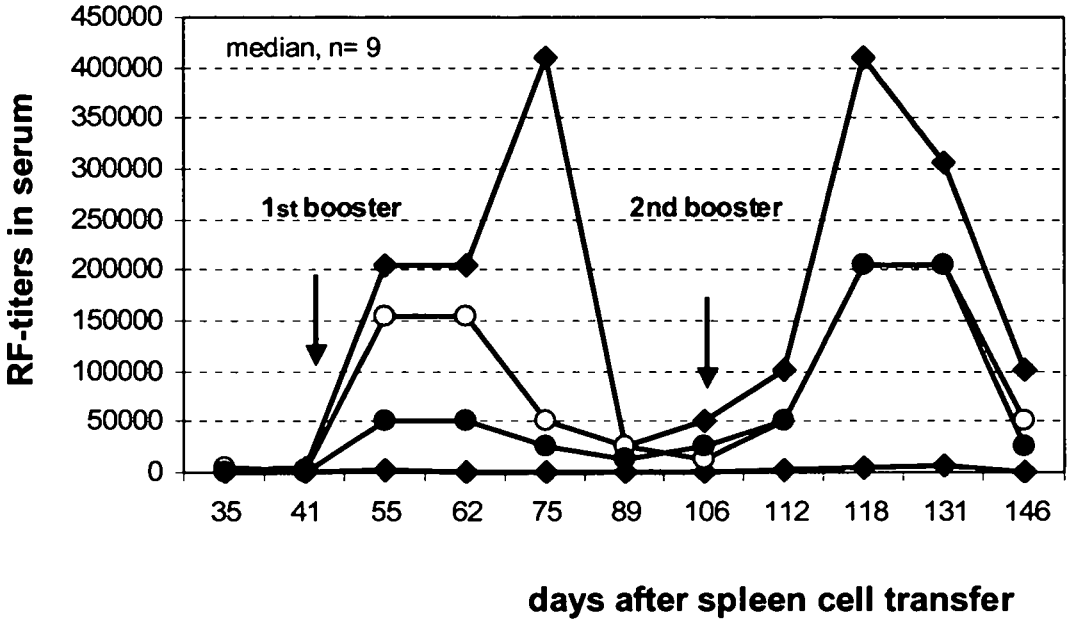
transfer of spleen cells

Figure 6. Primary immune response from transfer experiments shown in Fig. 5

RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (6a) or the areas under the curve (AUC) for the development of ELISA titers from days 0-41 (6b). AUCs were calculated for each individual animal. Means and standard deviations for AUCs were calculated for each group. ***$p<0.001$

Figure 7.

7a



7b

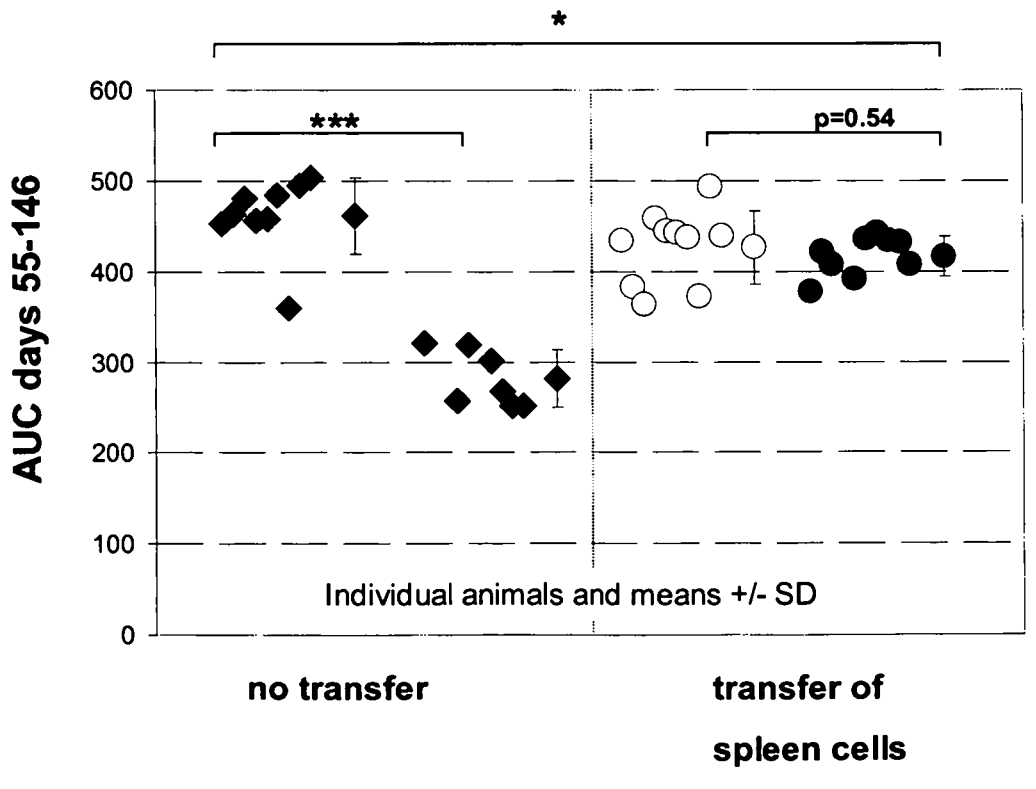
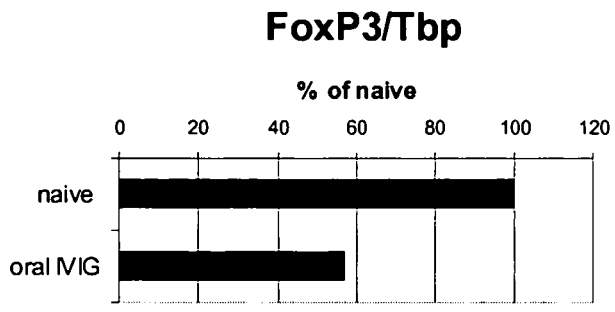


Figure 7. Memory responses after two booster immunizations of the transfer experiment shown in Fig. 5

RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (6a) or the areas under the curve (AUC) for the development of ELISA titers from days 55-146 (6b). AUCs were calculated for each individual animal. Means and standard deviations for AUCs were calculated for each group. *$p<0.05$; ***$p<0.001$

Figure 8.

8a



8b

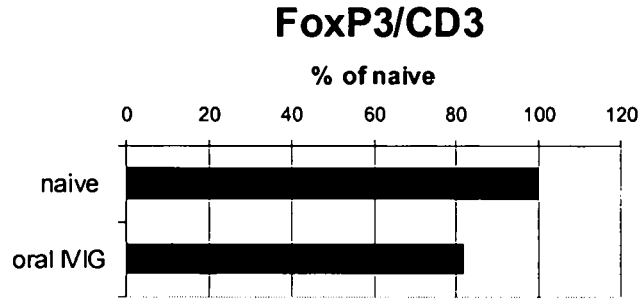


Figure 8. FoxP3 expression of spleen cells obtained from naïve or IVIG treated mice

Forkhead box P3 (FoxP3) expression normalized to T TATA-box binding protein (Tbp), a housekeeping gene (8a). FoxP3 expression normalized to CD3, a T cell marker (8b).

4.1.7. References

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4.2. Induction of oral tolerance on rheumatoid factor responses by treatment with human IgG (IVIG)

4.2.1. Abstract

Intravenous immunoglobulins (IVIG) are indicated as the treatment of choice in a number of inflammatory and autoimmune diseases. In the present study we investigated whether oral treatment with IVIG can induce systemic tolerance to experimentally induced rheumatoid factors (RFs). Balb/c mice were fed with IVIG before and after immunization with insoluble immune complexes (ICs) for the induction of RFs. The serum antibody titers of RFs were significantly reduced after both primary and booster immunization when IVIG was administered as a single oral dose or as continuous feeding starting 5 days before primary induction of RFs. This effect was inhibited when treatment with oral IgG was started 5 days after RF induction. However, continuous feeding with IVIG prevented booster effects on antibody titers even when treatment was started after primary immunization. Treatment with fragments of IgG, provide evidence that the observed effect is mediated by the Fc-part and not the Fab part of IgG. Furthermore we were able to show that transfer of spleen cells from oral IVIG treated animals, suppressed systemic RF responses in recipient mice with established RF response. We conclude that oral tolerance against primary and memory RF responses in mice can be induced by oral application of IVIG. The results also indicate, that the immunomodulatory activity of oral IVIG might be mediated by regulatory cells.

4.2.2. Introduction

Intravenous immunoglobulins (IVIG) are polyvalent human IgG preparations that are purified from large plasma pools obtained from thousands of healthy donors. They are indicated as the treatment of choice in primary immune deficiencies and in a number of inflammatory and autoimmune diseases (1, 2, 3). The immunomodulatory effects of IVIG have been attributed to a range of different biological functions of the polyvalent human IgG that are mediated by either the Fc- part or the antigen-binding $F(ab`)_2$ part of the IgG molecule (3, 4). Recently Krause et al. presented evidence that feeding of mice with IVIG or $F(ab`)_2$ fragments of IVIG induced a reduction in the antibody response against ß2 glycoprotein I (ß2GPI) and a significant attenuation of clinical symptoms in a murine model of experimental antiphospholipid syndrome. The therapeutic effect of oral IVIG in this model was due to an induction of oral tolerance by anti-idiotypic antibodies present in IVIG that were directed against anti-ß2GPI autoantibodies. The anti-idiotypic antibodies may bear the internal image of the eliciting antigen (5) and, therefore, would be able to induce oral tolerance against the antigen. The authors concluded that IVIG might be used to induce oral tolerance in various autoimmune diseases (5).

We studied the immunmodulatory potential of oral IVIG in a murine model of experimentally induced human rheumatoid factors (RF). In this model, a single intravenous injection of insoluble immune complexes (IC) containing the antigen human IgG induce a sustained systemic IgG response with RF like properties (6, 7). RFs are autoantibodies that recognize the Fc part of the IgG molecule (8, 9). They can be detected in several autoimmune diseases, such as Rheumatoid Arthritis (9, 10), Systemic Lupus Erythematosus (10), Mixed Cryoglobulinemia and Sjögren Syndrome (11, 12). Although the significance of RF in the pathogenesis of these autoimmune diseases is still controversial, several studies provided evidence that the severity and activity of Rheumatoid Arthritis can be correlated with systemic levels of RFs (9). Furthermore, it was shown that RF-expressing B cells act as antigen-presenting cells that internalize any IgG-containing immune complex, process the antigens contained in the immune complexes and present the antigenic peptides to T cells (13, 14). This could induce the activation of autoreactive T cells. RFs themselves may be the stimulus for self-perpetuating B

cell activation by binding of antibody complexed antigens. Therefore, both RF-expressing B cells and RFs are considered to contribute synergistically to severe Rheumatoid Arthritis (15, 16).

In the present study we asked the question whether oral application of IVIG can inhibit the induction of systemic responses of RF-like antibodies. In this approach, the Fc-part of the IgG molecule should provide the antigen that is required for the induction of oral tolerance. If successful, oral IVIG could be used for the treatment of autoimmune diseases that are associated with the development of RFs.

4.2.3. Material and Methods

Animals:

Female Balb/c were obtained from Harlan Winkelmann GmbH (Borchen, Germany) and maintained in our own animal facility (Baxter AG, Vienna, Austria). All animals were 8-10 weeks old at the start of the experiments and housed in 5 mice per cage. All studies were carried out in accordance with Austrian federal Law (Act BG 501/1989) regulating animal experimentation.

Induction of rheumatoid factors:

Human rheumatoid factors (mouse antibodies specific for the Fc-part of human IgG) were induced in Balb/c mice by i.v. treatment with 100 µg of insoluble immune complexes (IC) containing human IgG (Pierce, Rockford, Illinois, USA) and mouse anti-human IgG (Jackson Lab. Bar Habor, Maine, USA) according to a protocol described by Abedi-Valugerdi (5, 6).
At several time points after the injection of IC, blood samples were taken by retro-orbital puncture. Serum was prepared and stored at -20°C until further analyses. Titers of human RF were measured by ELISA as described below. At several time points after primary immunization, mice received i.v booster injections with 100 µg IC.

Detection of rheumatoid factors by ELISA :

Polysorb microplates (Nunc, Roskilde, Denmark) were coated with Fc fragments of human IgG (Jackson Lab, Maine, USA) in carbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed and unbound sites were blocked with 1% bovine serum albumin (BSA, Aurion, Vienna, Austria) in Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Paisley, Scotland). Serum samples obtained from mice were added at serial dilutions for 2 hours at room temperature (RT). Human RFs (mouse IgG specific for the Fc-part of human IgG) bound to the

plates were incubated with horseradish peroxidase (HRP) labelled rat anti-mouse IgG1 (Pharmingen Int. Heidelberg, Germany) or HRP-labelled goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA). Substrate development was done using O-phenylene diamine dihydrochloride (Sigma-Aldrich, Steinheim, Germany) in citrate puffer (pH 5.0). The reaction was stopped with 2 N H_2SO_4 (Merck, Darmstadt Germany) after 30 min and analyzed using an ELISA reader at 492 nm (620 nm reference wave-length). Titers of RF are expressed as the highest sample dilution showing a positive result (optical density > 0.2) in the ELISA.

Induction of anti-factor VIII antibodies and blood sampling:

Treatment with human factor VIII (FVIII) was performed according to a protocol described by Hausl et al. (17).
Mice received 4 i.v. doses of 200ng recombinant human FVIII diluted in 200 µl DPBS at weekly intervals. The recombinant human FVIII was an albumin free bulk material obtained from Baxter BioScience (Thousands Oaks, CA.). At several time points after the first dose of FVIII, blood samples were taken by retro-orbital puncture. Serum was prepared and stored at -20°C until further analyses. Titers of anti-FVIII antibodies were measured by ELISA.

Detection of anti-FVIII antibodies by ELISA:

Serum titers of anti-FVIII antibodies were measured by ELISA as described previously (17).

Oral treatment with IVIG:

Groups of 10 mice were orally treated by either single dose application or continuous treatment with IVIG (Endobulin S/D, Baxter AG, Vienna, Austria).

Single dose application was done by intragastric intubation using a syringe fitted with a 18-gauge ball point needle. Mice were orally treated with one dose of 10 mg

IVIG. 30 min before treatment with IVIG, mice received an oral application of 300 µl soybean trypsin inhibitor (20 mg/ml, Sigma-Aldrich, Vienna, Austria) containing 0,15M sodium bicarbonate (Merck, Darmstadt, Germany) to neutralize enzyme activities and stomach acidity.

Continuous treatment with IVIG: IVIG was dissolved in drinking water. The daily water consumption per cage (5 mice) was measured to estimate the water uptake per mouse per day. The amount of IVIG in the drinking water was adjusted for a daily uptake of 10 mg IIVIG per mouse. Drinking water was replaced every day.

Isolation of spleen cells:

Spleens obtained from mice were minced and passed through an 70-µM nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ.) The cells were then washed in RPMI 1640 (Life Technologies, Paisley, Scotland) containing 5% fetal calf serum (Hyclone, Logan, UT). Red blood cells were removed by hemolysis using a lysis buffer (pH 7.2) containing 0.15M ammonium chloride, 10M potassium bicarbonate (both from Merck, Darmstadt, Germany) and 0.1M ethylene-diaminetetraacetic acid (Life Technologies, Paisley, Scotland).

After hemolysis cells were washed and resuspended in RPMI 1640 supplemented with 10% fetal calf serum, 2mM L-glutamine (Hyclone, Logan, UT), 100 U/ml penicillin, 100 mg/ml streptomycin (both from Life Technologies) and $5x10^{-5}$ M ß-mercaptoethanol (Sigma-Aldrich, Irvine, UK).

In some experiments B cells were depleted from spleen cells using mouse pan B (B220) Dynabeads (Dynal Biotech ASA, Oslo, Norway). Depletion was done following the manufacture's instructions. The purity of the cell populations was analyzed by flow cytometry.

Adoptive cell transfer:

$1x10^7$ cells in 200 µl DPBS were i.v. injected into recipient mice that were pretreated with two doses of ICs.

Flow cytometric analysis:

The purity of different cell populations was analyzed by flow-cytometry. Phycoreythrin-labelled hamster anti-mouse CD3e (clone 145-2C11) and fluorescein isothiocyanate labeled rat anti-mouse CD19 (clone 1D3) were used for cell staining. Isotype matched antibodies were included as negative controls for antibody staining. All antibodies were obtained from PharMingen International (Heidelberg, Germany).

Human monoclonal IgG1:

Human purified monoclonal IgG1 specific for an unrelated human antigen was obtained from Baxter BioScience (Orth, Austria).

Preparation of IgG1-fragments:

Purified human monoclonal IgG1 was digested with papain immobilized to agarose beads (Sigma-Aldrich, Steinheim, Germany) in the presence of the reducing agent cystein (Sigma-Aldrich, Steinheim, Germany). The digestion was performed in batch mode at pH: 5.5, at 2-8 °C for 24 hours. 0.3 µg of immobilized papain were added to 1mg IgG1. The fragmentation was stopped by filtration through a glas fibre (Satorius, Göttingen, Germany). Fc and Fab fragments from IgG1 were separated chromatographically using a cation exchanger (Source S, Amersham, Upsala, Sweden). Samples were diluted in 2-N-Morpholinoethanesulfonic acid (MES) loading buffer containing 20 mM MES (Sigma-Aldrich, Irvine, UK) and 2% Mannit (Merck, Darmstadt, Germany) at pH: 5.0 and subsequently applied to the Source S column. The fragments were eluted using a linear sodium (Na^+) gradient ranging from 25 mM to 250 mM at pH: 5.5. Fractions containing highly purified Fc fragments or Fab fragments were identified by size-exclusion (SEC) and SDS-PAGE electrophoresis combined with western blot analysis. Purified fragments were further concentrated using the cation exchanger Source S. Elution was performed with Dulbecco's phosphate buffered saline (DPBS, pH 7.0, Life

Technologies, Paisley, Scotland). The final preparations were stored in PBS at 2-8°C.

Statistical analyses:

Results for antibody titers are expressed as means +/- standard deviation SD. Statistical analyses were performed based on calculations of areas under the curve (AUC). The AUC data were assessed for normal distribution using the Anderson-Darling method. Means, SD, minimum, maximum, least sqare means (LSMEANS) and 95% confidence limits (95% CI) were calculated for each group using the program SAS. Comparisons between the treated groups and the control groups were performed by variance analyses using the program SAS.

4.2.4. Results

Induction of systemic rheumatoid factor (RF) responses

Intravenous treatment of Balb/c mice with a single dose of IC induced a sustained antibody response against the Fc-part of human IgG (Fig.1a). The antibody response was almost exclusively of the IgG1 isotype. The results confirm previous findings published by Abedi-Valugerdi et al. (6, 7).
Antibodies against the Fc-part of human IgG are subsequently called RF because their specificity resembles RF found in patients with autoimmune diseases.
After decline of antibody titers, further i.v. treatment with IC boostered titers of RF (Fig.1b).

Single oral dose of IVIG and continuous oral treatment with IVIG prevents induction of systemic RF responses

IVIG is a human polyvalent IgG preparation and therefore contains the antigen recognized by RF. We asked the question, whether oral treatment with IVIG prevents the induction of RF in mice. A single oral dose of IVIG given 5 days before the induction of RF prevented the formation of RF almost completely (Fig.2). When we compared different doses of IVIG (0.1 mg; 1 mg and 10 mg). 10 mg showed the most dramatic effect. Oral exposure to IVIG at 4 days after induction of RF responses did not cause any inhibition of antibody development (Fig.2). These results indicate that the timing of oral IVIG treatment is very important. Once the RF response is established, it is no longer sensitive to oral IVIG.
We next asked the question whether feeding with IVIG in drinking water would induce similar immunomodulatory effects as treatment with a single dose of IVIG. IVIG was dissolved in drinking water at an amount sufficient to provide a daily uptake of 10 mg IVIG per mouse. We used two feeding protocols, one protocol started 5 days before induction of RF and the other protocol started treatment 5 days after induction of RF. When we started treatment 5 days before induction of IC, development of RF was almost completely inhibited (Fig. 3a, 3c). However,

when we started treatment 5 days after induction of RF, no inhibition of RF responses was observed (Fig. 3b, 3c). Therefore, we conclude that results observed after treatment with IVIG in drinking water were comparable with findings obtained with a single oral dose of IVIG given by intragastric intubation.

Continuous oral treatment with IVIG suppresses booster of systemic RF responses

Autoimmune diseases in patients are usually diagnosed after the autoimmune process has established itself. Therefore, it would be elusive to believe that preventive treatment with oral IVIG could be done in patients with autoimmune diseases. The course of most autoimmune diseases is characterized by a series of stable phases and relapses. Relapses are caused by booster effects of the immune system and present a particular problem for the patient. We asked the question whether continuous feeding with oral IVIG would prevent booster effects of RF responses. Preventive treatment with oral IVIG did not only prevent primary RF responses (Fig. 3a, 3c) but did also prevent booster effects of RF responses (Fig. 4a, 4c). Significant differences in RF titers were observed between control groups and treatment groups. Therapeutic treatment with oral IVIG did not modulate primary RF responses (Fig. 3b, 3c) but did significantly reduce booster effects of RF responses (Fig. 4b, 4c). The immunomodulatory effect of oral IVIG after therapeutic treatment was maintained during all three booster immunizations tested (Fig. 4b, 4c). These results indicate that oral treatment with IVIG modulates RF responses even when given after the onset of the immune response.

Immunomodulation by oral IVIG is antigen-specific

Oral treatment with IVIG induces suppressive effects on systemic RF responses. These effects could be of interest for potential clinical application if they would be antigen-specific and would not reflect a general immunosuppressive potential of oral IVIG.

Therefore, we asked if the immunomodulatory activity of oral IVIG is specific for the antigen investigated. To address this question, we compared the immunmodulatory activity of oral IVIG in mice i.v. immunized with IC with the effects in mice i.v. immunized with an irrelevant antigen.

We chose human factor VIII as irrelevant antigen because we could previously establish that it induced detectable antibody responses after i.v. application in mice (17). When we treated mice with four doses of IC, oral IVIG completely prevented development of RF (Fig. 5b). When we treated mice with four doses of human factor VIII, however, oral IVIG did not induce any suppression of antibody responses (Fig. 5a).

These findings indicate, that treatment with oral IVIG did not influence the immune response to the irrelevant antigen human FVIII.

Immunomodulatory activity of oral IVIG is mediated by the Fc-part and not by the Fab-part of IgG

We could show that oral treatment with IVIG prevents the systemic induction of RF responses. These RFs are antibodies that recognize the Fc-part of human IgG. We wanted to know whether the antigen itself would be sufficient to induce the immunomodulatory effects observed after oral application of IVIG. At first, we investigated a human monoclonal IgG preparation. Human IgG contains the antigen recognized by RFs induced in our mouse model. Balb/c mice were treated with a single oral dose of either IVIG or monoclonal human IgG1 and subsequently treated with i.v. IC. Both primary and booster RF responses were down-modulated by oral treatment with IVIG or monoclonal human IgG1 (Fig. 6a, 6b). These results indicate that the Fc part of human IgG might be responsible for the down regulation of RF responses in our mouse model. To further support this hypothesis we prepared Fc- and Fab-fragments of human monoclonal IgG1 and tested their efficiency in preventing RF responses. Whereas IgG1-Fc induced similar effects as total IgG1, IgG1-Fab did not show any immunomodulatory effects (Fig. 6c, 6d). These results suggest that the Fc part of the IgG molecule is the essential part in mediating the tolerogenic activities of oral IgG in our model.

Immunomodulation by oral IVIG is mediated by regulatory cells

Oral application of IVIG in drinking water down-modulates systemic induction of RFs and prevents booster effects on RFs (Fig. 3a, 3c, 4a, 4c). Many previous studies provided evidence that regulatory cells, in particular regulatory T cells, are involved in the induction of oral tolerance (18). Therefore, we were interested to know whether the immunomodulatory effects of oral IVIG are due to the induction of some form of regulatory cell. To address this question we treated mice with oral IVIG by continuous application in drinking water and induced and boostered RFs as described. Development of RFs in these mice is shown in figures 3a and 4a. We prepared total spleen cells and spleen cells depleted of B cells from these mice and transferred these cells into mice that had been pretreated with two doses of IC. As a control we transferred spleen cells from naïve mice. Before transfer, recipient mice had titers of RFs between 1:1600 and 1:25,600 (data not shown). We asked the question whether transferred cells would be able to prevent booster effects on RF in mice that had an established RF response after two doses of IC. Both spleen cells from naïve mice as well as spleen cells from mice treated with oral IVIG down-modulated booster responses in mice with an established RF response (Fig. 7a, 7c). However, spleen cells obtained from mice treated with oral IVIG showed a significantly higher capacity to prevent these booster effects (Fig. 7a, 7c). When we used B-cell depleted spleen cells instead of total spleen cells, these immunomodulatory effects were even more obvious (Fig. 7b, 7d). These results clearly indicate that oral IVIG induces regulatory cells (possibly regulatory T cells) that are responsible for the immunosuppressive effects observed.

4.2.5. Discussion

In the current study the immunomodulatory activity of orally applied IVIG on systemic RF immune responses was investigated. For these investigations we established a mouse model in which human RF were experimentally induced by application of ICs containing human IgG. Administration of one single dose of IC induced a strong and sustained antibody response against the Fc part of human IgG and these antibodies were mostly of the IgG1 isotype. This experimental system for RF induction was previously described by other authors (6, 7) The data we obtained are in agreement with their earlier findings.

We first asked the question if oral application of IVIG, given either by single dose injection or by permanent treatment, influences the systemic induction of RF immune responses. Our results show clearly that IVIG treatment by oral route down-modulates the RF activity. This effect was observed when IVIG was given as one single dose or by continuous IVIG treatment when treatment started 5 days before RF induction. These data are in agreement with many other studies of oral tolerance in which successful induction of immune tolerance was achieved when oral exposure to the appropriate antigen started before primary antigen immunization was performed (19, 20). We next wanted to know if the tolerogenic activity of orally applied IVIG can also be achieved when treatment starts after the induction of the systemic RF response. Previous studies in other experimental models have shown that feeding with the after primary antigen immunization, was less or not effective in tolerance induction (21, 22). Our data show that the suppressive effect of oral IVIG on primary RF response was lost when IVIG was applied after induction of the primary response. However, oral IVIG was able to significantly downmodulate RF booster responses when treatment with oral IVIG started after primary induction of RF responses. These results are very promising with respect to potential clinical applications of oral IVIG in diseases associated with RF.

Previously, Krause et al reported data showing immunomodulatory activities of orally applied IVIG in an experimental model of anti-phospholipid syndrome (5). In these experiments oral tolerance was induced when mice were fed with IVIG before the onset of the disease. When treatment started after disease onset, IVIG treatment was not effective. Furthermore, Krause et al could show that the $F(ab)_2$ part of IVIG was

responsible for the induction of oral tolerance in their model. They presented evidence that anti-idiotypic antibodies present in IVIG were responsible for the immunomodulatory activity of oral IVIG. Anti-idiotypic antibodies are very similar to the original antigen and therefore act like the antigen itself in the induction of oral tolerance.

In our experimental model, the Fc fragment of IgG represents the antigen which is recognized by the RF. Therefore, we hypothesized that the Fc fragment might be responsible for the immunomodulatory activities of oral IVIG. We compared the effectiveness of Fc fragments and F(ab)2 fragments of IgG and found that it was indeed the Fc fragments that were responsible for the immunomodulatory effects of oral IVIG.

A very important question is whether the immunosuppressive effects observed after treatment with oral IVIG were antigen-specific or simply represented a more general immunosuppressive effect of oral IVIG. This question is of great importance with respect to a potential clinical application of oral IVIG. Our data show very clearly that the immunomodulation by oral IVIG is antigen-specific. Continuous treatment with IVIG in drinking water inhibits induction of systemic RF responses but does not influence immune responses against an unrelated antigen such as human factor VIII.

To address the question about the underlying mechanisms of the immunomodulatory activity of oral IVIG, we performed in vivo transfer experiments using spleen cells from mice treated with oral IVIG. Adoptive transfer of immunosuppressive activities provides clear evidence of the involvement of regulatory cells in oral tolerance induction (23). We transferred spleen cells from mice that had been treated with oral IVIG for a long period into recipients that had already a well established RF response and asked the question whether the cell transfer would prevent further booster effects on RF responses. Our data demonstrated that transfer of spleen cells significantly reduced RF booster responses. Transfer of B-cell-depleted spleen cells was even more efficient. We conclude that immune cells, probably T cells were responsible for the immunomodulatory effects of oral IVIG.

Several studies in RA animal models demonstrated that Treg cells, induced by oral antigen treatment, play a key role in the inhibition of arthritis development (24, 25, 26). The most intensively investigated Treg cells are the $CD4^+CD25^+$ population and

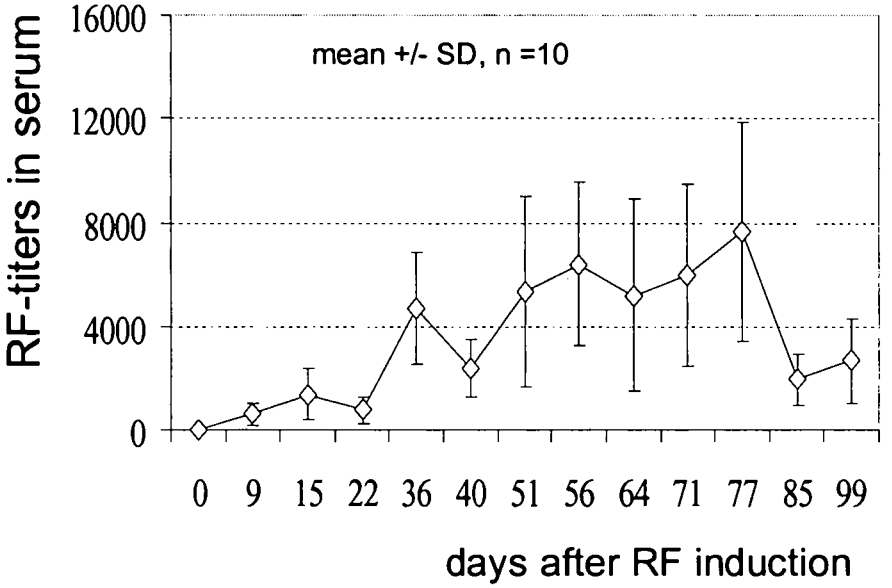
it is assumed that this subset of T cells play a major role in the induction of oral tolerance in RA (20 26). Further studies will clarify whether CD4+CD25+ are responsible for the immunomodulatory activities of oral IVIG, too.

Taken together our data presented in this study demonstrate that orally delivered IVIG induces immunosuppressive activities on primary and memory RF responses.
We successfully showed that continuous feeding with IVIG prevented booster effects on antibody titers even when treatment was started after primary immunization. This effect can be considered to be antigen-specific, because immune response to an irrelevant antigen was not down- regulated. Adoptive transfer experiments provided evidence that oral IVIG might induce regulatory cells, probably T cells.
The findings obtained in this study may provide new insights in immunomodulatory activities of orally delivered IVIG and contribute to new therapeutic approaches in treatment of disorders that are associated with the formation of RFs.

4.2.6. Figures

Figure1.

1a



1b

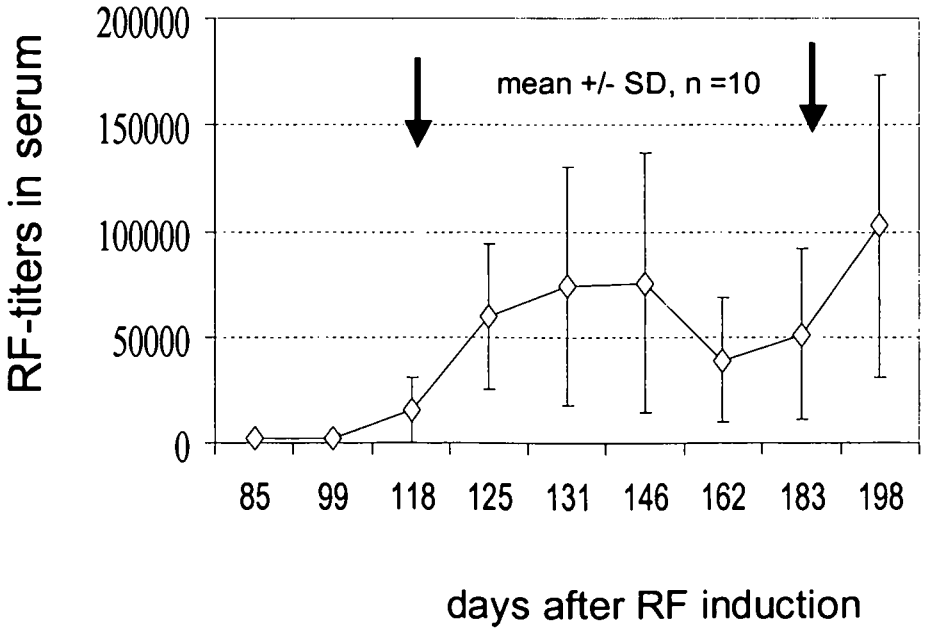


Figure 1. <u>Induction of systemic rheumatoid factor responses</u>

Female Balb/c mice were immunized by i.v. injection with one single dose of 100 µg IC (◇) on day 0 (1a).

Booster immunizations (1b) with 100 µg IC were performed on days 110, 182 (↓).

RF-titers in serum samples were measured by ELISA. Presented are the means +/- standard deviations (SD) for ELISA titers, calculated for each group.

Figure 2.

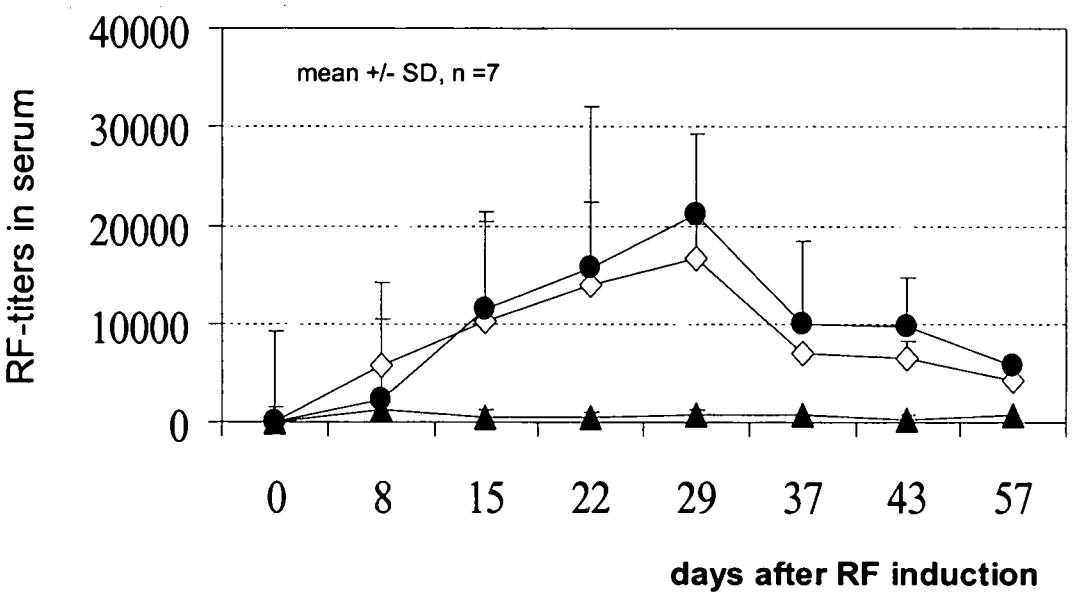
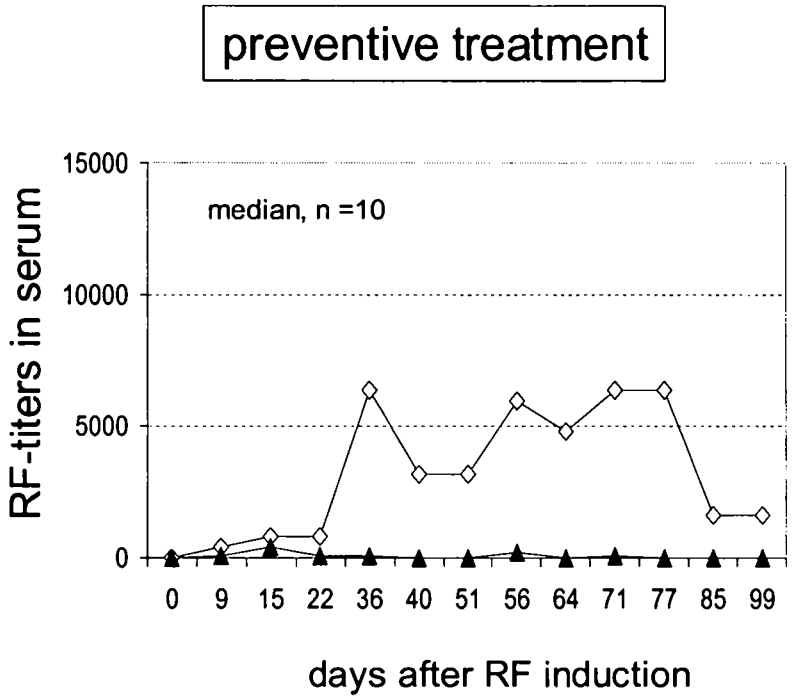


Figure. 2 Oral treatment with one single dose of IVIG prevents induction of human rheumatoid factors in Balb/c mice.

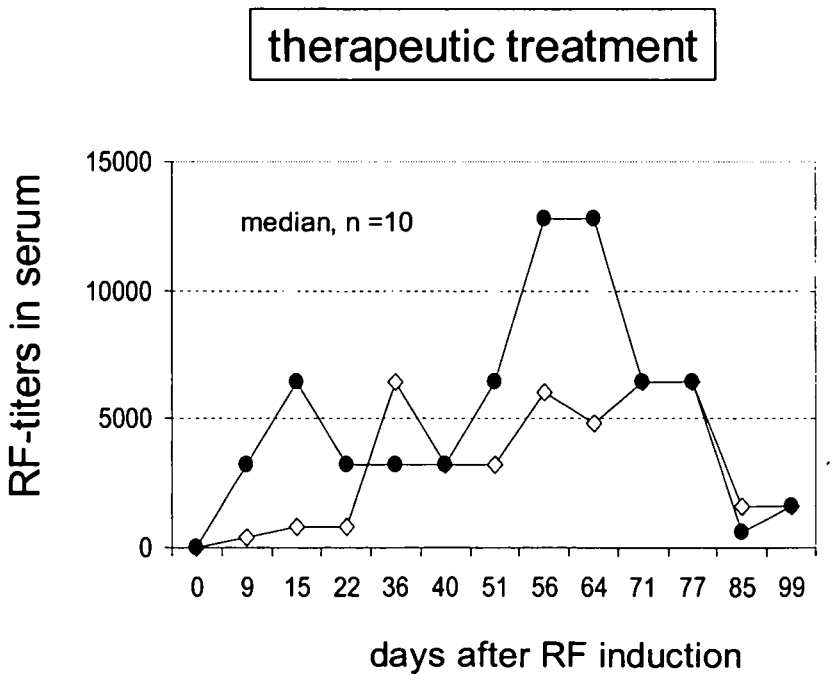
Female Balb/c mice were fed with one single dose of 10 mg IVIG by gavage. Treatment was performed either 5 days before (▲) or 4 days after (■) intravenous injection of a single dose of 100 µg IC, defined as day 0.
A control group (◇) received only 100 µg IC on day 0. RF-titers in serum samples were measured by ELISA. Presented are the means +/- SD for ELISA titers, calculated for each group.

Figure 3.

3a



3b



3c

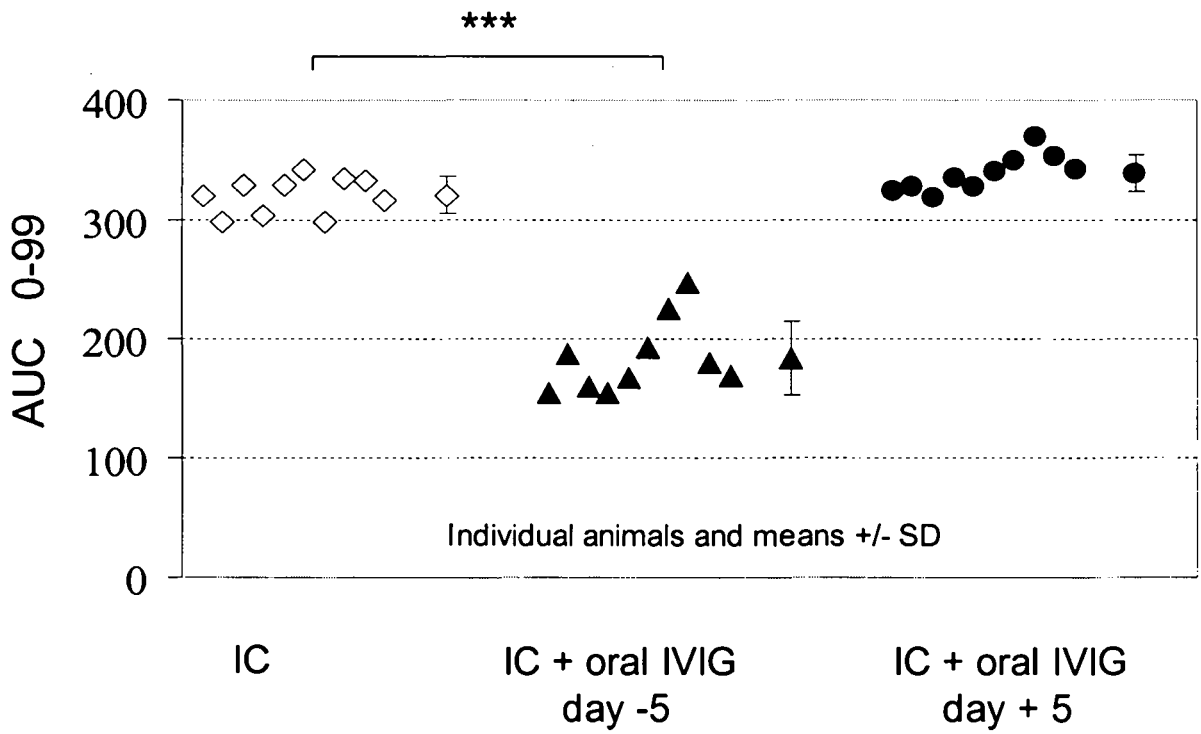
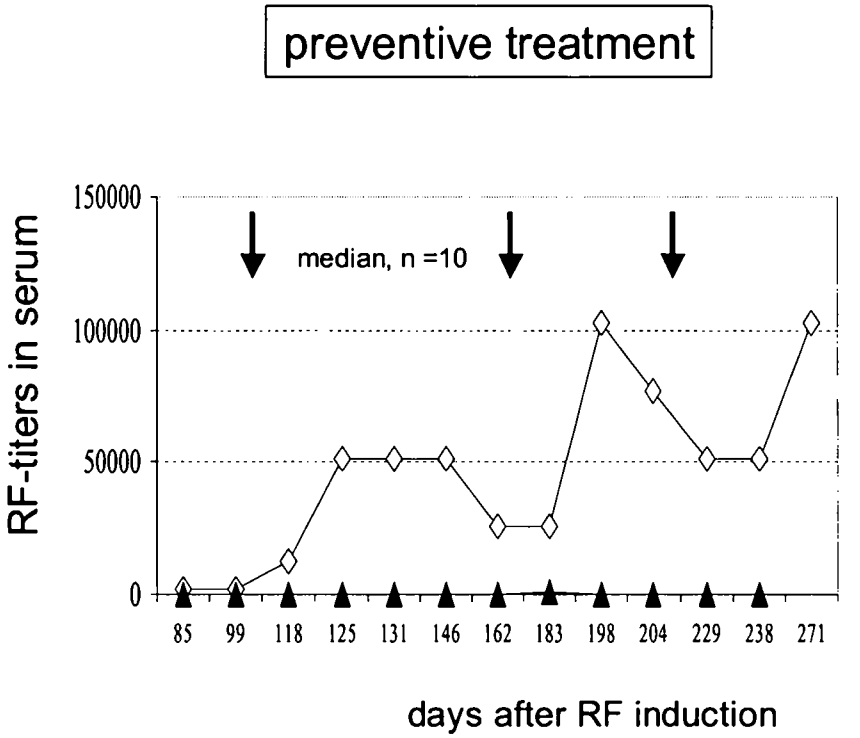


Figure 3. Permanent oral IVIG treatment prevents primary induced rheumatoid factors in Balb/c mice

For the induction of RF female Balb/c mice received 100 µg IC (◇) on day 0. Mice were daily fed with 10 mg IVIG in drinking water. Treatment was started either (3a) 5 days before (▲) or (3b) 5 days after (●) i.v. injection of one single dose of 100 µg IC. RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (3a, 3b). Areas under the curve (AUC) from days 0-99 (3c) were calculated for each individual animal. Means and SD for AUCs were calculated for each group.
***$p < 0.001$

Figure 4.

4a



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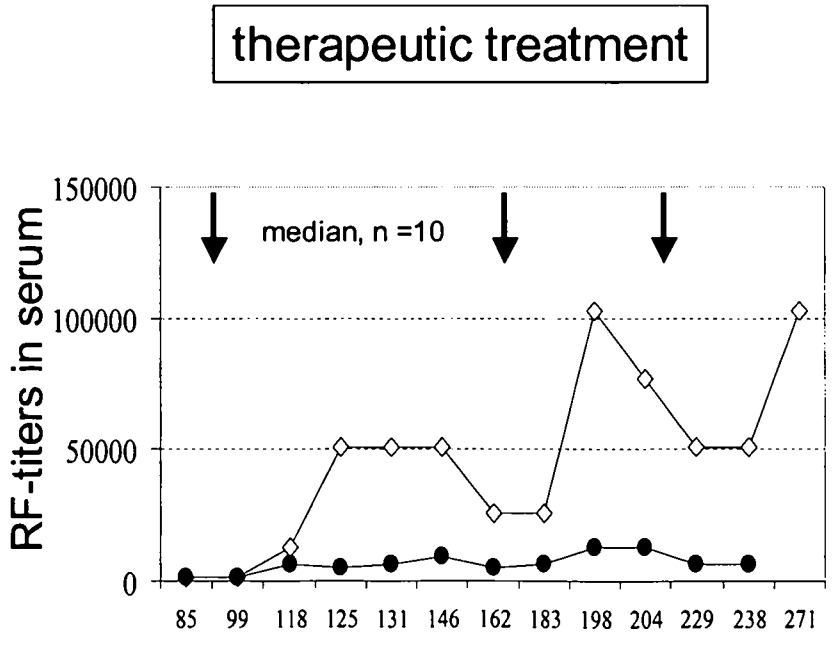


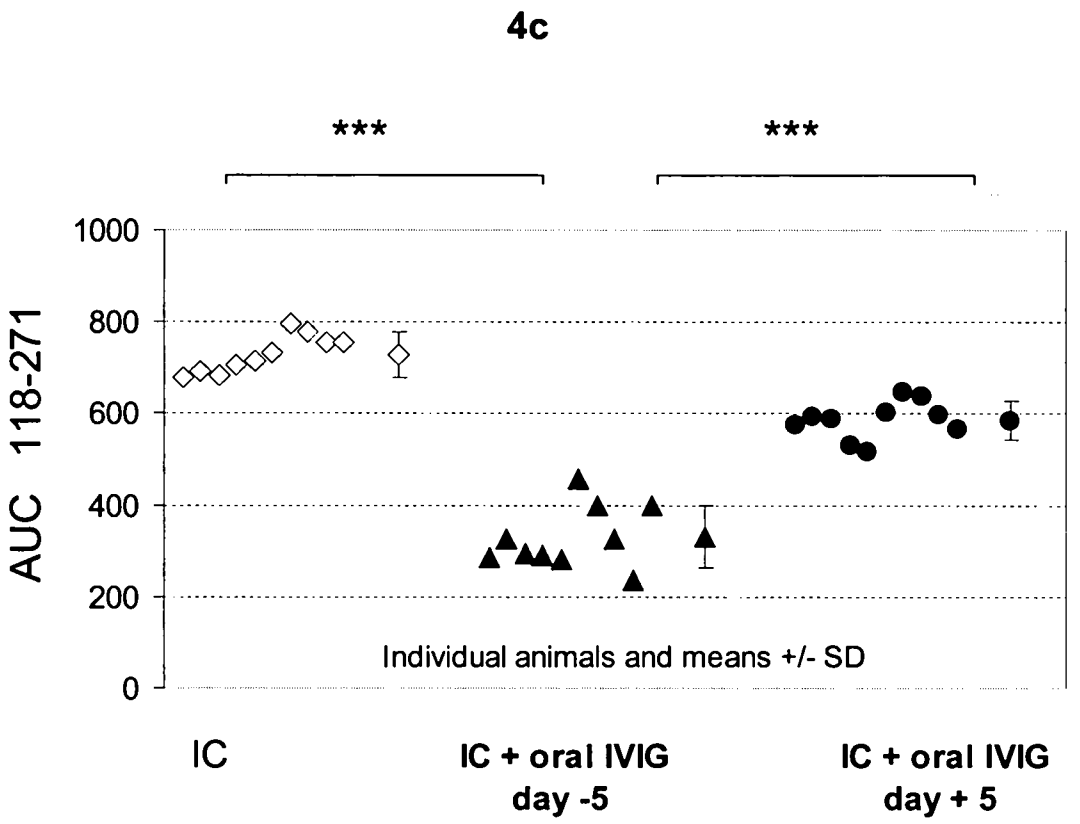


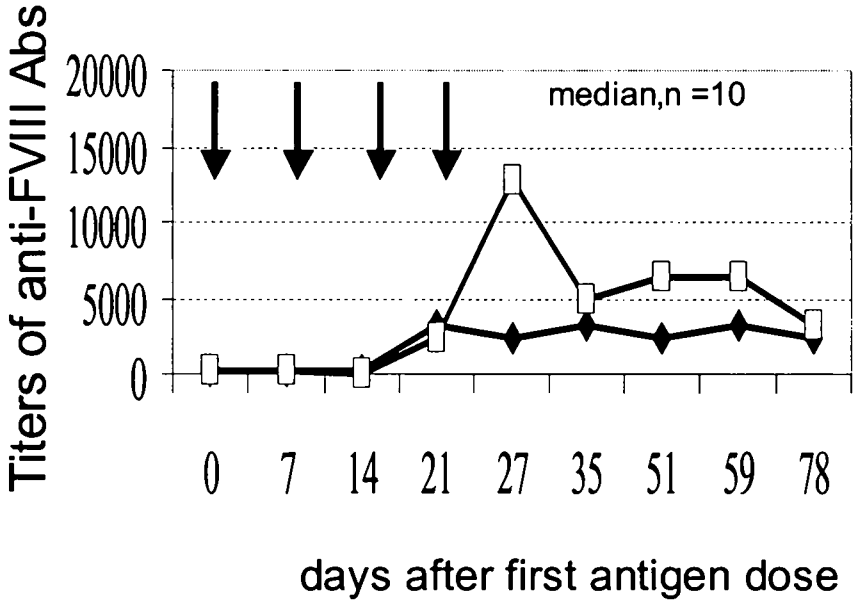
Figure 4. <u>Permanent oral IVIG treatment prevents RF booster effects in Balb/c mice.</u>

Female Balb/c mice were daily fed with 10 mg IVIG in drinking water. Treatment was started either (4a) 5 days before (▲) or (4b) 5 days after (●) intravenous injection of a single dose of 100 µg ICs. A control group (◇) received only 100 µg human immune complexes on day 0.Booster immunizations with 100 µg ICs were performed on days 110, 182 and 228 after RF induction (indicated as ↓ in figure). RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (4a, 4b).

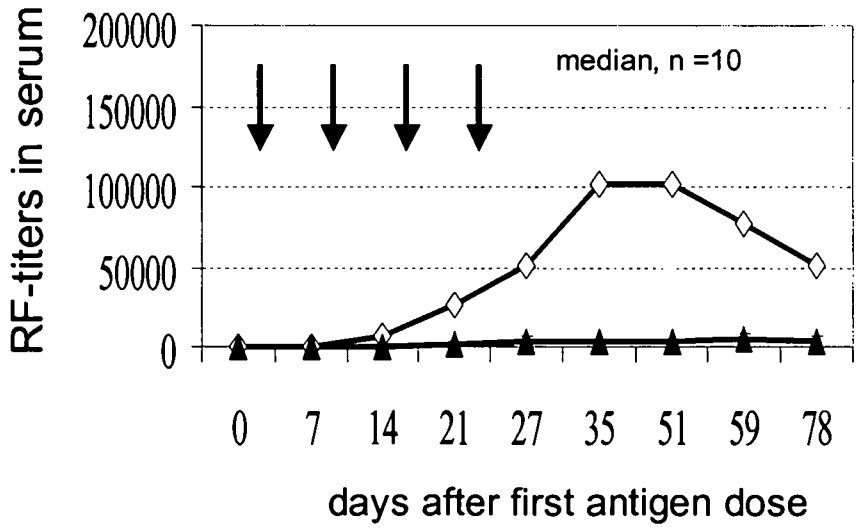
AUCs from days 118-271 (4c) were calculated for each individual animal. Means and SD for AUCs were calculated for each group. ***$p < 0.001$

Figure 5.

5a



5b



5c

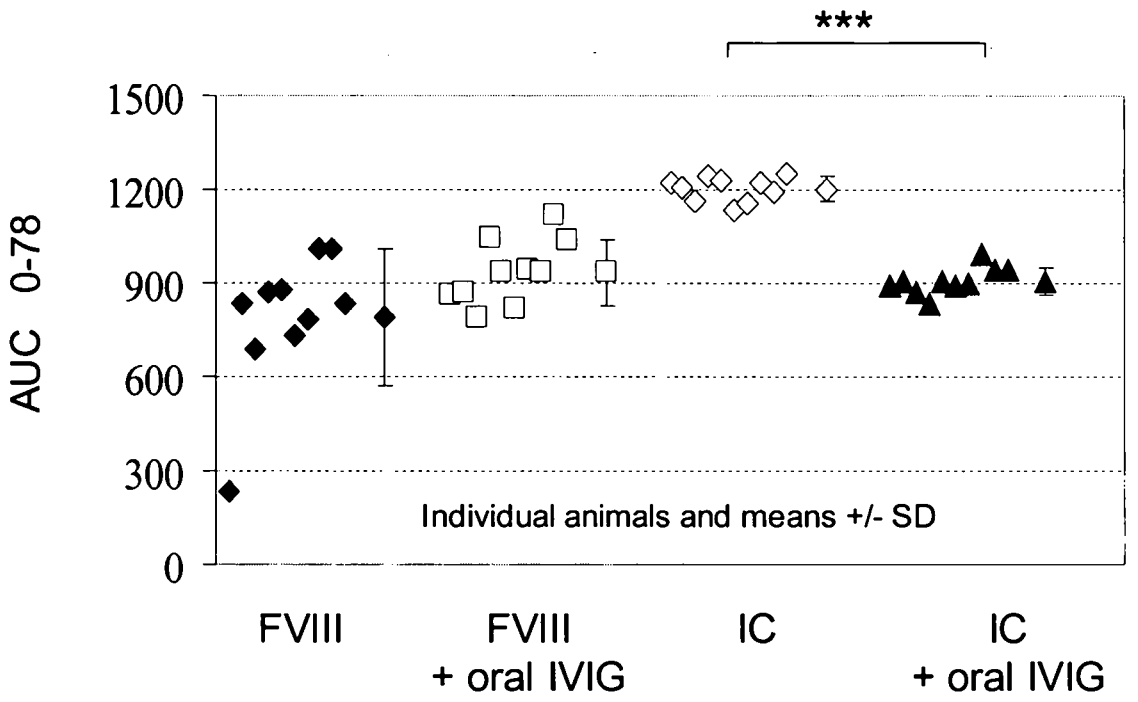


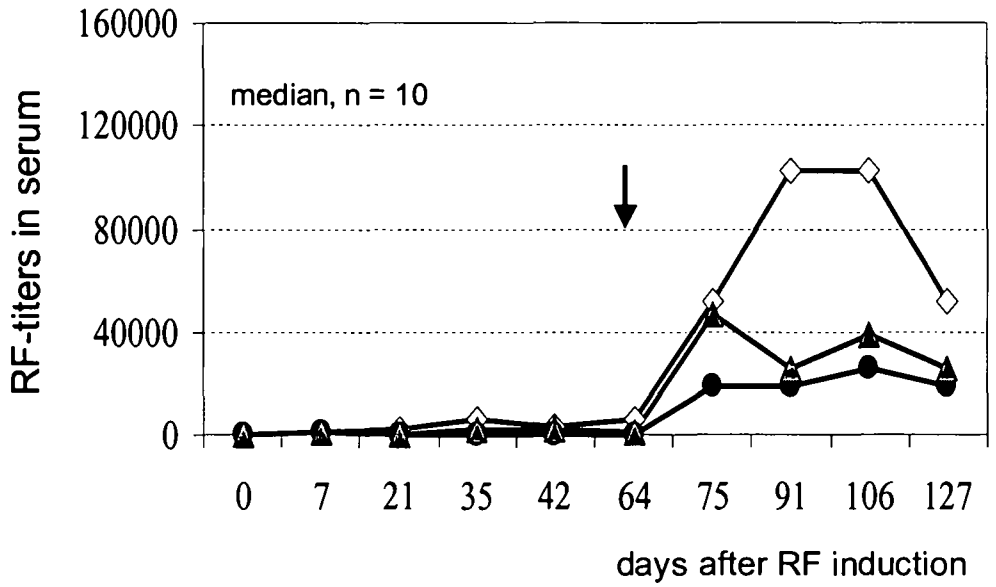
Figure 5. Down modulation of immune responses in prophylactically oral IVIG treated Balb/c mice is antigen specific.

Female Balb/c mice were immunized i.v either 4 times (indicated as ↓ in figure). with 200 ng FVIII (◆) or 100 µg IC (◇) in weekly intervals, starting on day 0 (5a). Further treatment groups (5b) were fed with 10 mg IVIG via drinking water, starting 5 days before (day -5) application of FVIII (□) or human IC (▲) respectively (5b). Anti-factor VIII antibody titers (5a) and RF-titers (5b) in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (5a, 5b).

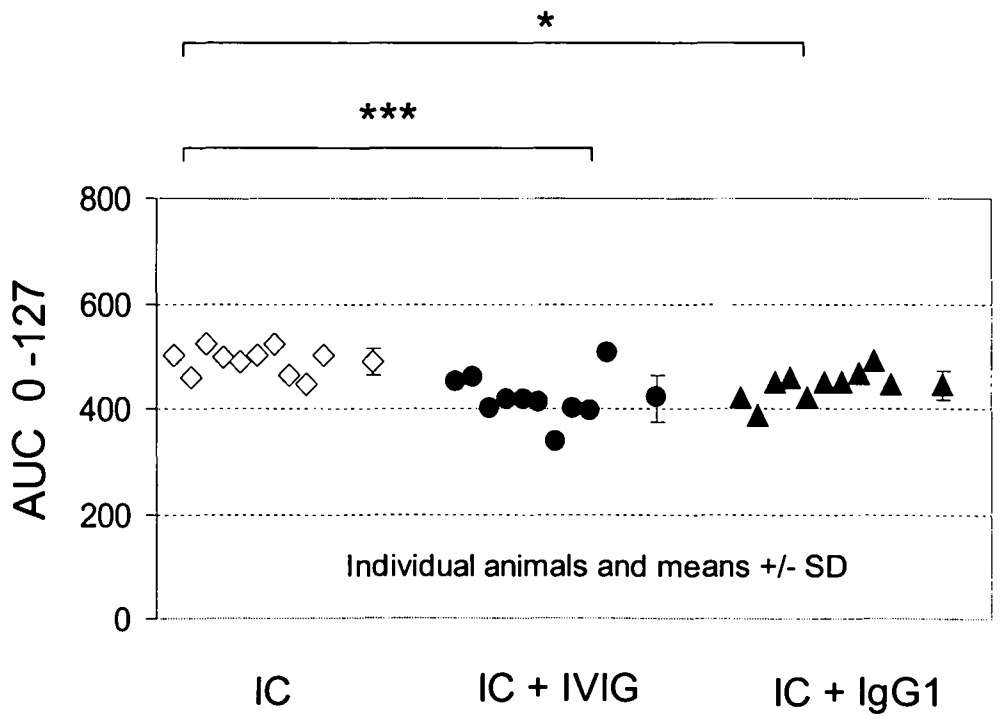
AUC from days 0-78 (5c) were calculated for each individual animal. Means and SD for AUCs were calculated for each group.***$p < 0.001$

Figure 6.

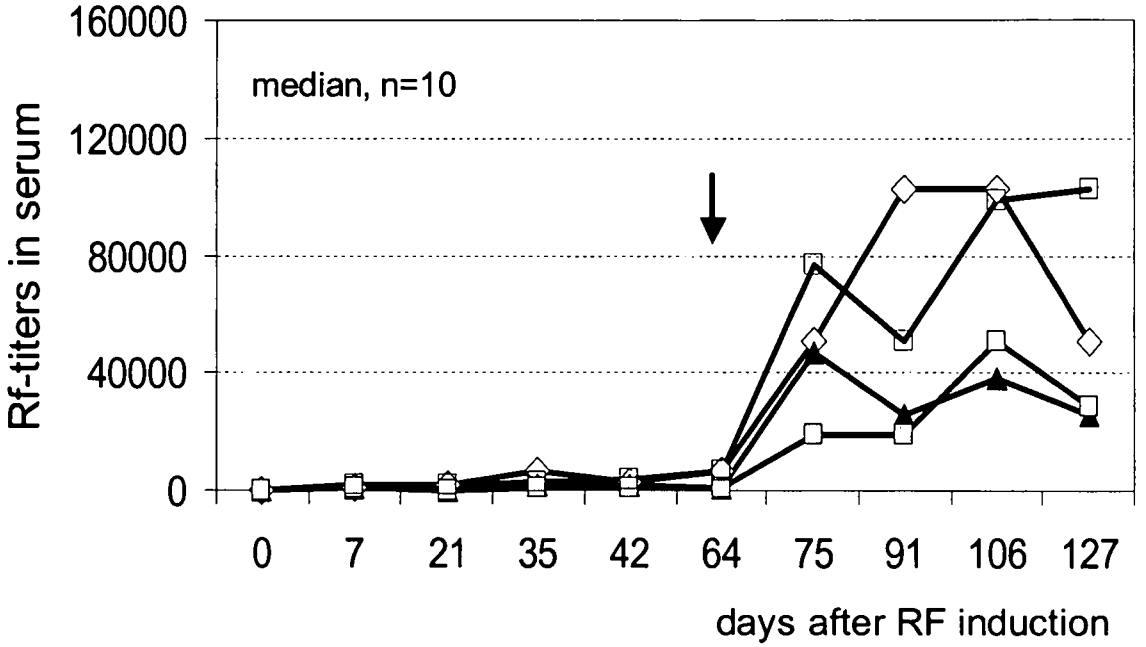
6a



6b



6c


160000
120000
80000
40000
0
median, n=10
Rf-titers in serum
0
7
21
35
42
64
75
91
106
127
days after RF induction

6d

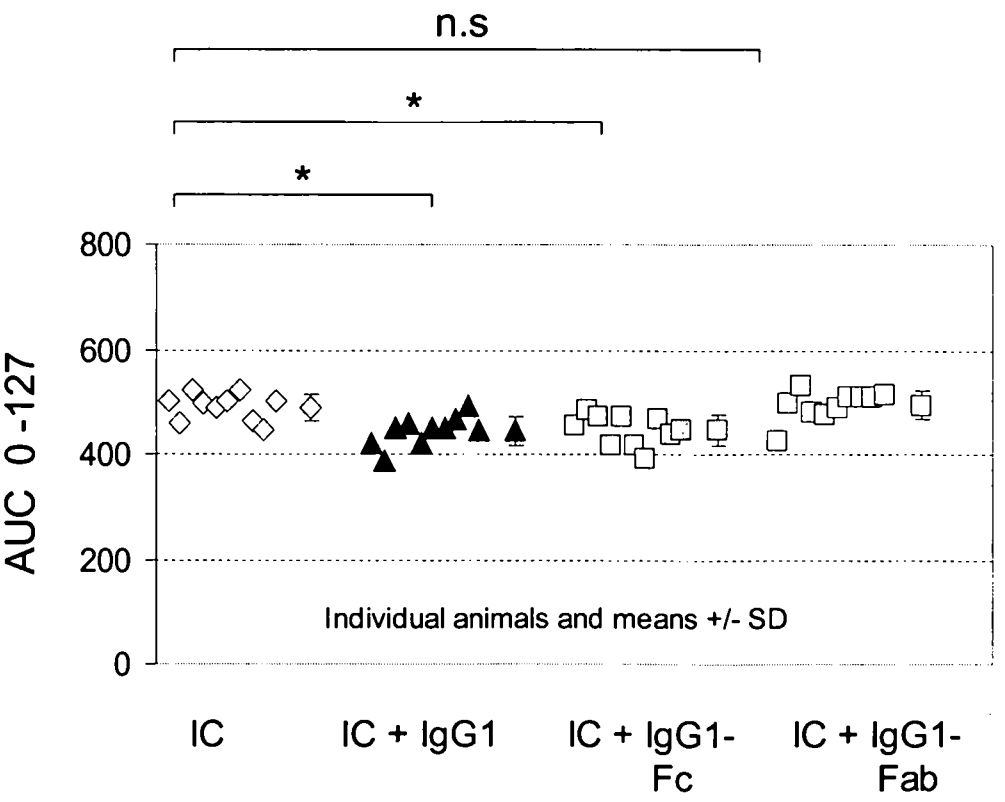

n.s
*
*
800
600
400
200
0
AUC 0 -127
Individual animals and means +/- SD
IC
IC + IgG1
IC + IgG1-Fc
IC + IgG1-Fab

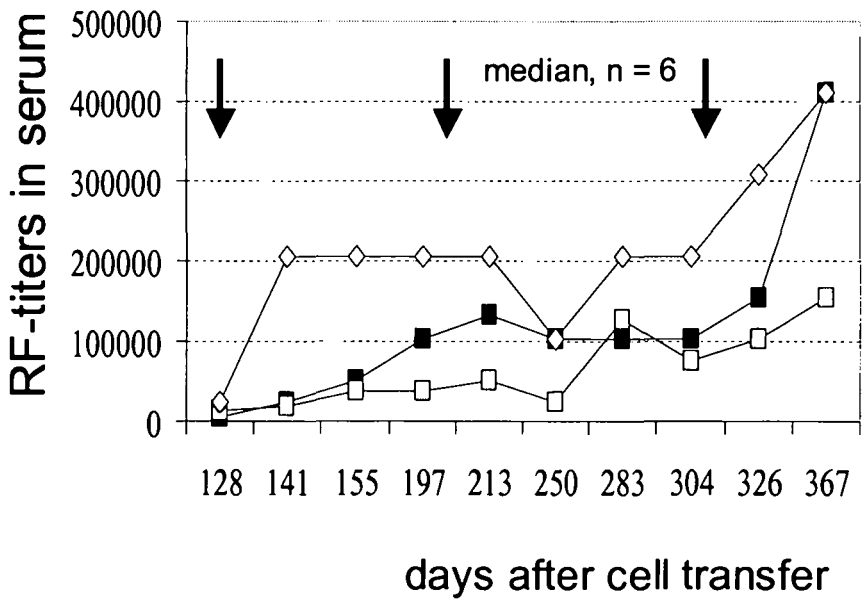
Figure 6. <u>Modulation of rheumatoid factor responses by oral treatment with monoclanal IgG1 and IgG1-fragments in Balb/c mice.</u>

Female Balb/c mice were treated with one single oral dose of either 10 mg IVIG (●),10 mg monoclonal IgG1 (▲), 5 mg IgG1-Fc fragments (▣), or 5 mg IgG1-Fab fragments (□) 5 days before immunized i.v with 100 µg of ICs. Control mice were immunized i.v with 100 µg of ICs (◇). Booster immunizations i.v. with 100 µg ICs were performed on day 64 (indicated as ↓). RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (6a, 6c).

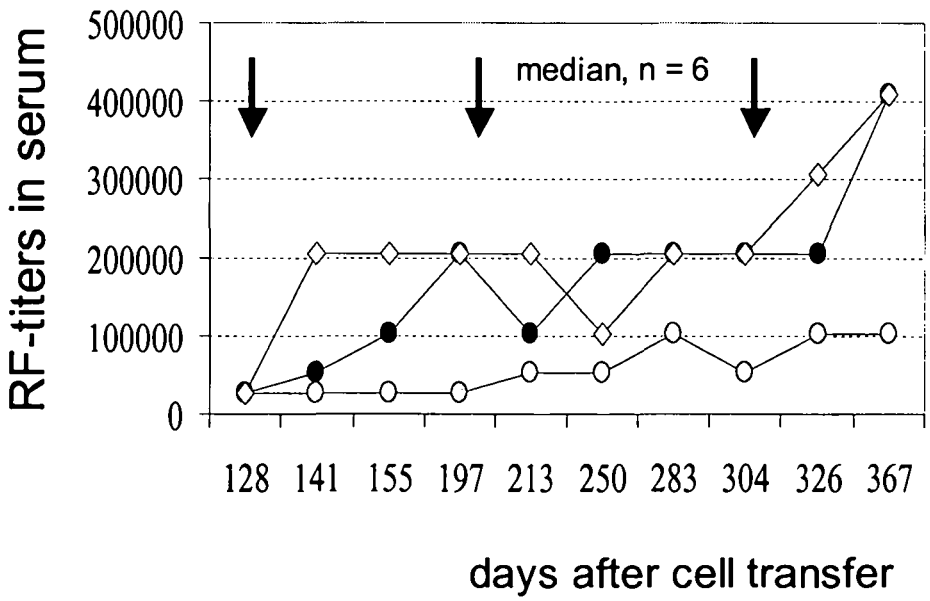
AUC from days 0-127 (6b, 6d) were calculated for each individual animal. Means and SD for AUCs were calculated for each group. *$p < 0.05$, ***$p < 0.001$, n.s. non significant

Figure 7.

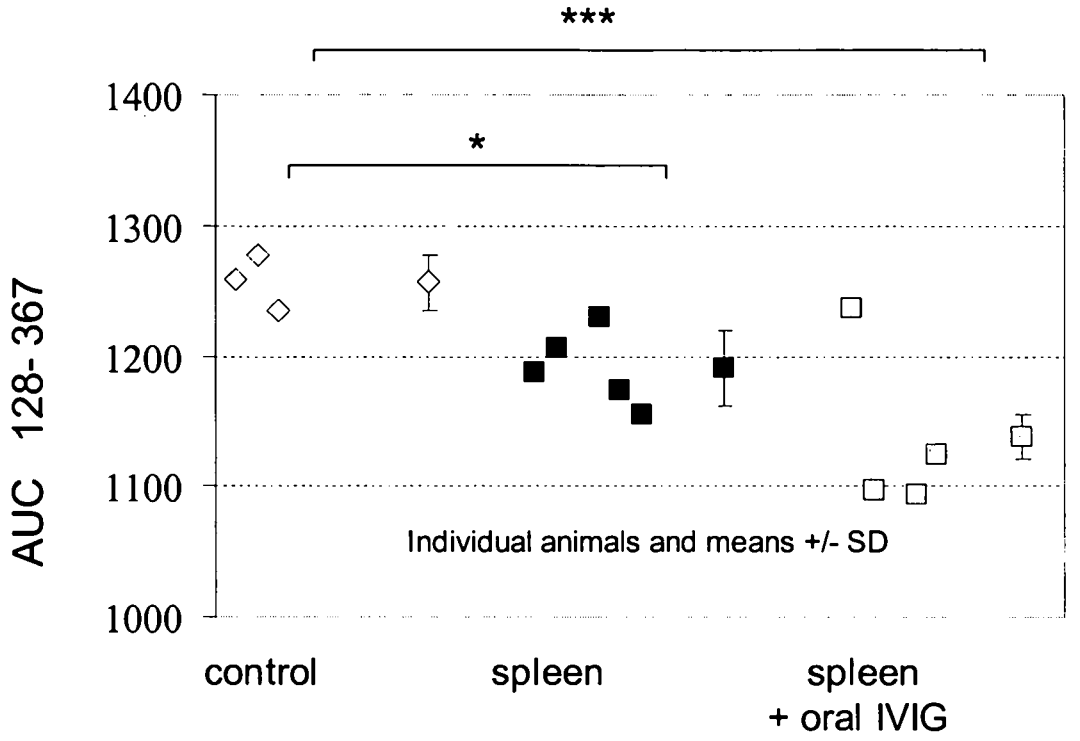
7a



7b



7c


***
*
1400
1300
1200
1100
1000
AUC 128-367
Individual animals and means +/- SD
control
spleen
spleen
+ oral IVIG

7d

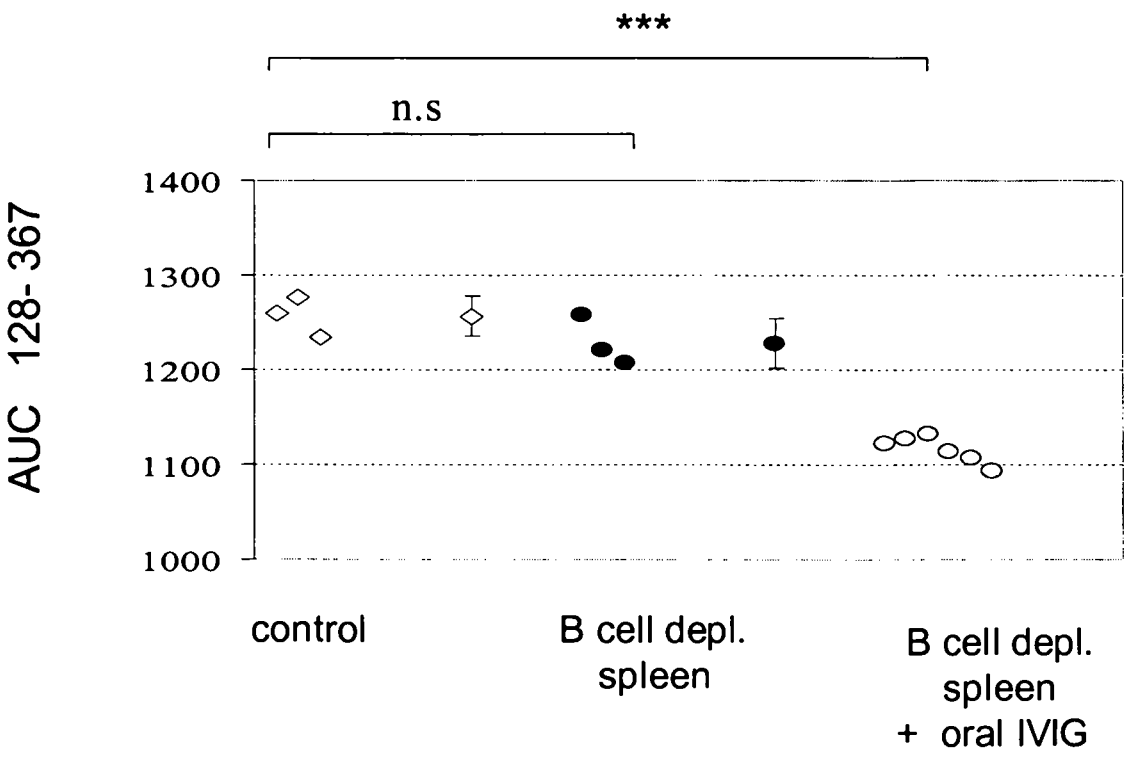

***
n.s
1400
1300
1200
1100
1000
AUC 128-367
control
B cell depl.
spleen
B cell depl.
spleen
+ oral IVIG

Figure 7. <u>Adoptive transfer of spleen cells from long term oral IVIG treated Balb/c mice into rheumatoid factor induced recipient mice</u>

1x107 spleen cells from donor mice were transferred into recipient mice, which were treated (i.v.) in weekly intervals two times with 100 µg human ICs. Day of transfer was defined as day 0. Transfer was performed with total spleen cells (7a) derived from naïve mice (■) or total spleen cells derived from IVIG treated mice (□) or with B-cell depleted spleen cells (7b) derived from naïve mice (●) or derived from IVIG treated mice (○). (The donor cells derived from untreated mice or IVG treated mice as described in Fig. 3a and 4a). Control mice received no treatment (◇). Booster immunizations i.v. with 100 µg ICs were performed on days 123, 204 and 323 (indicated as ↓). RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (7a, 7b).

AUC from days 128-367 (7c, 7d) were calculated for each individual animal. Means and SD for AUCs were calculated for each group. ***$p < 0.001$, n.s. non significant

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4.3. Significance of regulatory T cells in the suppression of rheumatoid factor responses induced by oral immunoglobulin

4.3.1. Abstract

In previous studies we have already demonstrated (see 4.2) the capacity of oral IVIG to induce unresponsiveness to immune complexes. In our recent studies we asked the question, whether these immunomodulatory activities of by oral IVIG involve the activation of regulatory T cells. In adoptive transfer experiments we transferred spleen cells and B-cell depleted spleen cell fractions derived from naïve mice and spleen cells derived from mice that received oral IVIG into naïve recipients. Afterwards we induced rheumatoid factor (RF) responses by the immunization with human immune complexes (ICs). B-cell depleted spleen cells derived from IVIG treated mice significantly prevented RF primary responses. Both transfer of spleen cells and B-cell depleted spleen cell fractions derived from IVIG treated mice showed a significant down-modulation of RF memory responses. We were not able to transfer tolerance into recipient mice by the transfer of T cell subpopulations, which seemed to be due to the duration of the preparation procedure. Nevertheless, by in vivo depletion of $CD25^+$ cells we were able to prevent the effects of oral IVIG and we therefore speculate that $CD4^+CD25^+$ cells are involved in immunomodulatory activities of oral IVIG.

4.3.2. Introduction

Oral administration of protein antigens has been shown to be effective in inducing immune tolerance in a number of experimental studies (1). It has been reported that oral administration of protein antigens before systemic immunization suppresses the immune response in different autoimmune diseases (2, 3, 4, 5). In preliminary studies (see 4.2) we investigated the immunomodulatory potential of oral IVIG in a murine model of experimentally induced human rheumatoid factors (6, 7). Rheumatoid factors (RF) are autoantibodies that can be detected in several autoimmune diseases, such as Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus and Sjögren Syndrome (8). RFs are produced by plasma cells in inflamed synovial tissues and they recognize the Fc part of the IgG molecule (9, 10, 11, 12, 13). Furthermore it has been shown that RF-expressing B cells act as antigen-presenting cells that internalize any IgG-containing immune complex and present the antigen to T cells. This could induce the activation of autoreactive T cells (14). Rheumatoid factors are associated with the severity of RA. Although there is a correlation between RFs and severity of RA, the contribution of RFs to the pathogenesis of the disease itself remains unclear (12).

Intravenous immunoglobulins (IVIG) are polyvalent human IgG preparations that are purified from large plasma pools obtained from thousands of healthy donors. They are indicated as the treatment of choice in primary immune deficiencies and in a number of inflammatory and autoimmune diseases (15, 16, 17). We reported previously (see 4.2) that oral administration of IVIG prevented and down-modulated systemic RF responses in mice. Even the administration of a single dose of oral IVIG could inhibit the induction of systemic responses of when given before immunization.

Based on these results we wanted to examine the mechanisms of action of the immunomodulatory activities of oral IVIG. In addition to previously described mechanisms, such as clonal deletion or anergy of antigen specific cells, there is accumulating evidence that active suppression by the induction of regulatory T cells (Tregs) represents an important mechanism for maintaining immunological tolerance. Many studies in experimental models have demonstrated the involvement of so called regulatory T cells in the prevention of autoimmune

diseases (18, 19). Today CD4+ T cells are believed to contain an important population of regulatory cells (1). In addition, it is assumed that $CD4^+CD25^+$ T cells play a major role in the induction of oral tolerance in autoimmune diseases like RA (20, 21). This consideration is supported by the findings that adoptive transfer of antigen-specific $CD4^+CD25^+$ Tregs into recipient mice decreases the severity of autoimmune disease, when recipients were immunized after the transfer (21). Furthermore, experimental studies have shown that in vivo depletion of CD25+ T cell accelerates the parthenogenesis of autoimmune disease (21). On the other hand, there are indications that Tregs are also contained in the $CD4^+CD25^-$ T cell population of normal animals. In different experiments it has been shown that $CD4^+CD25^-$ T cells also bear the ability to control autoimmune diseases in animal models (18).

The aim of this study was to find out whether the induction of unresponsiveness to immune complexes induced by oral IVIG involves the activation of regulatory T cells, in particular $CD4^+CD25^+$ T cells.

4.3.3. Material and methods:

Animals:

Female Balb/c mice were obtained from Harlan Winkelmann GmbH (Borchen, Germany) and maintained in our own animal facility (Dep. Of Exp. Pharmacology and Transgenic Animals, Vienna, Austria). All animals were aged 10-12 weeks at the beginning of the experiments.

Preparation of insoluble human immune complexes:

The preparation of insoluble human immune complexes was according to a protocol described by Abedi-Valugerdi (6, 7). Human IgG (Pierce, USA) and mouse anti-human IgG (Jackson Immuno Research Lab., USA) were mixed in a ratio 1:6 and incubated over night at 4°C. Thereafter, the antibody mixture was spun down at 17400 x g for 15 min. The supernatant was removed and the remaining pellet that contained the insoluble human immune complex was resuspended in 0,9% sodium chloride (Mayrhofer Pharmaceutics, Austria).

Induction and booster of human rheumatoid factors:

Human rheumatoid factors (mouse antibodies specific for the Fc-part of human IgG) were induced in Balb/b mice by i.v. treatment with 100 µg of insoluble immune complexes (IC) containing human IgG (Pierce, Rockford, Illinois, USA) and mouse anti-human IgG (Jackson Lab. Bar Habor, Maine, USA) according to a protocol described by Abedi-Valugerdi (6, 7). At several time points after the injection of IC, blood samples were taken by puncture of the retro-orbital vein. Serum was prepared and stored at -20°C until further analyses. Titers of human RF were measured by ELISA as described below. At several time points after primary immunization, mice received i.v booster injections with 100 µg IC.

Oral treatment with human IgG:

Balb/c mouse received human IVIG (Endobulin S/D, Baxter AG, Vienna, Austria) in drinking water for 45 days. The daily water consumption per cage was measured to estimate the water uptake per mouse per day. The amount of IVIG in the drinking water was adjusted for a daily uptake of 10 mg IVIG per mouse. Drinking water was replaced every day.

Isolation of spleen cells:

Spleens obtained from mice were minced and passed through an 70-µM nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ.) The cells were then washed in RPMI 1640 (Life Technologies, Paisley, Scotland) containing 5% fetal calf serum (Hyclone, Logan, UT).

Cell isolation by density gradient centrifugation (Lympholyte-M):

After single cell preparation, cells were purified from erythrocytes, apoptotic cells and debris by density gradient centrifugation using Lympholyte-M (Cedarlane, Ontario, Canada). For density gradient centrifugation, cells were resuspended in RPMI-1640 with 2% FCS.

After centrifugation cells were washed in RPMI 1640 supplemented with 5% fetal calf serum.

Depletion of B cells:

B cells were depleted from spleen cells using mouse pan B (B220) Dynabeads (Dynal Biotech ASA, Norway). Depletion was performed following the manufacturers instructions. The purity of the cells was confirmed by FACS-analysis. Cells were analyzed using a FACS Calibur and CellQuest pro software (both from BD Bioscience, Heidelberg, Germany).

The B-cell depleted cells were used in adoptive transfer experiments.

Enrichment of CD4+CD25+ cells:

Enrichment of CD4+CD25+ cells was done using a Magnetic cell sorting (MACS) separation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturers protocol.
The purity of the cells was analyzed by FACS-analysis using a PE-labeled anti-25 antibody and FITC-labeled anti-CD4 antibody (3C7, Pharmingen, BD Becton Dickinson, Heidelberg, Germany). Cells were analyzed using a FACS Calibur and CellQuest pro software (both from BD Bioscience, Heidelberg Germany).
CD4+CD25+ T cells and CD4+CD25- T cells were collected and used for adoptive transfer experiments.

Adoptive cell transfer of spleen cell fractions :

$2x10^7$ cells (total spleen cells or B-cell depleted spleen cells) or $2x\ 10^6$ cells (CD4+CD25+ or CD4+CD25- T cells) in 300µl Dulbecco`s PBS (DPBS, Sigma-Aldrich Chemie GmbH, Germany) were i.v. injected into recipient mice on day 0. Control groups received DPBS only. One day after cell transfer (day 1) mice were immunized with insoluble human immune complex.

In vivo depletion of CD25+ cells:

Mice were injected intraperitoneally with 0,4mg purified anti-CD25 antibody (NA/LE, PC61) on day –5 and –3. Polyclonal rat anti-mouse IgG1κ (NA/LE, A110-1) was used as isotype control.
Mice were treated with oral IVIG in drinking water for 5 days (day –5 to day 0) and immunized with insoluble human immune complexes on day 1.

In vivo depletion of CD25+ cells was confirmed by FACS analysis using a PE-labeled anti-CD25 antibody (3C7) and a FITC-labeled anti-CD4 antibody (L3T4) The PE-labeled anti-CD25 antibody used for FACS-analysis recognizes a different epitope on the CD25 molecule than the anti-CD25 antibody used for cell depletion.

We obtained all antibodies from Pharmingen (BD Becton Dickinson, Heidelberg, Germany). Cells were analysed using a FACS Calibur and CellQuest pro software (both from BD Bioscience, Heidelberg, Germany).

Detection of human rheumatoid factors by ELISA:

Titers of rheumatoid factors were by ELISA as desribed (see 4.2).

Preparation of total-RNA and Reverse Transcription:

Total RNA of spleen cells was isolated using TRIzol reagent (Invitrogen Life Technologies, Lofer, Austria) following the manufacturers protocol. The amount of total RNA was measured using a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Inc, Montchanin, USA).
Reverse transcription was carried out with 1µg total RNA using Hexamer primers (MBI Fermentas GMBH, St.Leon-Rot, Germany). 5x reaction buffer, 10mM dNTP mix Ribonulease inhibitor and RevertAid^{TM} M-MuLV were obtained from MBI Fermentas.

Real Time PCR:

Real Time PCR analysis was performed using TaqMan real-time reverse transcriptase–polymerase chain reaction (RT-PCR) assays on the AB Prism 7000 (Applera Austria Handels GMBH, Brunn am Gebirge) sequence detection system.
Gene expression levels were measured using pre-developed assays ("Assay-on-demand^{TM}" Gene expression products) for forkhead box P3 (FoxP3), CD3 and the TATA-box binding protein (TBP) as described by the manufacturer (Applera Austria Handels GMBH, Brunn am Gebirge). TBP a housekeeping gene was used for internal control.
Calculation was done using the ^^CT-calculation-method and Microsoft Exel software (Mircosoft office 2000 SR-1 Premium, USA).

Statisical analyses:

Results for antibody titers are expressed as means +/- standard deviation (SD). Statistical analysis was performed based on calculations of areas under the curve (AUC). The AUC data were assessed for normal distribution using the Anderson-Darling method. Means, SD, minimum, maximum, least sqare means (LSMEANS) and 95% confidence limits (95% CI) were calculated for each group using the program SAS. Comparisons between the treated groups and the control groups were performed by variance analyses using the program SAS.

For statistical analyses of antibody titers, areas under the curve (AUC) were calculated for each individual animal. Means and standard deviations for AUC were calculated for each group. Comparisons between groups were performed by variance analysis using the program SAS. Differences between groups were considered to be statistically significant if $p < 0.05$.

4.3.4. Results:

Induction of systemic rheumatoid factor (RF) responses

Intravenous injection with a single dose of insoluble human immune complexes into naive mice induced a sustained antibody response (rheumatoid factors) against the Fc-part of human IgG (Fig.1-3). These results confirm previous findings published by Abedi-Valugerdi et al. (6, 7). After decline of antibody titers, mice were again i.v. treated with IC to booster the titers of RF (Fig.1-3).

Prevention of rheumatoid factor responses after oral IgG

IVIG is a human polyvalent IgG preparation and therefore contains the antigen recognized by RF-expressing B cells. We asked the question, whether oral treatment with IVIG prevents the induction of RF in mice. Here, we show that oral treatment with IVIG in drinking water for 45 days prevents the induction of RFs in naïve mice (Fig. 4-7). Next, we were interested to know whether oral treatment with IVIG also has a positive influence on memory responses after oral booster injections. In our experiments we could show that preventive treatment with oral IVIG for 45 days did not only prevent primary RF responses (Fig. 1-3) but did also prevent booster effects of RF responses (Fig. 1-3).

Adoptive transfer of spleen cells fractions prevents RF responses

To address the question whether regulatory T cells are involved in the immunmodulatory activities of oral IVIG we transferred spleen cells or B-cell depleted spleen cell fractions from either naïve mice or mice treated with oral IVIG for 45 days into naïve recipients. The spleen cells were injected intravenous into naïve donors. Subsequently we induced systemic RF responses by immunizing with human IC. Control groups without transfer received PBS instead.
Transfer of spleen cells obtained from mice that were treated with IVIG showed a tendency in preventing the induction of primary RF responses (Fig. 2a, 2b) and

significantly down-modulated the immune responses after booster injections (Fig. 1a, 1b, 3a, 3b). B-cell depleted spleen cells also significantly down-modulate RF responses. When compared to the controls, B-cell depleted spleen cells obtained from mice that received oral IVIG were even more effective than total spleen cells (Figs. 1-3).

FoxP3 expression of spleen cells from naïve mice and IVIG treated mice did not show any differences

Another way to address the question whether the immunmodulatory activities of oral IVIG are due to regulatory T cells was to investigate the expression of FoxP3. FoxP3 is considered to be a potential marker for regulatory T cells. However, using real time PCR we examined the expression of FoxP3 in spleen cells and in B-cell depleted spleen cells obtained from naïve mice as well as from mice that received IVIG for 45 days in drinking water. Results from real time PCR showed that the expression level of FoxP3 in spleen cells from naïve mice and in spleen cells from IVIG treated mice did not show significant differences (Fig. 4a). B-cell depleted spleen cell show a slight increase in the expression of FoxP3, which might be due to the enrichment of T cells in these spleen cell fractions. By adjusting the expression of FoxP3 to the amount of T cells in the cell populations, we saw no difference in the expression of FoxP3 in spleen cell or B-cell depleted spleen cell fractions independent of the way mice were treated (Fig.4b).

Neither Adoptive transfer of CD4+CD25+ T cells nor the transfer of $CD4^+CD25^-$ T cells did prevent RF responses

Based on our results from adoptive transfer experiments with B-cell depleted spleen cells, we suggested that T cells are involved in the immunomodulatory activities of oral IVIG. Since $CD4^+CD25^+$ T cells are believed to have regulatory features, we decided to do transfer experiments with this T cell subpopulation.
Again we transferred spleen cells from either naïve mice or mice treated with oral IVIG for 45 days into naïve recipients. $CD4^+CD25^-$ T cells were transferred as a

control, because some publications have demonstrated that these cells have regulatory functions, too (14). Purity of the spleen cell fractions was analyzed by FACS-analysis (Fig. 5a)

Neither the transfer of CD4+CD25+ T cells nor the transfer of CD4+ CD25- T cells from naïve or oral IVIG treated mice prevented the primary RF response or the memory response in our system (Fig. 5b). Cell transfer induced an amplification of the immune response what might be due to a high number of apoptotic cells in the cell preparation transferred.

Depletion of CD4+ CD25+ T cells reverses the immunmodulatory effects of oral IgG

Transfer experiments with T cell subpopulations obtained from of IVIG treated mice failed to down-modulate systemic RF responses what might be due to preparation procedures and stress factors associated with these procedures. Therefore, we examined the potential significance of regulatory T cells in the immunosuppressive activities of oral IVIG by in vivo depletion of CD25+ T cells. We injected naïve mice or mice that received oral IVIG in drinking water with a depleting anti-CD25 antibody. Control groups received an isotype control antibody. After depletion of CD25+ cells mice were injected with human ICs to induce systemic RF responses. Depletion of CD25+ cells was confirmed by FACS-analysis using an antibody that recognizes a different epitope on the CD25 molecule than the antibody used for in vivo depletion. Figure 6a shows two representative FACS-dot plots from spleen cells obtained from mice treated with the depleting antibody and from mice treated with the isotype control antibody.

Mice that received an anti-CD25 antibody showed a depletion of CD25+ cells for about four weeks (Fig. 6b). Afterwards, CD25+ cells started to appear again. Mice injected with the isotype control antibody showed no depletion of CD25+ cells (Fig.6b). Mice treated with oral IVIG and anti-CD25 antibody did not show any down-modulation of RF response in comparison to mice that received oral IVIG and the isotype control antibody (Fig.7a, 7b, 7c, 7d). These results indicate that the immunmodulatory effects of oral IVIG depend on CD25+ cells.

4.3.5. Discussion:

The present study investigated the immunomodulatory activities of oral IVIG. For our studies we used a mouse model were we induced an antibody response against the Fc-part of human IgG by a single dose injection of insoluble human immune complexes. We asked the question, whether oral treatment with IVIG prevents the induction of systemic RF responses in mice. In several experiments we could show that administration of IVIG in drinking water for a time period of 45 days prevents the induction of RF responses and RF memory responses in naïve mice.

Further on we tried to characterize the mechanisms of action that were responsible for the immunmodulatory activities of oral IVIG. For this approach we used adoptive transfer experiments, were we transferred spleen cells or B-cell depleted spleen cell fractions from naïve mice or cells obtained from mice that received IVIG in their drinking water for 45 days. Transfer of spleen cells obtained from mice that received oral IVIG showed a tendency to prevent the induction of primary RF responses and significantly inhibited RF memory responses. B-cell depleted spleen cells also significantly down-modulated RF responses. When compared to the controls, B-cell depleted spleen cells obtained from mice that received oral IVIG were even more effective than total spleen cells. These findings seem to confirm our theory that some kind of regulatory cells that are part of the T-cell compartment contribute to the effects of oral IVIG.

Furthermore, we wanted to examine if cells involved in the immunmodulatory activities of oral IVIG express forkhead box P3 (FoxP3). FoxP3 is a transcription factor that is considered to be a possible genetic marker for regulatory T cells (22, 23). We compared the FoxP3 expression of cells from naïve mice and cells obtained or from mice that received oral IVIG for 45 days in drinking water. Our results showed no significant difference in the expression of FoxP3. We also examined B-cell depleted spleen cell fractions for the expression of FoxP3. In the B-cell depleted fraction we saw a higher amount of FoxP3 expression independent of the way mice were treated. This might be due to the T cell enrichment in the B-cell depleted spleen cell fractions. When we adjusted the expression of FoxP3 to the amount of T cells in the preparation, we saw no difference in the expression of

FoxP3 in spleen cells or B-cell depleted spleen cell fractions independent of the way mice were treated. There are publications that described regulatory T cells that do not express FoxP3 (24). Therefore it is possible that regulatory T cells involved in the immunmodulatory activities of oral IVIG do not express FoxP3.

Further on we wanted to find out if these regulatory T cells belong to the $CD4^+CD25^+$ subpopulation. $CD4^+CD25^+$ are often considered to be the mediators of oral tolerance (25, 26). So we transferred $CD4^+CD25^+$ T cells of either naïve or IVIG treated mice into naïve mice before induction of RF responses. In contrast to our expectations, transfer of this subpopulation did not prevent or down-modulate the systemic RF responses. As a control, we transferred $CD4^+CD25^-$ T cells into naive mice. Some publications (18) indicate that CD4+CD25- T cells might also express regulatory function similar to $CD4^+CD25^+$ cells. The transfer of $CD4^+CD25^-$ cells did not induce prevention or down-modulation of RF responses. Surprisingly, this was also true for transfer of total spleen cells. In contrast, we saw amplification rather than a prevention of the immune response. As we have applied all spleen cell fractions at the same time point to treat them equally, we assume that the amplification of the immune response was due to the preparation procedure. Obviously, the duration of the preparation procedure has a negative influence on cell quality.

We believe that the amplification of the immune response of RF was due to apoptotic cells that resulted from the preparation procedures used. In different publications it has already been published that apoptotic cells are immunogenic and that they might release natural endogenous adjuvants, which stimulate antigen presenting cells (APCs) to mature and become immunostimulatory cells (27, 28). Some papers also suggest that injection of damaged cells in combination with an antigen leads to an immune response against the antigen, even cells and antigen are physically separated (27, 28). Based on these results we believe that in our model the high amount of apoptotic cells caused by the preparation method might lead to an amplification of the immune response against the IC.

In previous studies (see 4.1) we have already shown that the preparation procedure has an influence on the cell quality. We used density gradient centrifugation to remove apoptotic cells and cell debris. Although this preparation

method seemed to be promising for stable transfer experiments as we have seen before, there might still be a lot of early apoptotic cells that are not eliminated by density gradient centrifugation. It was already published that cells injured during experimental manipulations might dye by injection. In addition, experiments showed when cells were stressed or injured for several hours before dying, the amount of adjuvants increase about 10-fold (28).

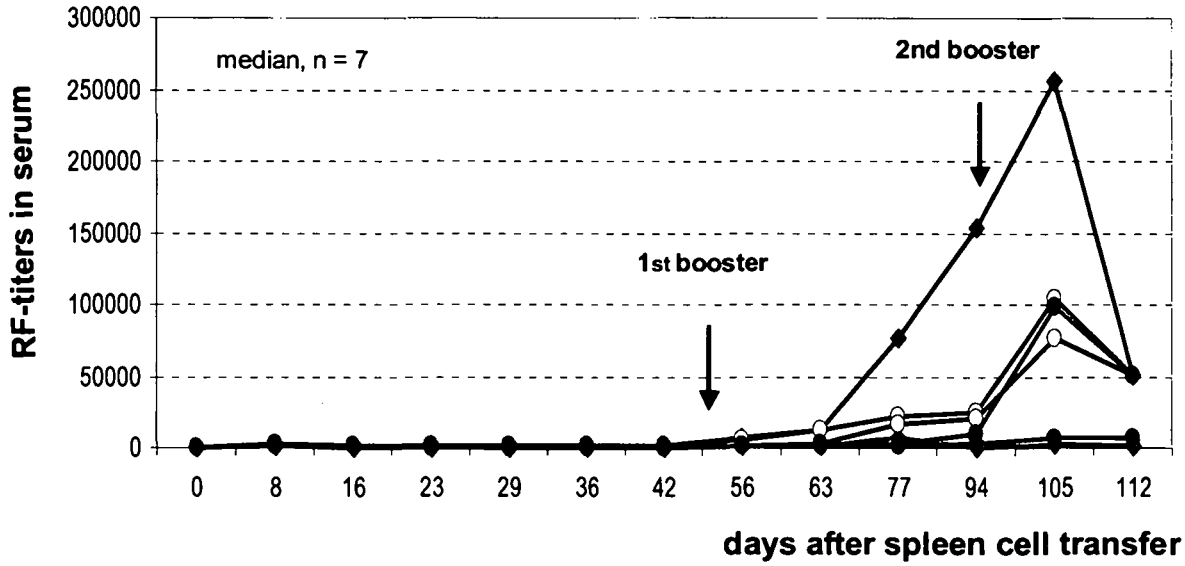
We concluded that transfer of $CD4^+$ subpopulations might not be the ideal way to examine the mechanisms of action of the immunomodualtory activities of oral IVIG. Therefore, we chose another experimental approach to characterize the mechanisms of action of oral IVIG. We performed in vivo depletion of $C25^+$ cells either in naïve mice or in mice fed with IVIG in drinking water. With this experimental approach we could show that depletion of $CD25^+$ prevents the immunmodulatory effect of oral IVIG.

Based on our results we conclude that $CD25^+$ regulatory cells are involved in the immunosuppressive effects that are induced by oral IVIG. These regulatory cells are likely to belong to the T-cell compartment. Therefore, we can speculate that $CD4^+CD25^+$ regulatory T cells are responsible for the immunomodulatory effects observed after oral treatment with IVIG.

4.3.6. Figures

Figure 1.

1a



1b

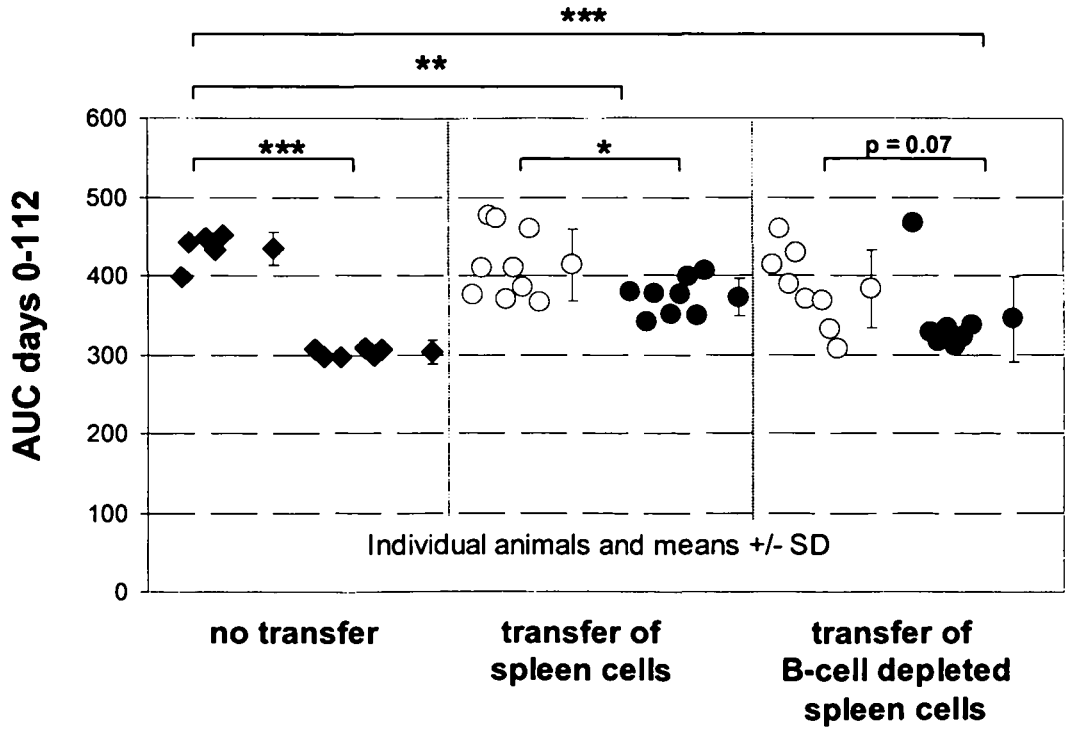
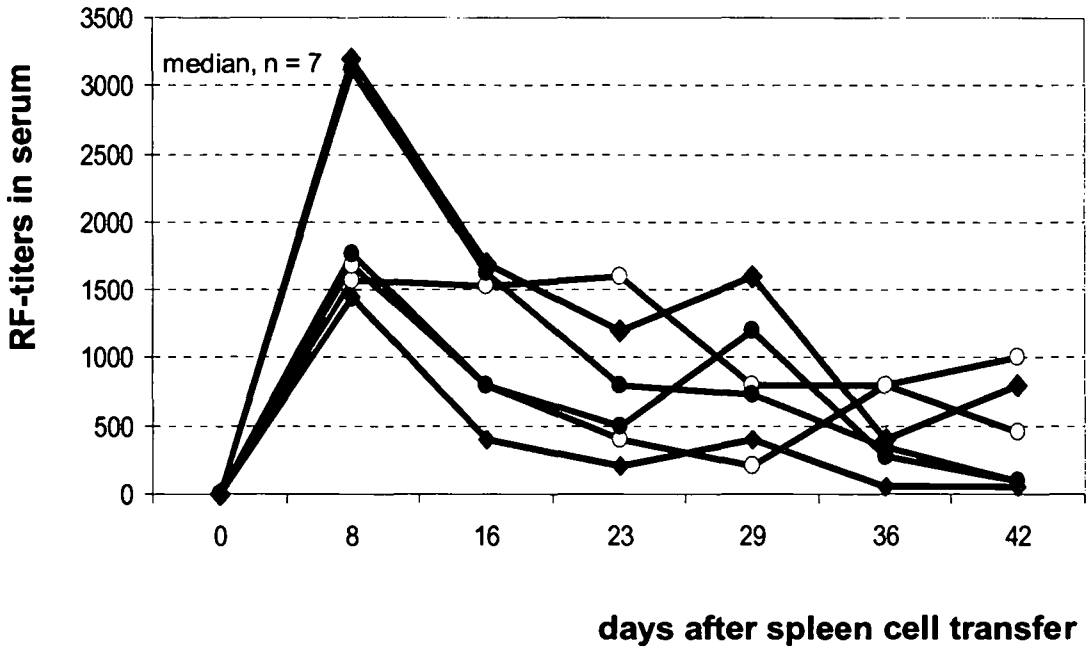


Figure 1. Transfer of total spleen cells and spleen cells depleted of B cells

Spleen cells were prepared using density gradient centrifugation. Total spleen cells (○) and B-cells depleted spleen cells (○) from naïve mice or total spleen cells (●) and B-cells depleted spleen cells (●) from mice that had received oral IVIG for 45 days in drinking water were transferred on day 0. Control mice received DPBS buffer instead of cells and either no further treatment (◆) or IVIG in drinking water for 45 days (◆). All groups were first immunized with IC on day 1 and received booster injections on day 50 and 97. RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (1a) or the areas under the curve (AUC) for the development of ELISA titers from days 0-122 (1b). AUCs were were calculated for each individual animal. Means and standard deviations (SD) for AUCs were calculated for each group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Figure 2.

2a



2b

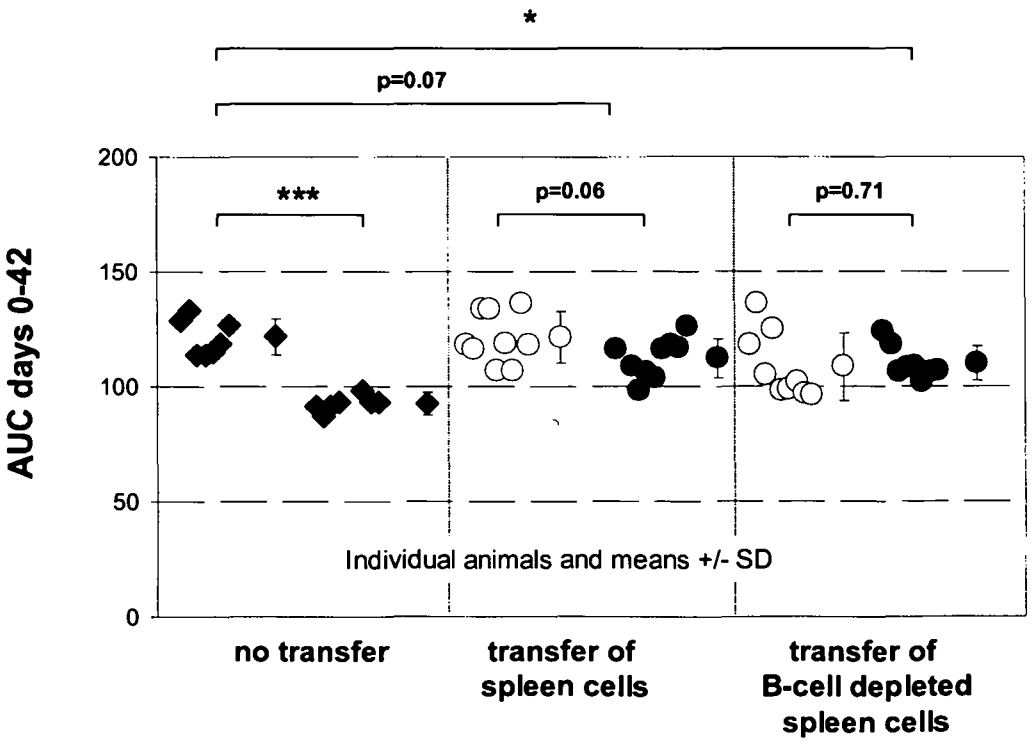
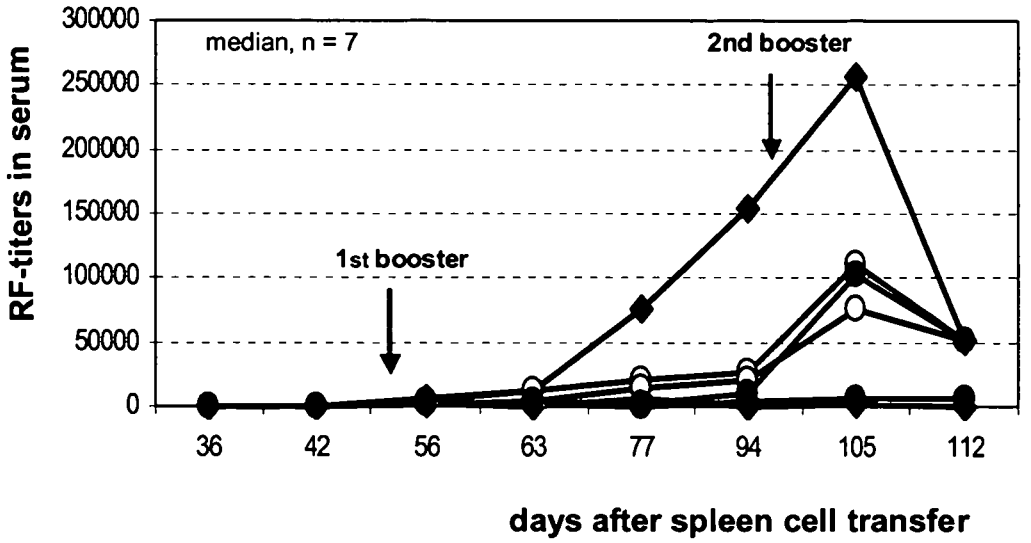


Figure 2. Primary response (days 0-42) of the transfer experiment shown in Fig. 1

Spleen cells were prepared using density gradient centrifugation. Total spleen cells (○) and B-cells depleted spleen cells (○) from naïve mice or total spleen cells (●) and B-cells depleted spleen cells (●) from mice that had received oral IVIG for 45 days in drinking water were transferred on day 0. Control mice received DPBS buffer instead of cells and either no further treatment (◆) or IVIG in drinking water for 45 days (◆). All groups were first immunized with IC on day 1. Presented are the medians for ELISA titers calculated for each group (2a) or the AUC for the development of ELISA titers from days 0-42 (1b). AUCs were were calculated for each individual animal. Means and SD for AUCs were calculated for each group.
* $p<0.05$; *** $p<0.001$

Figure 3.

3a



3b

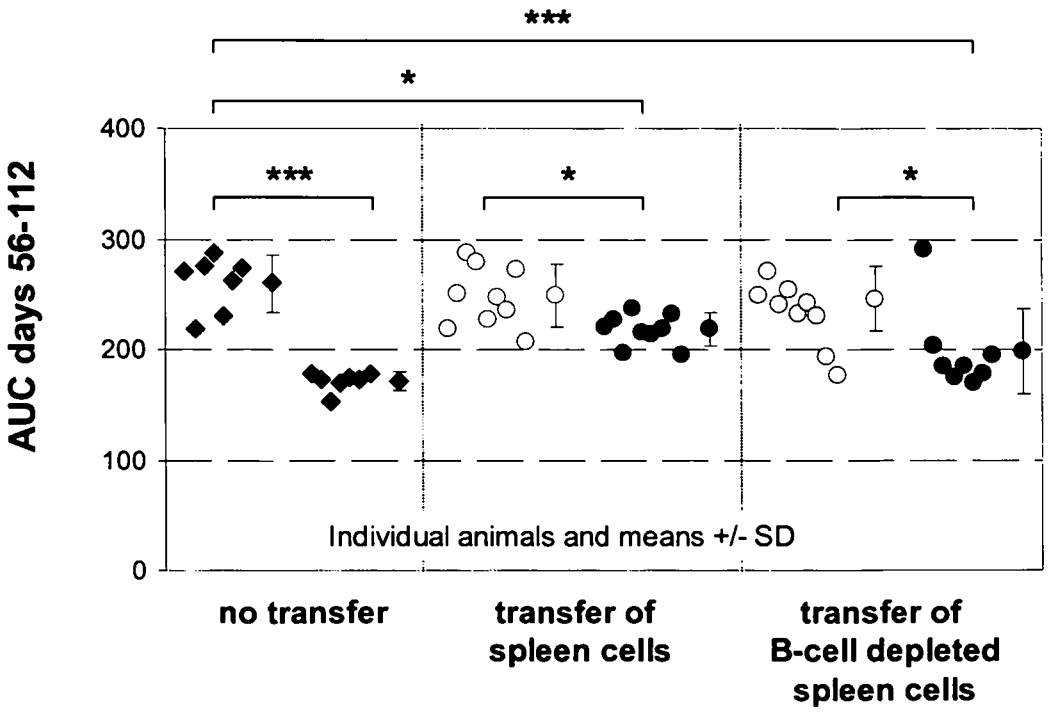
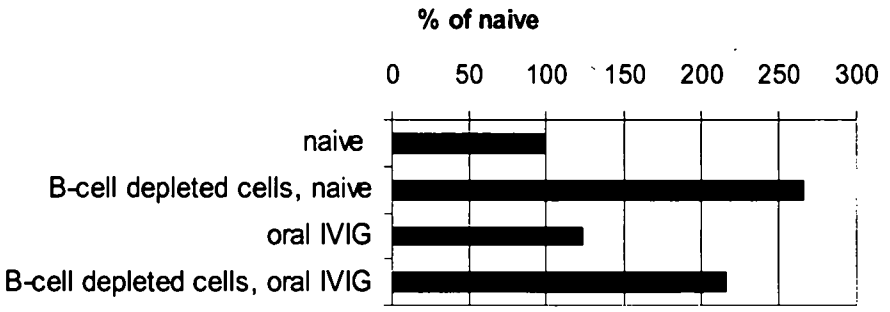


Figure 3. <u>Memory response (days 55-112) after two booster immunizations of the transfer experiment shown in Fig. 1.</u>

Spleen cells were prepared using density gradient centrifugation. Total spleen cells (○) and B-cells depleted spleen cells (○) from naïve mice or total spleen cells (●) and B-cells depleted spleen cells (●) from mice that had received oral IVIG for 45 days in drinking water were transferred on day 0. Control mice received DPBS buffer instead of cells and either no further treatment (◆) or IVIG in drinking water for 45 days (◆). All groups were first immunized with IC on day 1 and received booster injections on day 50 and 97. RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (3a) or the areas under the curve (AUC) for the development of ELISA titers from days 0-122 (3b). AUCs were were calculated for each individual animal. Means and standard deviations (SD) for AUCs were calculated for each group. * $p<0.05$; ***$p<0.001$

Figure 4.

4a



4b

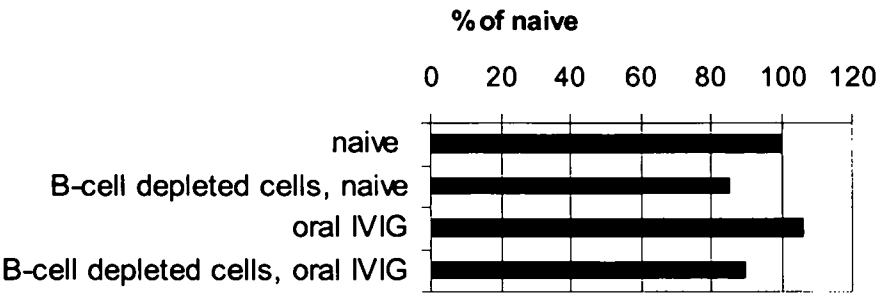


Figure 4. FoxP3 expression of spleen cells or spleen cell fractions obtained from naïve or IVIG treated mice

Forkhead box P3 (FoxP3) expression normalized to T TATA-box binding protein (Tbp), a housekeeping gene (4a). FoxP3 expression normalized to CD3, a T cell marker (4b).

Figure. 5

5a

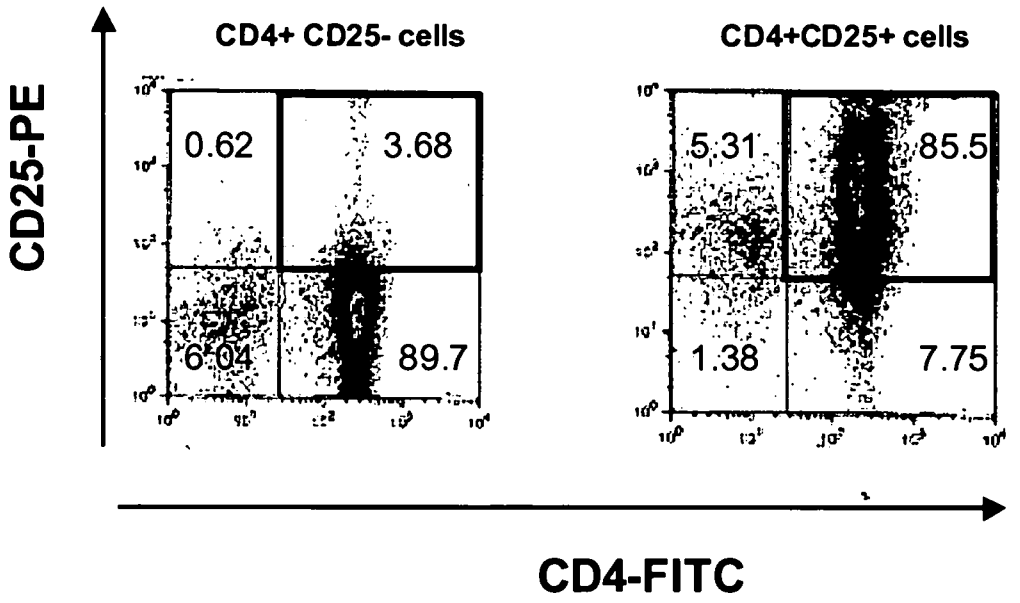


Figure 5.

Figure 5a. FACS analysis of CD4CD25 expression of T cell subpopulations

FACS analysis of spleen cells using anti-CD4-FITC and anti-CD25-PE antibodies. The left picture examines the CD4CD25 expression of enriched CD4+ CD25- cells. The right picture examines the CD4CD25 expression of enriched CD4+ CD25+ cells.

5b

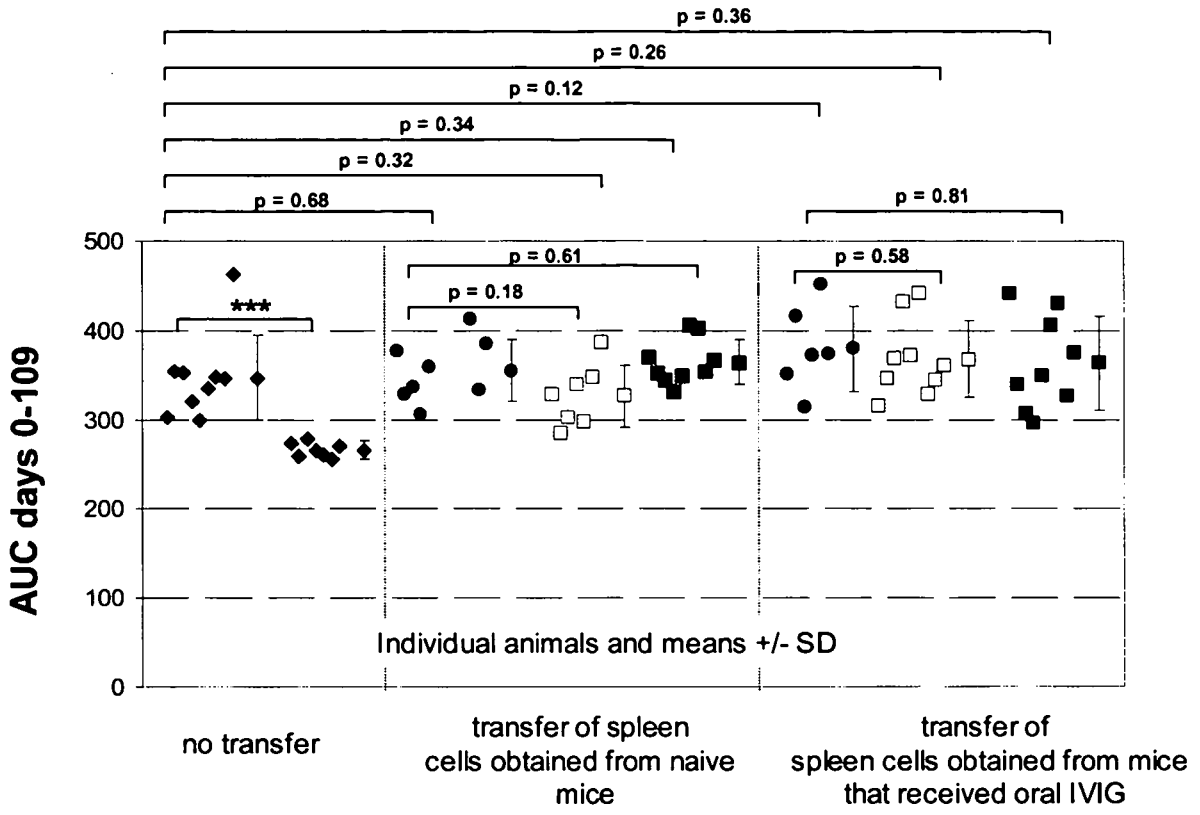
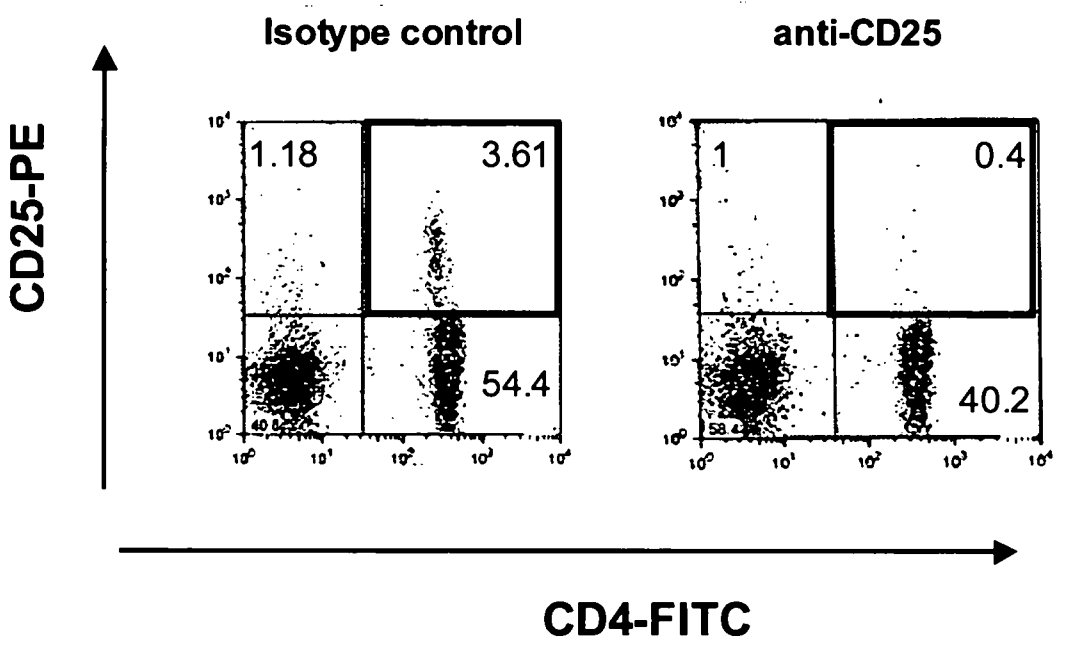


Figure 5b. Transfer of CD4+CD25+ or CD4+ CD25- T cells subpopulations

Spleen cells were prepared using density gradient centrifugation. Total spleen cells (●), CD4CD25- spleen cells (□) and CD4CD25+ spleen cells (■) from naïve mice or total spleen cells (●), CD4CD25- spleen cells (□) and CD4CD25+ spleen cells (■) from mice that had received oral IVIG for 45 days in drinking water were transferred on day 0. Control mice received DPBS buffer instead of cells and either no further treatment (◆) or IVIG in drinking water for 45 days (◆). All groups were first immunized with IC on day 1 and received booster injections on day 42 and 84. RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (5a) or the AUC for the development of ELISA titers from days 0-109 (5b). AUCs were were calculated for each individual animal. Means and SD for AUCs were calculated for each group. ***p<0.001 ; s.n. non significant

Figure 6.

6a



6b

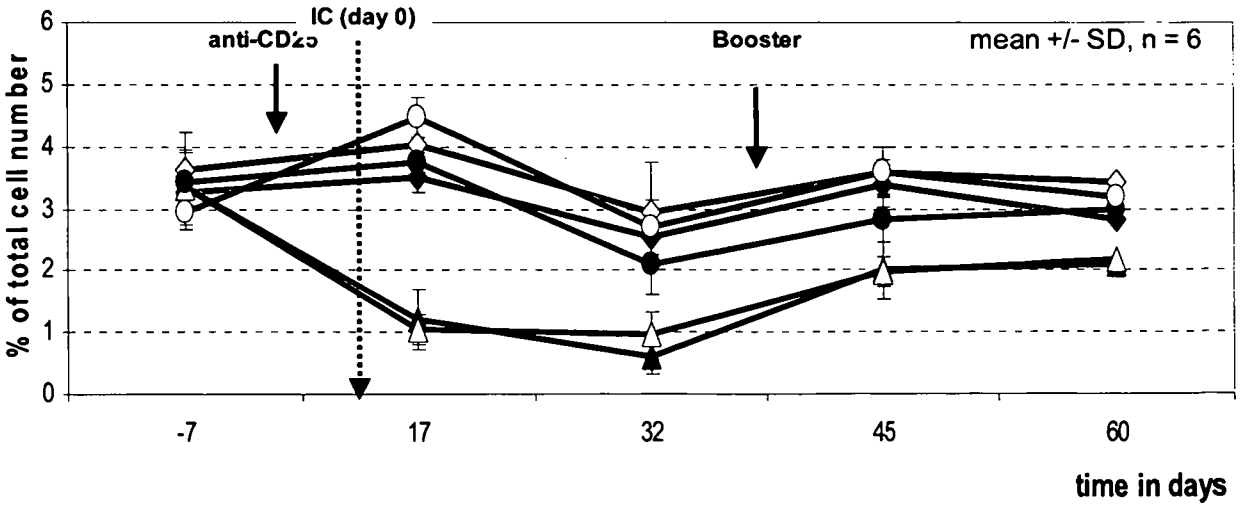


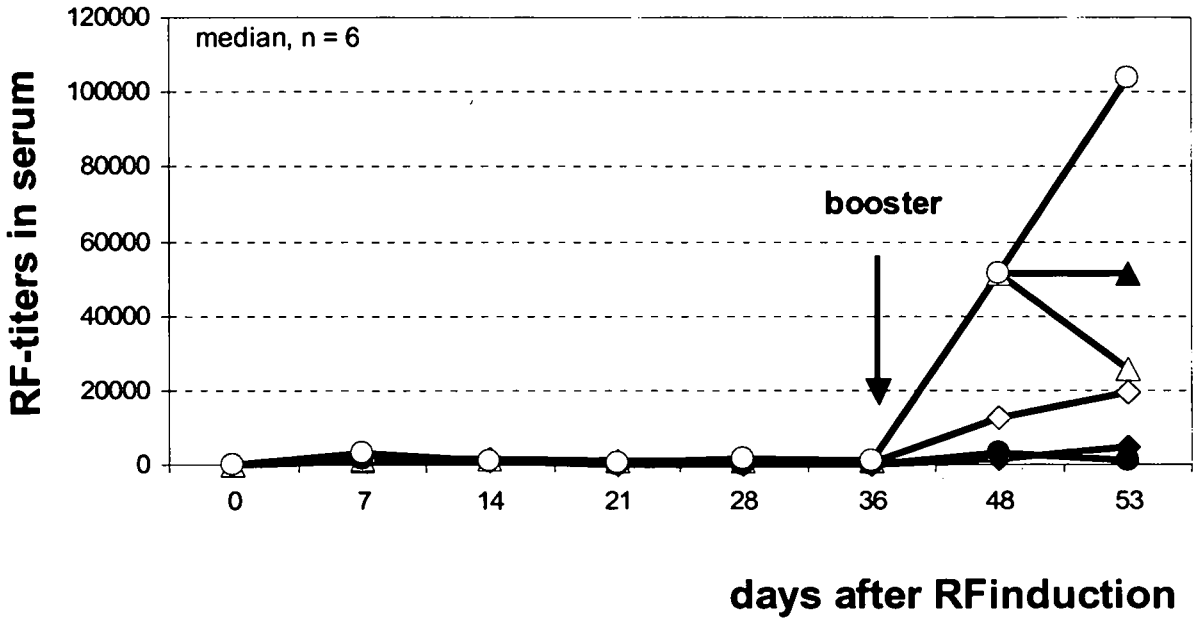
Figure 6. Depletion of CD25+ cells.

Fig. 6a represents FACS analysis of CD4+CD25+ cells in whole blood from mice that received isotype control antibodies (left picture) and mice that received a depleting anti-CD25 antibody (right picture).

Figure 6b shows the kinetic of CD25+ cells in whole blood. Cells are obtained from mice that received a depleting anti-CD25 antibody and no further treatment (△) or a depleting anti-CD25 antibody and IVIG in the drinking water for 5 days (▲). Cells were also obtained from mice that received an isotype control antibody and no further treatment (○) or received an isotype control antibody and IVIG in the drinking water for 5 days (●). Control mice received no treatment (◇) or IVIG in drinking water for 5 days (◆). All groups were first immunized with IC on day 1 and received booster injection on day 38. Means and SD of CD25+ cells normalized to the percentage of total cell number were calculated for each group.

Figure 7.

7a



7b

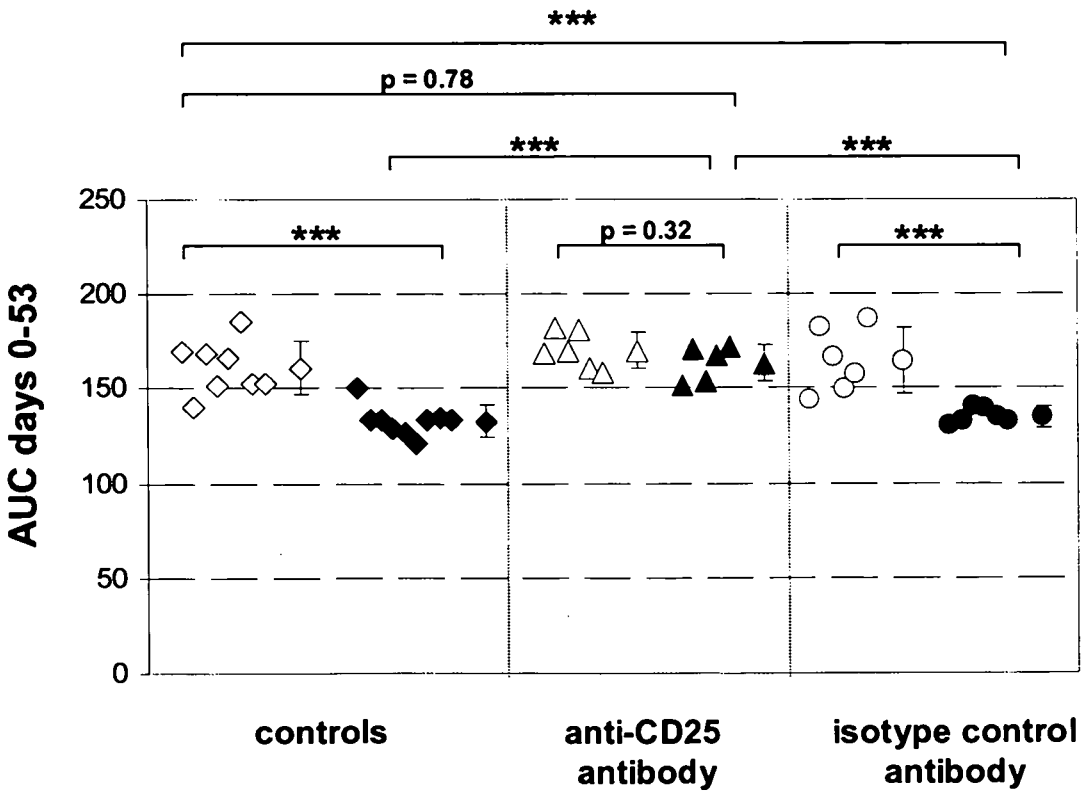
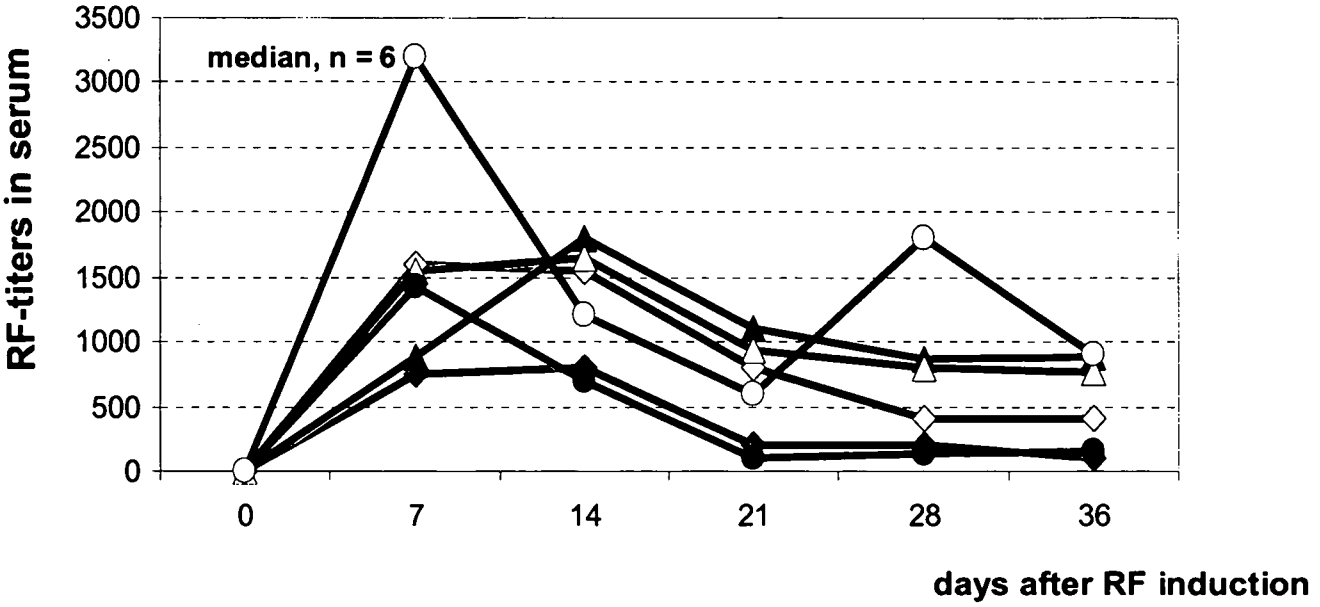


Figure 7. <u>The influence of the depletion of CD25+ cells on the immunmodualtory activities of oral IVIG</u>

RF-titers of mice that received a depleting anti-CD25 antibody and no further treatment (Δ) or a depleting anti-CD25 antibody and IVIG in the drinking water for 5 days (▲)as well as RF-titers of mice that received an isotype control antibody and no further treatment (○) or received an isotype control antibody and IVIG in the trinking water for 5 days (●). Control mice received no treatment (◇) or IVIG in drinking water for 5 days (◆). All groups were first immunized with IC on day 1 and received booster injection on day 38. RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (7a) or the AUC for the development of ELISA titers from days 0-53 (7b). AUCs were were calculated for each individual animal. Means and standard deviations for AUCs were calculated for each group. ***$p<0.001$

7c


RF-titers in serum
3500
3000
2500
2000
1500
1000
500
0
median, n = 6
0
7
14
21
28
36
days after RF induction

7d

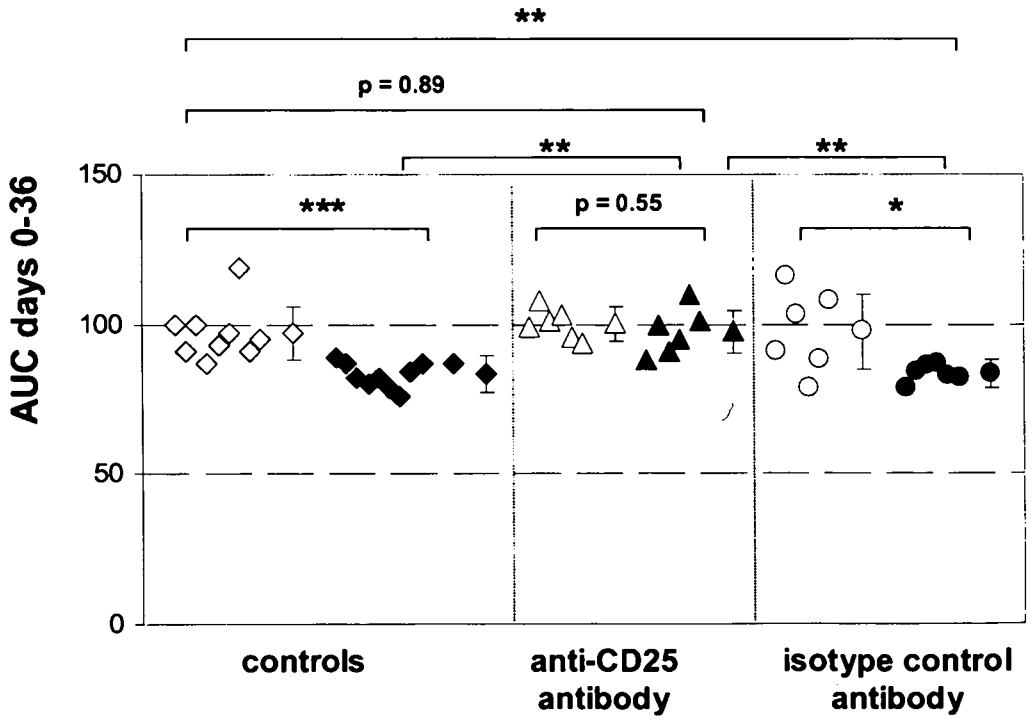

**
p = 0.89
**
**
AUC days 0-36
150
100
50
0
***
p = 0.55
*
controls
anti-CD25 antibody
isotype control antibody

Fig. 7c, 7d Primary immune response (days 0-36) of the experiment shown in Fig. 6.

RF-titers in serum samples were measured by ELISA. Presented are the medians for ELISA titers calculated for each group (7c) or the AUC for the development of ELISA titers from days 0-36 (7d). AUCs were were calculated for each individual animal. Means and standard deviations for AUCs were calculated for each group.
* $p<0.05$; ** $p<0.01$,***$p<0.001$

4.3.7. References

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5. Discussion

Autoantibodies are characteristic for autoimmune diseases such as rheumatoid arthritis (RA). Rheumatoid factors (RFs) are autoantibodies that appear in different autoimmune diseases as well as in a number of non-autoimmune conditions (31, 32). Rheumatoid factors are produced by plasma cells in inflamed synovial tissues and they recognize the Fc-region of human IgG (31, 32). Although there is a correlation between RFs and severity of RA, the contribution of RFs to the disease itself remains unclear (31).

In the studies presented in this thesis we asked the question whether oral application of intravenous immunoglobulins (IVIG) has the potential to inhibit the induction of systemic responses of RF-like antibodies. Based on the fact that the Fc-part of the IgG molecule is the autoantigen that is recognized by RF-expressing B cells, we assumed that IVIG might provide a suitable antigen for the induction of oral tolerance. For our studies we used a mouse model were we induced an RF-like antibody response against the Fc-part of human IgG by a single dose injection of insoluble human immune complexes (ICs) as described by Abedi-Valugerdi (39, 40).
Our results show that even a single dose of oral IVIG given by intragastric intubation, is able to prevent RF responses, but only if oral IVIG is administered before the induction of RF responses. Short-term preventive application of oral IVIG is able to prevent primary immune responses and to down-modulate memory responses. Continuous preventative treatment with oral IVIG in the drinking water does not only prevent the primary immune response but also prevents memory responses after booster injections. Administration of IVIG after the induction of RF responses does not prevent the primary immune response but it still down-modulates memory responses. Krause et al. (41), already reported immunomodulatory activities of oral IVIG in an experimental model of anti-phosphospholipid syndrome. The authors found immunomodulatory activities of oral IVIG only when IVIG was administered before the onset of the disease. In contrast to these data, we could show in our model that continuous therapeutic treatment with oral IVIG is able to reduce booster effects of RF memory responses. These findings are very interesting with regard to the fact that autoimmune diseases in patients are usually diagnosed after the autoimmune process has established itself. Besides, the course of autoimmune

diseases is often characterized by alternating stable phases and relapses. Relapses are caused by booster effects of the immune system and present a particular problem for the patient. Since we showed that continuous therapeutic administration of oral IVIG reduces RF memory responses after booster injections in a mouse model with experimentally induced RF factor responses, it is conceivable that oral administration of IVIG might have similar effects in clinical application for patients suffering from relapse phases of an autoimmune disease associated with RF responses.

Since the Fc-fragment of IgG represents the autoantigen recognized by RFs, we asked the question whether the Fc-fragment is the active part for mediating the tolerogenic effects of oral IVIG. We found evidence that the Fc-fragment but not the Fab-fragment of a monoclonal IgG1 preparation was capable to modulate primary and memory RF responses. These results indicate that the Fc-protein might be sufficient for new therapeutic approaches targeting the down-modulation of RF activities.

Another point that might be interesting for a clinical application of oral IVIG is that the immunomodulatory effects of oral IVIG are antigen-specific. When we immunized mice that received oral IVIG, with an irrelevant antigen (human factor VIII), the anti-factor VIII immune response was not modulated by oral IVIG. Based on these results we conclude that IVIG does not bear any general immunosuppressive potential and the tolerogenic effects of oral IVIG are only directed against RF-like antibody responses in our particular animal model.

In further investigations we characterized mechanisms of action that might be responsible for the immunomodulatory activities of oral IVIG. WE used adoptive transfer experiments to examine whether the immunomodulatory activities of oral IVIG are mediated by cellular mechanisms. At the beginning we transferred spleen cells from naïve mice or cells obtained from mice that received IVIG in their drinking water for 45 days. During the establishment of my transfer experiments we found that the quality of the cell preparation method has a significant influence on the outcome of the transfer experiments. Spleen cells prepared using hemolysis of the red blood cells resulted in a high amount of apoptotic cells, whereas preparation using density gradient centrifugation resulted in a sufficient amount of viable cells. Transfer of spleen cells prepared using hemolysis resulted in an increase of the RF-

titers rather that in a down-modulation. In different publications it has already been demonstrated that apoptotic cells are immunogenic (42, 43). Some authors also suggest that injection of damaged cells in combination with an antigen leads to an immune response against the antigen, even when cells and antigen are physically separated (43). This suggests that in our model the high amount of apoptotic cells might have increase the immune response against the IC. This is confirmed by the finding that the transfer of spleen cells from preparations using density gradient centrifugation contained a sufficient proportion of viable cells and did not increase the RF responses in recipient mice. In some of our transfer experiments spleen cells obtained from mice that had received oral IVIG showed a tendency of preventing primary RF responses. Furthermore, spleen cells obtained from mice that received oral IVIG significantly down-modulated RF memory responses after booster injections. In some experiments this effect was also seen after transfer of spleen cells from naïve mice. This suppression of RF responses after transfer of spleen cells obtained from naïve mice might be due to the presence of natural regulatory T cells in the spleen of naïve mice.

Based on our results obtained in transfer experiments we would like to conclude that oral IVIG is likely to induce regulatory cells. However, the concentration of regulatory cells might not be high enough. Therefore, we also transferred B-cell depleted spleen cell fractions. Here, we demonstrated that the transfer of B-cell depleted spleen cell fractions obtained from both naïve and IVIG-treated mice significantly down-modulated RF responses. When compared to the controls, B cell depleted spleen cells obtained from mice orally treated with IVIG were significantly more effective in suppressing the RF responses than total spleen cells. These findings indicate that oral IVIG induces some form of regulatory cell that is probably part of the T-cell compartment.

We next asked the question whether transfer of spleen cells obtained from IVIG treated mice is able to prevent booster effects in recipient mice that had already an established RF response. For this experiment we transferred spleen cells obtained from mice that were treated with oral IVIG for an extended period of time using continuous application and immunized with three doses of IC. As controls we transferred spleen cells from naïve mice. The spleen cells were transferred into

recipients that were already immunized with two doses of IC and had developed a sustained RF response.

Spleen cells from naïve mice down-modulated booster responses in mice with an established RF response, what might be due to the immunomodulatory activities of natural regulatory cells, as we have already indicated above. However, spleen cells obtained from mice treated with oral IVIG showed a significantly higher capacity to prevent these booster effects when compared to cells obtained from naïve mice. In this set of experiments, transfer of spleen cells induced, a more significant down-modulation of RF responses in recipients, than we have seen in other experiments. Reasons for this might be that in this experiment the donor mice received IVIG in drinking water for continuous period before the transfer, whereas in our former experiments the administration time of oral IVIG was limited to 45 days. We cannot exclude that 45 days of oral administration of IVIG are not enough for the induction of a sufficient proportion of regulatory cells in the spleen. Another point might be that in this transfer experiment the donor mice received three doses of IC during the application of oral IVIG. This early encounter with the antigen might induce a more sufficient proportion of regulatory cells than the administration of IVIG alone. Furthermore, it might induce chemo tactic signal for regulatory T cells to migrate into the spleen.

As $CD4^+CD25^+$ T cells are considered as one population of potentially regulatory T cells, we decided to examine whether the transfer of these T cell subpopulation enhances the immunomodulatory activities of oral IVIG in the recipients (15, 16, 17). Some authors argue that regulatory T cells might also be found in the $CD4^+CD25^-$ T cell subpopulation (16, 27) transferred this subpopulation, too.

We transferred these subpopulations as well as the total spleen cells obtained from naïve mice or mice that received oral IVIG for 45 days into naïve recipients. After the transfer we induced RF responses in the recipients. Our results demonstrated that the transfer of both subpopulations did amplify rather than prevent RF responses. This is also true for total spleen cells. Since we transferred the total spleen cells at the same time point as the subpopulations we suggest that the duration of the cell preparation procedure might have had an influence on the cell quality. Before transfer of these cells, we used density gradient centrifugation to get rid of apoptotic cells. Density gradient centrifugation seemed to be a useful method for depleting

apoptotic cells in earlier transfer experiments. Several authors already demonstrated that damaged cell in combination with an antigen lead to an immune response against the antigen (42, 43) Although apoptotic cells were removed with this method, there might be a lot of early apoptotic cells that are not eliminated by density gradient centrifugation, but are able to stimulate the immune response of the recipient animals when they die.

To circumvent the problem of long-lasting in vitro cell preparation, we used in vivo depletion of $CD25^+$ cells to characterized the role of $CD25^+$ cells in the immunmodulatory activities of oral IVIG. Our results demonstrate that depletion of $CD25^+$ cells prevents the immunomodulatory effect of oral IVIG on RF responses of in mice that were fed with oral IVIG. In contrast to anti-CD25 antibody, injection of an isotype control antibody did not affect the immunosuppressive effects of oral IVIG. These results suggest that $CD25^+$ T cells are involved in the immunomodulatory activities of oral IVIG.

Eventually, we wanted to examine if cells involved in the immunmodulatory activities of oral IVIG have a higher expression of forkhead box P3 (FoxP3). FoxP3 is a transcription factor that is considered to be a possible genetic marker for regulatory T cells (25, 26). We compared the FoxP3 expression of cells from naïve mice and cells obtained from mice that had received oral IVIG for 45 days in drinking water. We could not detect any significant difference in the expression of FoxP3 between both. We also examined spleen cells that were depleted of B cells. In the B-cell depleted fraction we saw a higher amount of FoxP3 expression independently of the way mice were treated. This might be due to the T cell enrichment in the B-cell depleted spleen cell fractions. When we adjusted the expression of FoxP3 to the amount of T cells in the preparation, we saw no difference in the expression of FoxP3 in spleen cells compared to B-cell depleted spleen cell fractions.

As we have already speculated before, 45 days of treatment with oral IVIG might not be enough for the induction of a sufficient proportion of antigen specific regulatory cells in the spleen or maybe they need an encounter with the antigen for proliferation and recruitment to the spleen. Some authors have already shown that not all regulatory T cells express FoxP3 (27). Therefore it is possible that regulatory T cells involved in the immunomodulatory activities of oral IVIG do not express FoxP3.

Summarizing our results, we can conclude that a single dose of oral IVIG is able to prevent the induction of RF responses. Continuous treatment with IVIG prevents both primary and booster effects of RF responses in mice that were immunized with insoluble human ICs. The immunomodulatory activities of oral IVIG are antigen specific and are mediated by the Fc-part of the human IgG. Continuous therapeutic administration of oral IVIG that started 5 days after the induction of RFs does not prevent the induction of RF responses but is able to reduce booster effects. The mechanisms of action of oral IVIG seem to be mediated by cellular mechanisms. We can show that these mechanisms are mediated by $CD25^+$cells. Therefore, we speculate that $CD4^+CD25^+$ regulatory T cells are responsible for the observed immunomodulatory effects observed after treatment of mice with oral IVIG.

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7. Appendix

7.1. Section 4.1

In this section I did all experiments myself. The actual animal work was done by our specialists. This work is not going to be published.

7.2. Publications

The data presented in the result section

are to be submitted to Blood

4.2. Induction of oral tolerance on rheumatoid factor responses by treatment with human IgG (IVIG). Maier E, Novy-Weiland T, Reipert BM, Auer W, Schwarz HP (2005)

My part of the data was concerned with preparation and transfer of immune cells and detection of antibodies by ELISA.

are to be submitted to Eur J Immunol.

4.3.Significance of regulatory T cells in the suppression of rheumatoid factor responses induced by oral immunoglobulin. Novy-Weiland T, Maier E, Baumgartner B, Muchitsch E, Schwarz HP, Reipert BM (2005)

In this section I did all experiments myself. The actual animal work was done by our specialists.

7.3. Posters

7.3.1. Mechanisms of action of immunomodulatoy activities of oral immunoglobulin
Presented at Immune tolerance workshop, June 2005, University of New Castle upon Tyne, UK

7.2.3. Significance of regulatory T cells in the suppression of rheumatoid factor responses induced by oral immunoglobulin
Presented at "33. Kongress der Deutschen Gesellschaft für Rheumatologie", September 2005, Dresden, Germany

MECHANISMS OF ACTION OF IMMUNMODULATORY ACTIVITIES OF ORAL IMMUNGLOBULIN

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7.3.1.

INTRODUCTION

Oral administration of protein antigens has been shown to be effective in inducing immune tolerance in a number of experimental studies (1). Oral tolerance can be mediated by different mechanisms such as clonal deletion, anergy of antigen specific cells, or active suppression by the induction of regulatory T cells (2). Recently, we could show that oral administration of human IgG to Balb/c mice prevented and down-regulated the systemic induction of rheumatoid factor responses (Maier et al., this meeting). Rheumatoid factors (RFs) are autoantibodies that are directed against the Fc regions of IgG molecules (3). They can be detected in several autoimmune diseases such as rheumatoid arthritis and lupus erythematosus (4). Here, we asked the question whether the induction of unresponsiveness to immune complexes induced by oral IgG involves the activation of regulatory T cells. In order to approach this question we performed adoptive cell transfer studies using spleen cells and spleen cell fractions from mice orally treated with IgG. Initially, we compared different procedures for cell isolation to obtain cells of sufficient quality for transfer.

MATERIAL AND METHODS

Animals:

Female Balb/c mice were obtained from Harlan Winkelmann GmbH (Borchen, Germany) and maintained in our own animal facility. All animals were aged 10-12 weeks at the beginning of the experiments.

Induction and booster of human rheumatoid factors:

Human rheumatoid factors (RF) were induced by treatment with insoluble human immune complexes (IC) according to a protocol described by Abedi-Valugerdi et. al. (3). Titers of RF Serum were measured by ELISA assays.

Oral treatment with human IgG:

Mice received approximately 10mg human IgG (Endobulin, Baxter AG, Vienna, Austria) in drinking water per day for 45 days.

Preparation of spleen cells:

Single cell suspensions of spleen cells were prepared as recently described (5).
Removal of red blood cells was performed either by hemolysis (using a lysis buffer containing 0.15 M ammonium chloride, 10 m potassium bicarbonate and 0.1M ethylene-diamnetetraacetic acid pH 7.2) or by density gradient centrifugation using Lympholyte M (Cedarlane, Canada). For density gradient centrifugation, cells were resuspended in RPMI-1640 with 2% FCS (contrary to the the manufacturers protocol).
B cells were depleted using mouse pan B (B220) Dynabeads (Dynal Biotech ASA, Norway) following the manufacturers protocol.

Adoptive transfer of spleen cells:

2 x 107 spleen cells were resuspended in Dulbecco's PBS (DPBS, Sigma-Aldrich Chemie GmbH, Germany) and injected into the tail vein as indicated. Control groups received DPBS only.

Flow cytometry (FACS):

Cells were analysed using a FACS Calibur and CellQuest pro software (both from BD Bioscience, Germany).

Statisical analyses:

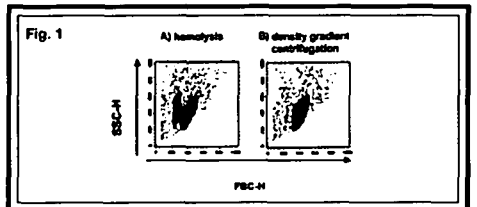
For statistical analyses of antibody titers, areas under the curve (AUC) were calculated for each individual animal. Means and standard deviations for AUC were calculated for each group. Comparisons between groups were performed by variance analysis using the program SAS. Differences between groups were considered to be statistically significant if $p < 0.05$.

RESULTS

Quality of spleen cell preparation depends on the procedure used for isolation of cells

The quality of spleen cells prepared by hemolysis (Fig. 1A) was compared with the quality of cells prepared by density gradient centrifugation (Fig. 1B). Results demonstrate that only cells prepared by density gradient centrifugation contained a sufficient proportion of viable cells.

Spleen cells prepared by different isolation procedures

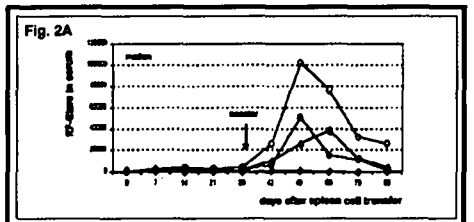


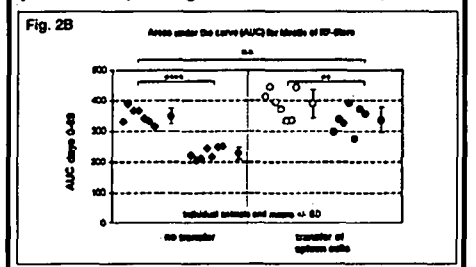
A) FSC-H/SSC-H analysis of spleen cells prepared by using hemolysis
B) FSC-H/SSC-H analysis of spleen cells prepared by using density gradient centrifugation

Prevention of rheumatoid factor responses after oral IgG

Intravenous injection of insoluble human immune complexes into Balb/c mice causes the development of RFs in naïve mice (Figs. 2A, 2B). Oral treatment with IgG prevents the induction of RFs in naive mice as well as memory responses after booster injection (Figs. 2A, 2B).

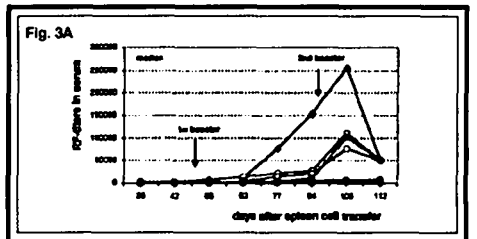
Transfer of spleen cells prepared by using hemolysis

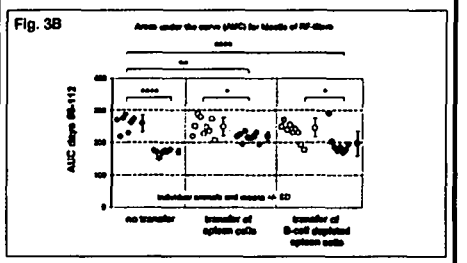




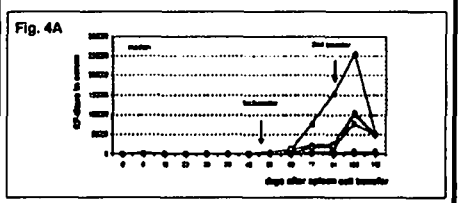
Spleen cells were transferred on day 0. All groups were first immunized with IC on day 1 and received booster injection on day 36.
A) Median of RF-titers
B) AUC for kinetic of RF-[redacted]y 0-99)
** p<0.01; ***p<0.000[redacted]e not significant

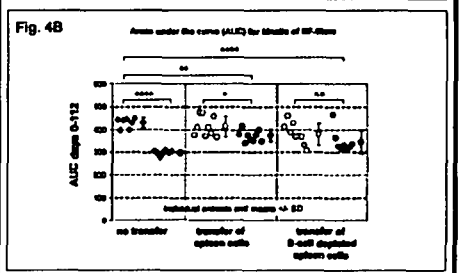
Transfer of spleen cells and B-cell depleted spleen cells prepared by using density gradient centrifugatio





Spleen cells were transferred on day 0. All groups were first immunized with IC on day 1 and received booster injections on days 50 and 97.
A) Median of RF-titers
B) AUC for kinetic of RF-titers (days 56-112)
* p<0.05; ** p<0.01; ***p<0.0001





Spleen cells were transferred on day 0. All groups were first immunized with IC on day 1 and received booster injections on days 50 and 97.
A) Median of RF-titers
B) AUC for kinetic of RF-titers (days 0-112)
* p<0.05; ** p<0.01; ***p<0.0001, n.s.= no[redacted]icant

Adoptive transfer of spleen cells prepared by hemolysis does not prevent RF responses

Transfer of spleen cells from either naïve mice or mice treated with oral IgG for 45 days does not prevent the induction of RF responses if spleen cells are prepared using hemolysis of red blood cells. Cells obtained from naïve mice even amplify the RF response (Figs. 2A, 2B). The amplification of the immune response might be due to a stimulation of the immune system by apoptotic cells.

Adoptive transfer of spleen cells prepared by density gradient centrifugation prevent RF responses

Transfer of spleen cells prepared by density gradient centrifugation significantly inhibits the induction of RF responses and the RF memory responses after booster. Spleen cells obtained from mice treated with oral IgG for 45 days induce significantly stronger inhibition than spleen cells obtained from naïve mice (Figs. 3A, 3B, 4A, 4B). Furthermore, B-cell depleted spleen cells are significantly more effective than total spleen cells (Figs. 3A, 3B, 4A, 4B).

CONCLUSIONS

Oral administration of human IgG prevents the induction of RFs in naive in Balb/c mice and RF memory responses after booster. Transfer of spleen cells and in particular transfer of B-cell depleted spleen cells into naïve mice inhibits the induction of RF responses. Spleen cells obtained from mice orally treated with IgG for 45 days are significantly more effective in suppressing the immune response than cells obtained from naïve mice. The quality of cell preparation significantly influences the outcome of the transfer experiment. These results indicate that oral IgG induces some form of regulatory cell that is probably part of the T-cell compartment. Further investigations will elucidate the exact nature of this regulatory cell. Suppression of immune responses after transfer of cell obtained from naïve mice indicates the presence of natural regulatory cells, e.g. natural regulatory T cells. However, at this point we do not exclude that other mechanisms in addition to regulatory T cells contribute to the observed immunmodulatory effects of oral IgG.

ACKNOWLEDGEMENT

We thank Irmgard Schindler, Elisabeth Hopfner, Martin Matzinger, Michaela Waller and for technical assistance and Eva Attakpah and Bettina Bischetsrieder for animal experiments.

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5. Hausl, C et al. Blood 2004; 104:115

7.

SIGNIFICANCE OF REGULATORY T CELLS IN THE SUPPRESSION OF RHEUMATOID FACTOR RESPONSES INDUCED BY ORAL IMMUNGLOBULIN

Novy-Weiland T.[1], Maier E.[2], Baumgartner B.[2], Muchitsch E.[2], Schwarz H.P.[1,2], Reipert B.M.[1,2]

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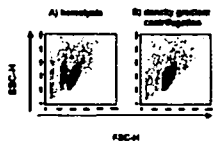
Spleen cells prepared by different isolation procedures

Fig. 1



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Transfer of spleen cells prepared by using hemolysis

Fig. 2A

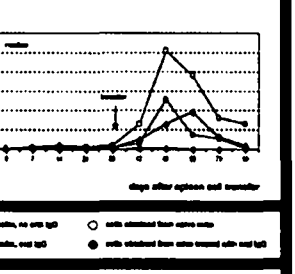
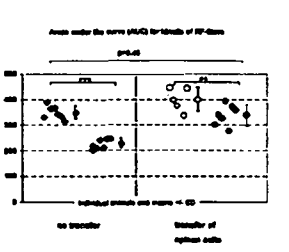


Fig. 2B



Spleen cells were transferred on day 0. All groups were first immunized with IC on day 1 and received booster injection on day 36.
A) Median of RF-titers
B) AUC for kinetic of RF-titers (day 0-99)
** p<0.01; ***p<0.001

Adoptive transfer of spleen cells prepared by density gradient centrifugation prevent RF responses
Transfer of spleen cells prepared by density gradient centrifugation significantly inhibits the induction of RF responses and the RF memory responses after booster. Spleen cells obtained from mice treated with oral IgG for 45 days induce significantly stronger inhibition than spleen cells obtained from naïve mice (Figs. 3A, 3B, 4A, 4B). Furthermore, B-cell depleted spleen cells are significantly more effective than total spleen cells (Figs. 3A, 3B, 4A, 4B).

CONCLUSIONS

Oral administration of human IgG prevents the induction of RFs in naive in Balb/c mice and RF memory responses after booster. Transfer of spleen cells and in particular transfer of B-cell depleted spleen cells into naïve mice inhibits the induction of RF responses. Spleen cells obtained from mice orally treated with IgG for 45 days are significantly more effective in suppressing the immune response than cells obtained from naïve mice. The quality of cell preparation significantly influences the outcome of the transfer experiment. These results indicate that oral IgG induces some form of regulatory cell that is probably part of the T-cell compartment. Further investigations will elucidate the exact nature of this regulatory cell. Suppression of immune responses after transfer of cell obtained from naïve mice indicates the presence of natural regulatory cells, e.g. natural regulatory T cells. However, at this point we do not exclude that other mechanisms in addition to regulatory T cells contribute to the observed immunmodulatory effects of oral IgG.

Transfer of spleen cells and B-cell depleted spleen cells prepared by using density gradient centrifugation

Fig. 3A

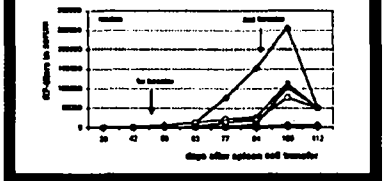
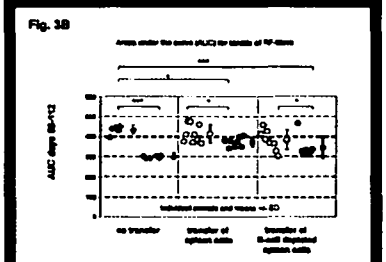


Fig. 3B



Spleen cells were transferred on day 0. All groups were first immunized with IC on day 1 and received booster injection on day 36.
A) Median of RF-titers
B) AUC for kinetic of RF-titers (day 56-112)
* p<0.05; ***p<0.001

Fig.4A

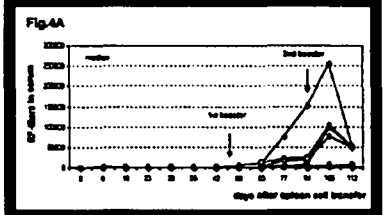
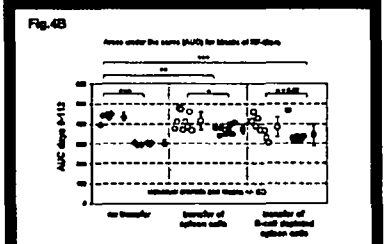


Fig.4B



Spleen cells were transferred on day 0. All groups were first immunized with IC on day 1 and received booster injections on days 50 and 97.
A) Median of RF-titers
B) AUC for kinetic of RF-titers (days 0-112)
* p<0.05; ** p<0.01; ***p<0.001

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-Danke-