Immunological aspects of antibody formation against recombinant human therapeutics

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Immunological aspects of antibody formation against recombinant human therapeutics

Immunologische aspecten van antilichaam productie tegen recombinant humane therapeutische eiwitten

(met een samenvatting in het Nederlands)

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geboren op 28 november 1980 te Krefeld, Duitsland

Promotoren: Prof.dr. H. Schellekens
Prof.dr. W. Jiskoot

Do not worry about your difficulties in Immunogenicity.
I can assure you mine are still greater.
- Melody Sauerborn, adapted from Albert Einstein,

Table of Contents **Page**

Page

Part I

...the weird (recombinant human therapeutics), the bad (anti-drug antibodies), and the good (mouse models)!

Chapter 1

Immunological mechanism underlying the immune response to recombinant human protein therapeutics

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Abstract

Recombinant human (rhu) protein therapeutics are powerful tools to treat many severe diseases such as multiple sclerosis and diabetes mellitus, among others. A major drawback of these proteins is the production of anti-drug antibodies (ADAs). In some cases, these ADAs have neutralizing capacity and can interfere with the efficacy and safety of the drug. Unfortunately, little is known about the immunological mechanisms underlying the unwanted immune response against human homologue protein therapeutics. This hampers prevention and prediction of immunogenicity. Current research mainly focuses on the structural aspects of therapeutic proteins and how these might influence the course of the immune response in the patient. This article aims to provide current insights into recent immunological developments and tries to link this in regards to production of ADAs.
A special focus is given to aggregates being present in a rhu protein formulation and their impact on the immune system, subsequently leading to breakage of tolerance and formation of ADAs. Aggregation is one of the key factors in immunogenicity and by reducing aggregation one can reduce immunogenicity and make drugs safer and more efficient.

Keywords: recombinant human protein therapeutics; self-tolerance; breakage of tolerance; Marginal zone B cells; T independent antibody responses

Introduction

One of the reasons for developing therapeutic proteins that are as identical as possible to their endogenous counterparts was to avoid recognition and activation of the immune system. Due to their similarity to endogenous proteins, rhu protein therapeutics were not expected to cause formation of ADAs. Nevertheless, the formation of antibodies against human homologue protein therapeutics in patients was recognized when reports appeared about the loss of efficacy of the therapeutic drug due to ADAs [1-2]. ADAs were also implied in severe side-effects due to cross-reactivity with the endogenous protein, leading to life-threatening conditions [3]. Taking into account the large number of rhu protein therapeutics in the pipeline of production, the formation of ADAs needs to be prevented. By what measures (e.g. epitope deleting, formulation adjustments, etc.) depends on the underlying immunological mechanisms.

In general the immunogenicity of a therapeutic protein is categorized into two broad mechanisms: (a) low or non-existing tolerance with induction of a classical response dependent on T cells, and (b) breakage of B cell tolerance. Immunological tolerance is defined as the capability of the immune system to precisely discrimination between self/tolerant and non-self/non-tolerant. Therapeutics of non-human origin (e.g. streptokinase) are recognized as non-self/non-tolerant and the classical T-cell-dependent B-cell activation is initiated leading to xenoantibodies [1]. In the case of rhu proteins functioning as a replacement therapy (e.g. rhu Factor VIII) for patients carrying genetic defects in the endogenous protein, it is most likely the low-tolerance that will activate the immune system [4].

Nevertheless, even immune tolerant patients develop ADAs against human homologues, and therefore an explanation other than a lack of tolerance needs to be considered. In the recent years the dogma of immunological tolerance has been refined by the ‘danger theory’. The danger theory states that the immune system has maturated to not only distinguish between self and non-self but also to raise an immune response against danger signals which can originate from both self and non-self proteins.

How the tolerance in patients is broken, and the exact mechanism leading to the breakage of tolerance is not well understood and both the tolerance as well as the danger theory need to be considered. This article will focus on the influence of presenting the rhu self-protein in a repetitive pattern (as in

aggregates) and thereby confusing the immune system into believing that the aggregated rhu protein is a foreign antigen.
Current immunological findings, like the human marginal zone (MZ) B cells, are incorporated into the possible mechanism involved in breakage of tolerance against aggregated rhu therapeutics. It is well accepted that aggregation is a major key factor in immunogenicity and reducing aggregation levels also lowers immunogenicity [5]. Understanding the immunological mechanism behind the breakage of tolerance will give profound insights into the problem of immunogenicity of rhu proteins and may help to develop reliable pre-clinical screening tools, leading to safer and more efficient drugs.

Thymus-dependent versus thymus-independent B-cell activation

Immunological mechanisms leading to the production of antibodies

The classical immune response against foreign antigens is dependent on T cell/B cell interaction to produce effective antibodies. Since rhu therapeutics are not supposed to be recognized as foreign by the immune cells the classical activation of T cell/B cell interaction is not sufficient to explain the formation of ADAs. This chapter describes the mechanisms of inducing B cell activation and maturation into antibody-secreting plasma cells.

(a)Thymus-dependent B-cell activation

Proteins that need the help of T cells to elicit antibody responses are termed T-cell- or thymus-dependent (TD) antigens and initiate thereby a so-called ‘classical’ immune response. The main characteristics of TD B-cell activation are summarized in Table 1 and Figure 1 is a schematic illustration of the TD pathway. Some therapeutic proteins, as is most likely in the case of Factor VIII, are given to patients with low or non-existing tolerance due to inherent deficiencies and therefore the rhu protein is considered ‘foreign’ and processed as a TD antigen. Processing of the antigen is induced by the uptake of the foreign protein by professional antigen-sensing dendritic cells (DCs) in the periphery [6]. DCs are specialists in the uptake and processing of

antigens, and in the presentation of fragments/peptides on major histocompatibility complex (MHC) class II molecules expressed on their cell surface. After antigen encounter, they migrate to the T-cell zones of draining lymph nodes [7]. Once a DC reaches the lymph nodes, it is continuously scanned by T cells to find a matching T-cell receptor (TCR) that fits in a 'key-lock' manner. After recognition of the peptide on the MHC class II molecule by the T cell, an immunological synapse [8] is formed between the T cell and the DC. The antigen-primed T cell will leave the T-cell zone and migrate to the follicles in which the B cells reside. These antigen-primed T cells that provide help for the B cell are termed T helper cells.
At the same time during T-cell antigen priming, protein antigens are also filtered by the draining system of the secondary lymphoid organs and thereby become accessible to B cells, which have the capability of recognizing foreign organisms via their B-cell receptor (BCR) [9]. The BCR is as specific as the TCR and will bind only one specific antigen. BCR-antigen interaction leads to clustering of signal transduction molecules on the B cell surface resulting in activation of the B cell. The activated B cell internalizes and processes the antigen to be finally presented via MHC class II molecules on the B-cell surface. This antigen-BCR interaction marks the first signal to induce B cell proliferation and differentiation. The second signal for the antigen-primed B-cell is usually provided by a T helper cell. Activated B cells migrate to the enriched T cell zone of the lymphoid organs. On the edge of the follicle and T cell zone, the antigen-primed T cell will encounter the antigen-primed B cell. During the second signal phase, B- and T cell interaction takes place in which the TCR and a CD40 ligand bind to the corresponding MHCII and CD40 receptor, respectively, on the B cell. This interaction also takes place in an immunological synapse, similar to the one between a DC and a T cell [10]. In addition, cytokines expressed by T helper cells and other surrounding cells will enhance the B cell response. It has been recently discovered that signal one and two is not sufficient to drive the B cell into isotype switching and hypermutation. A third signal is needed to perpetuate the process of B cell maturation into antibody-secreting plasma cells. This signal is provided by moieties on the foreign protein which are recognized by Toll like receptors. Activation of Toll like receptors finalizes the maturation of the B cell [11]. Eventually, antigen-specific and high-affinity antibodies are produced to clear the antigen from the system and a memory is formed to prevent further invasion of the antigen [12]. The significance of these signals with regard to

immunogenicity of human therapeutic proteins will be elaborated in more detail in the following paragraphs.

(a)Thymus-independent B-cell activation

Non-proteins, such as polysaccharides and lipids, activate B cells in the absence of Th cells and are thus considered T-cell- or thymus-independent (TI) antigens. TI antigens are categorized into TI-1 and TI-2 antigens based on their immunogenicity in mouse strains possessing an X-linked defect in B cell function. TI-1 antigens such as bacterial lipopolysaccharide are still immunogenic in these mice and lead to both non-antigen and antigen specific antibodies. TD and TI-2 cannot elicit any immune response. TI-1 antigens have no need for a second signal, whereas TI-2 antigens depend on it. Surface polysaccharides on encapsulated bacteria, such as *Streptococcus pneumoniae*, are typical TI-2 antigens. They can cross-link BCRs due to their repetitive manner and thereby activate downstream phosphorylation cascades and activating B cells [13]. A highly organized antigen such as the repetitive structure of polysaccharides is recognized as been foreign by the immune system.
One of the hallmarks of the activation of B cells via TI antigens is the predominant production of low affinity IgM together with little isotype switching to IgG (Table 1), and most TI-antigens do not induce formation of memory. However, some isotype switching and long-lasting protection takes place in the absence of T cells, although the underlying mechanism is not yet fully known [14-15]. Several mechanisms have been proposed to explain how TI antigens, mainly of type 2, can manipulate the adaptive immune response in stimulating somatic hypermutation and isotype switching of the B cell. Interferon gamma (IFNγ) is suspected to provide the necessary stimulus to initiate the constant region switch recombination [16]. Although the enhancing effect of IFNγ, GM-CSF and IL-6 on the response against TI antigens could be shown, the cellular sources of those cytokines are still controversially discussed, though natural killer cells are the most likely source [17]. But also the involvement of other cytokines and subsets of immune cells like MZ B cells are speculated to be competent to induce B cell maturation.

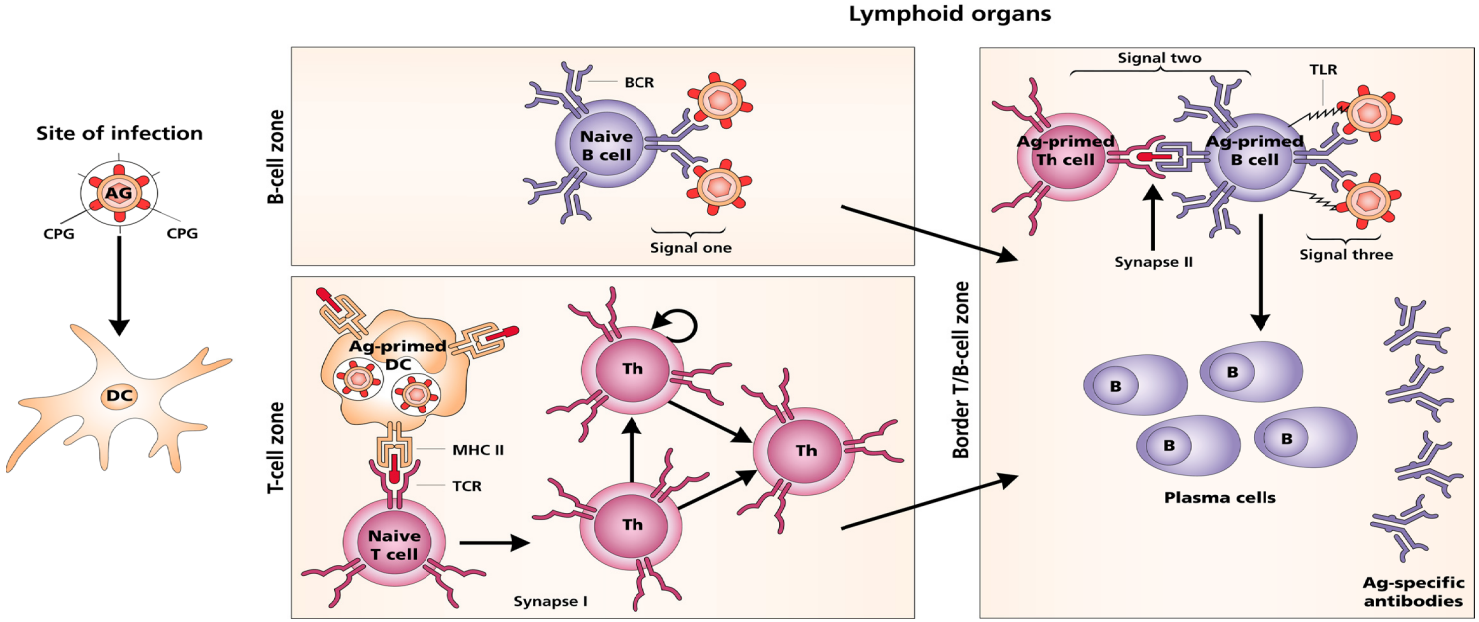

Figure 1. Illustration of the 'classical' immune response after exposure to a T cell independent antigen. The classical immune response starts with uptake of the invading antigen by the dendritic cell.. Activated dendritic cells migrate to the T-cell zone of the draining lymph node. Presentation of antigenic peptides via their MHC II molecules encounters the dendritic cells to activate naïve T cells expressing antigen-specific T-cell receptor and thereby initiating synapse I (Synapse I). B cells also have the capacity to recognize and uptake antigen via their B-cell receptor, being the first signal (Signal one) of B cell activation. After clonal expansion of the antigen-primed T helper cell, T helper cells migrate to the border of the T/B-cell zone where they encounter with antigen-primed B cells to form synapse II (Synapse II) and delivering signal two (Signal two) in the process of B cell activation. In parallel, Toll-like receptor moieties of the antigen like CpG is the final signal (Signal three) to initiate maturation and hypermutation of the antigen-specific B cell into an Ag-specific antibody-secreting plasma cell.
Abbreviations: Antigen (Ag); T helper (Th) cell; Toll like receptor (TLR)

Antigens can be divided into TD and TI. They both activate and mature B cells to become antibody-secreting plasma cells in different manners. In aspect to formation of ADAs one can speculate that rhu proteins can be seen as a TD or/and TI antigens and thus eliciting an immune response as seen in the following case studies.

Table 1 Hallmarks of TD versus TI immune reactions

Characteristics	TD	TI	References
No. of signals required for full response	3	1-2	[11, 18-19]
T cell help required	yes	no	[11, 16]
Isotype switching	yes	little : IgG2a, IgG1, IgG3	[17, 20]
Source of Ab production	[a]FO B cells	MZ B cells	[21]
Localization	B-cell follicles, circulating in blood	marginal zone of spleen & lymph nodes, circulating in the blood	[22]
Antibody affinity	high	low to intermediate	[23]
Cytokines influencing CRS	IL-4 (IgE), IFNγ(IgG), TGF-β & IL-5 (IgA)	IFNγ, GM-CSF, B cell activating factor	[16, 24]
Main source of cytokines	T helper cells	natural killer cells, γδ T cells, Macrophages, DCs	[16-17, 25]
Memory	yes	yes, but only in some cases, no general mechanism	[12, 15, 26]

[a]Abbreviations used in this table: Follicular (FO); Marginal Zone (MZ); granulocyte-macrophage colony-stimulating factor (GM-CSF); constant region switch recombination (CRS)

Case studies supporting the formation of ADAs against rhu proteins

There are many well documented cases of immunogenicity of rhu therapeutic products. A few case studies will be presented.

Rhu erythropoietin

Rhu erythropoietin (epoetin) is used as an erythropoiesis-stimulating agent, and for many years it was considered to be extremely well tolerated. There were, however, rare reports of the development of pure red cell aplasia (PRCA) exclusively among patients with chronic renal failure [27]. The immunological form of PRCA was mediated by circulating anti-epoetin antibodies. The severity of this disease manifested itself through neutralizing not only the drug but also by binding to endogenous erythropoietin, leading to severe side-effects [3]. From 1999 to 2002, there was an increase in antibody-mediated PRCA in Europe and Canada, which was related to the replacement of human serum albumin by polysorbate 80 as a stabilizer in 1998. This correlated with the fact that PRCA occurred in patients treated with epoetin-α (Eprex®; manufactured by Johnson & Johnson), where the formulation change from human serum albumin to polysorbate 80 had taken place to fulfill new European recommendations. Canada received epoetin from Switzerland. The upsurge of PRCA incidences only occurred in Europe and Canada, whereas in the US the number of PRCA patients remained the same [28]. Several explanations were presented to explain the sudden accumulation of antibody-mediated PRCA. One 'danger signal' under investigation with the potency to evoke an immune response were leachates in the formulation from the rubber syringe [29], although the theory had strong opponents. Nevertheless, the mystery of the Eprex-associated PRCA still fuels today's research, and a recent publication picked up on the leachates theory and used a DC-based assay to investigate the possible immuno-stimulatory potency of these leachates [30]; however, no correlation between leachates and activation of human DCs could be shown.
The leachate opponents presented another theory to explain the sudden high immunogenicity of the Eprex formulation. They claimed that the high concentration of polysorbate 80 leads to formation of micelles to which the

epoetin molecules associate. This might have led to protein aggregation and subsequently to a breakage of tolerance (see below) [31-32].

Insulin

Experience with insulin in patients with diabetes mellitus as a therapeutic exists for more than 80 years. The first therapeutics used in the 1920s were purified from porcine and bovine pancreas and immunogenicity was expected due to their non-human origin. Besides the source, purification methods were poor and also contributed to the fact that all patients developed anti-insulin antibodies. But insulin production has changed markedly with regard to source and purity, thus today's insulin preparations are almost all rhu. Nevertheless, rhu insulin also raises anti-insulin antibodies in patients. But, as long as there is no consensus on the assay used for detecting insulin antibodies, no precise percentage of patients developing ADAs against rhu insulin can be obtained.
Although rhu insulin is known to be a safe drug to use, ADAs including subclasses of Ig are observed in up to 50% of diabetic patients treated with insulin with controversial views about their clinical impact [33-34].
Moreover, the presence of insulin autoantibodies in diabetes Type I patients hampers interpretation of clinical data, though it could be shown that insulin autoantibodies and insulin antibodies (ADAs) differ in their binding characteristics and therefore it is possible to distinguish between them [35].
A lot of theories circulate with regard to why so many patients develop ADAs against insulin. One theory involves the high tendency of insulin to self-associate and form higher organized species (which is the very basis for long-acting insulin formulations) such as aggregates that contribute to its high immunogenicity [36].

Interferon beta (IFNbeta)

Rhu IFNbeta is the most common prescribed anti-inflammatory drug to treat relapsing-remitting multiple sclerosis. Despite its promising effects, it has been seen that many patients do not respond to this treatment [37]. This may in part be due to the production of neutralizing antibodies (nAbs) against IFNbeta, though there is a profound and conflicting discussion ongoing about

the significance of these nAbs on the efficacy of the treatment with rhu IFNbeta [38].
IFNbeta-1a (Avonex®, Rebif®) and IFNbeta-1b (Betaseron®) products on the market differ in source of production, glycosylation pattern, amino acid sequence and level of aggregation [39]. In addition to protein structure, all formulations are administered via different routes, follow different dosage and frequency regimes and can thereby influence the immunogenicity of the rhu therapeutic.
Interestingly, some patients who are positive for nAbs and are continuously treated with IFNbeta show spontaneous disappearance of the nAbs [2]. This is an indication that production of nAbs does not subsequently lead to the formation of memory and thus the involvement of a classical immune response is questionable. Besides that, there is an increasing interest to investigate and characterize the formation of aggregates in IFNbeta formulations and their potency to elicit an immune response in the patient by breaking immune tolerance [40]. It has been observed that more multiple sclerosis patients receiving IFNbeta-1b, as compared with those receiving IFNbeta-1a [41], develop nAbs, an observation that is most probably correlated to the levels of aggregates [40]. IFNbeta-1b has a higher degree of aggregation than IFNbeta-1a. The reason for the high tendency of IFNbeta-1b to cluster together might be the lack of glycosylation that normally keeps the protein in solution [42].
On one hand these case studies show that formation of ADAs is a well known phenomenon, on the other hand that the cause of formation is less well understood. Nevertheless, aggregation of the rhu therapeutic is considered to be contributing to immunogenicity in all cases and thereby making it worth having a closer look at.

Table 2 Factors influencing immunogenicity

Protein-dependent factors

- Primary sequence, Glycosylation [43]

Treatment-dependent factors

- Dose, Route of Administration, Duration of treatment [44]

Patient-dependent factors

- HLA allele [45], Genetic defects [4]

Product-dependent factors

- Compounds, Mechanical processing, Packaging, Storage, Handling, Impurities/Contaminants, Formulation → *Aggregates* [46]

Breakage of tolerance against rhu protein therapeutics

Can aggregation be the key?

Many product-related factors are considered to contribute to the immunogenicity of human therapeutic proteins, including primary structure, impurities (in particular protein aggregates), and protein modification such as PEGylation [1, 43]. Clinical data suggests that aggregation is a major contributor to the immunogenicity of rhu therapeutics [47] (Table 2). Furthermore, studies conducted in animal models immune tolerant for the human protein have provided convincing results that aggregates indeed enhance the immunogenicity of a therapeutic human protein [48] by breaking B-cell tolerance, thereby supporting the clinical observations. So, how can it be that aggregates break the tolerance and thereby raise the immunogenicity of a therapeutic protein?
It has been known for a long time that aggregated proteins are more competent in stimulating the immune system as compared with their monomeric forms [47]. The physical-chemical properties of proteins make them prone to aggregation, and all protein formulations contain low levels of aggregates [49-50]. Aggregates present multiple and repetitive epitopes of the therapeutic protein, and thereby mimic TI-2 antigens. The immune

system is likely to confuse the aggregated therapeutic self-protein with a foreign, bacterial TI-2 antigen and could in theory produce antibodies in a TI matter.
However, not every antigen carrying repetitive epitopes induces efficiently antibody production. Many additional factors influence the immunogenic properties of aggregates such as molecular weight, spacing of the repetitive epitopes, rigidity of the presented epitopes, minimum number of cross-linked BCRs and kinetics of the antigen binding to the receptors [19, 51]. This large variety of factors explains in part the diversity of immune responses against therapeutic proteins. Rhu therapeutics can form many different types of aggregate, e.g. non-covalent arrays of native like or denaturated proteins and arrays of covalently bound native-like or denaturated proteins [47]. In addition to BCR cross-linking by aggregates and activation of downstream cascades leading to B-cell proliferation, a second signal is still needed to induce isotype switching and further maturation of the B cell [19]. Rhu therapeutics can induce antibodies other than IgM [36, 52] and thus they also need to present a second signal in order to facilitate the switching of the B cell to other isotypes than IgM.

Human marginal zone B cells: a possible source of ADAs?

Humoral immunity relies on B cells as the source of antibody production. In response to TD antigens, 'conventional' B cells (also called follicular (FO) B cells or B-2 cells) were, until recently, the only properly defined and investigated B cell population. In the case of TI immune events, conventional B cells seemed to be an inappropriate population to investigate, and therefore in recent years more and more interest grew in human MZ B cells. For many years, human MZ B cells were misleadingly called IgM memory B cells. But experiments have shown that they are a separate lineage from the switched memory B cell created via a TD immune response, and they are indeed circulating splenic MZ B cells [53]. Although human MZ B cells carry somatic hypermutations in their BCR, they are created independently of germinal center reactions and most probably already undergo hypermutation during fetal development in contrast to switched memory B cells [54]. MZ B cells are the main effector cells in responses to TI antigens, but can also participate in TD immune reactions. Interestingly, when encountering a very immunogenic

TI antigen MZ B cells can become IgG-secreting plasma cells [26]. By which mechanism needs to be further explored.
With regard to human therapeutics, it is very likely that MZ B cells are the first cells to interact with administered therapeutics due to their strategic location in the marginal zone of the spleen and lymph nodes. Figure 2 schematically illustrates the possible interactions of therapeutic aggregates with MZ B cells and production of ADAs.
One can assume that MZ B cells will respond to aggregated therapeutics by raid production of IgM and in some cases might switch to IgG-secreting plasma cells, accounting for the neutralizing antibodies seen in some patients. The second signal needed to switch is probably provided by the B-cell-activating factor. This factor has recently been under investigation for having a profound impact on B-cell development and favoring the survival of B-cell subsets, such as the MZ B cells [55-56].

Conclusion: The 'waters' of immunogenicity of rhu protein therapeutics run deep and need further exploration – animal models as appropriate models

Immunogenicity of therapeutic proteins is an increasing problem with sometimes serious clinical consequences and safety concerns. The rejection of human homologues has challenged the widely accepted dogma that the immune system can distinguish precisely between foreign and non-foreign proteins. Other theories, such as the danger theory, need to be further explored. So far, much information and insight have been obtained by using mouse models. Because breakage of tolerance seems to be a very important mechanism by which ADAs are produced, animals have been genetically modified to be immune tolerant for the human therapeutic of interest by introducing the human gene into their genome. For examples, human interferon alpha and IFNbeta transgenic mice have been created to investigate the influence of aggregated protein to break immune tolerance in these animals [40, 48]. Aggregated rhu therapeutics are capable of breaking the tolerance in these animals, thus proofing a role of aggregation in immunogenicity.

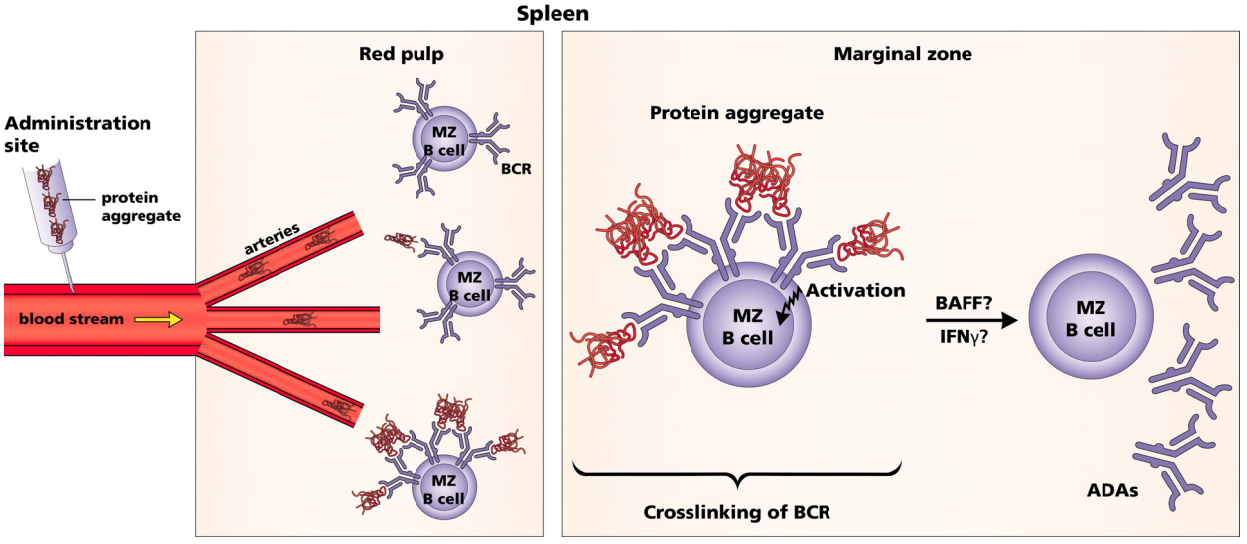

Figure 2. Illustration of the immune process activated after administration of aggregated recombinant human protein therapeutics. After administration the aggregated recombinant human therapeutic is transported via the blood stream to the spleen. The splenic arteries open up into the red pulp, a zone in very close proximity of the marginal zone. Marginal zone B cells come in contact with the aggregated therapeutic leading to cross-linking of their B cell receptor due to the repetitive epitopes of the aggregates. Signaling pathways in the marginal zone B cell are activated and production of anti-drug antibodies is initiated. By which mechanism the marginal zone B cells undergo isotype switching into IgG secreting plasma cells is still under investigation. It is speculated that the cytokines B cell activating factor and interferon gamma are involved.
Abbreviation: B cell activating factor (BAFF)

Although aggregation of therapeutic proteins raises their immunogenic potency, there are many unanswered questions in the field of immunogenicity, and even the very basic questions have not been answered as yet (Box 1). The lack of standardized assays also hampers the collection and interpretation of data. Regardless of the unanswered questions about the nature of immunogenicity of human homologues, many attempts have been made to decrease immunogenicity, e.g by PEGylation or elimination of T-cell/B-cell epitopes [57]. Identifying the major contributors to the immunogenicity of human therapeutics will aid our understanding of the mechanism by which immune tolerance is broken. By reducing the immunogenicity of human therapeutics in regards to aggregates, patients will benefit from safer and more efficient drugs.

Unanswered questions

• Which immune cells play a major role in a breakage of tolerance against human therapeutics? Conventional B cells, MZ B cells or a not yet identified population?

• Is the breakage of tolerance against human protein therapeutics T-cell independent?

• If so, which components of the immune system provide the necessary signals for isotype switching: IFNγ (from which source then - NK cells?)?, B cell activating factor?

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Chapter 2

Aggregated recombinant human interferon beta induces antibodies but no memory in immune tolerant transgenic mice

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Abstract

Purpose. To study the influence of protein aggregation on the immunogenicity of recombinant human interferon beta (rhIFNβ) in wildtype mice and transgenic, immune tolerant mice, and to evaluate the induction of immunological memory.
Methods. rhIFNβ-1b and three rhIFNβ-1a preparations with different aggregate levels were injected intraperitoneally in mice 15x during 3 weeks, and the mice were rechallenged with rhIFNβ-1a. The formation of binding (BABs) and neutralizing antibodies (NABs) was monitored.
Results. Bulk rhIFNβ-1a contained large, mainly non-covalent aggregates and stressed rhIFNβ-1a mainly covalent, homogeneous (ca. 100 nm) aggregates. Reformulated rhIFNβ-1a was essentially aggregate-free. All products induced BABs and NABs in wildtype mice. Immunogenicity in the transgenic mice was product dependent. RhIFNβ-1b showed the highest and reformulated rhIFNβ-1a the lowest immunogenicity. In contrast with wildtype mice, transgenic mice did not show NABs, nor did they respond to the rechallenge.
Conclusions. The immunogenicity of the products in transgenic mice, unlike in wildtype mice, varied. In the transgenic mice neither NABs nor immunological memory developed. The immunogenicity of rhIFNβ in a model reflecting the human immune system depends on the presence and the characteristics of aggregates.

Keywords: Antibodies; Immunogenicity; Immunological memory; Protein aggregates; Recombinant human interferon beta

Introduction

The ability of biopharmaceuticals to elicit an undesirable immune response in patients is a major concern. Despite the development of recombinant therapeutic homologues to human proteins, antibodies are still frequently observed in patients[1-2]. In general, antibodies against recombinant human therapeutic products only appear after prolonged treatment. They can have an effect on the clearance of the drug, decrease its therapeutic efficacy, or may lead to immune complex related diseases such as anaphylaxis and serum sickness [3]. Occasionally antibodies cross-react with the endogenous homologous protein leading to severe clinical consequences, e.g. in the case of epoetin [4]. An example of a therapeutic protein with high clinical immunogenicity is recombinant human interferon beta-1b (rhIFNβ-1b).
The rhIFNβ's are considered first-line disease-modifying therapies of relapsing-remitting forms of multiple sclerosis. They reduce relapse rates and brain lesions. A substantial proportion of relapsing-remitting multiple sclerosis (RR-MS) patients shows a decline in response over time, which can be attributed to the formation of neutralizing antibodies (NABs). This immunological response generally starts with the appearance of BABs after approximately 9 to 18 months of treatment, followed by NABs [5].
Neutralizing antibodies against rhIFNβ are tested in assays based on the inhibition of a cellular response to human interferon beta.
All four commercial rhIFNβ products, Betaferon/Betaseron® (Schering, Berlin, Germany and Berlex Laboratories, Montville, New Jersey, USA), Avonex® (Biogen Idec, Cambridge, Massachusetts, USA), Rebif® (Serono, Geneva, Switzerland) and Extavia® (Novartis, Basel, Switzerland), show immunogenicity in patients, but the level of BABs and NABs varies among the products [6]. The products differ with respect to formulation, and route and frequency of administration. Moreover, Avonex® and Rebif® (both rhIFNβ-1a) are glycosylated, produced in CHO cells and have amino acid sequences corresponding to that of natural hIFNβ, whereas Extavia® and Betaferon® (both rhIFNβ-1b) are produced in *E. coli*, are not glycosylated and have a slightly different amino acid sequence (Cys-17 is mutated to Ser-17 and the N-terminal methionine is deleted [7]). These product differences apparently affect immunogenicity. Likely the lower solubility due to the lack of glycosylation results in aggregation causing a high immunogenicity of rhIFNβ-1b [6-7]. Whether the relatively low immunogenicity of rhIFNβ-1a products is also associated with aggregates is unknown.

Structure and formulation of the protein as well as degree of aggregation and aggregate characteristics are generally recognized as important factors influencing the immunogenicity of therapeutic proteins [2, 8-9]. The immunogenicity of recombinant human interferon alpha was related to the level of aggregation [10]. Also clinical data with other therapeutic proteins, such as intravenous immune globulin, human growth hormone and interleukin-2, strongly suggest a direct correlation between aggregate levels and immunogenicity [11].

Transgenic, immune tolerant mouse models are valuable tools to study the influence of product-related factors such as aggregation on immunogenicity [9, 12-13]. Wildtype mice recognize recombinant human proteins as foreign and consequently exhibit a classical immune response. Mice transgenic for a specific human protein are, like humans, immune tolerant for this protein and provide the opportunity to study the factors that break immune tolerance. In addition, these mice enable us to study the immunological mechanism by which the antibodies to therapeutic proteins are induced. A classical immune response against a foreign protein leads to immunological memory, resulting in an enhanced response after rechallenge with that protein [14]. Observations in patients producing antibodies to therapeutic proteins who are retreated after a washout period suggest a low level or even lack of memory response [15-16].

In this work, rhIFNβ-1a samples with different aggregate levels were prepared and characterized. The immunogenicity of these samples was compared with that of rhIFNβ-1b. We tested immunogenicity by measuring BAB and NAB levels after repetitive administration and a rechallenge with rhIFNβ-1a in our hybrid hIFNβ transgenic, immune tolerant mouse model [17]. The aims of this study were to investigate the influence of aggregation on the immunogenicity of rhIFNβ-1a and to evaluate the formation of antibodies and the induction of immunological memory for the protein in wildtype mice and transgenic, immune tolerant mice.

Materials and Methods

RhIFNβ products

Bulk rhIFNβ-1a was supplied by Biogen Idec Inc. (Cambridge, MA, USA) as a 0.27 mg/ml solution in 100 mM sodium phosphate buffer and 200 mM sodium chloride at pH 7.2. Reformulated rhIFNβ-1a was produced by dialysis of bulk rhIFNβ-1a with a 3.5 kDa MWCO Slide-A-Lyzer Cassette (Perbio Science, Etten-Leur, the Netherlands) against a commercially used formulation containing 20 mM sodium acetate buffer, 150 mM L-arginine monohydrochloride and 0.04 mM Tween 20 (Sigma Aldrich, Zwijndrecht, the Netherlands) at pH 4.8 [18], and subsequent filtration through a 0.22 µm polyethersulfone membrane (Millipore, Amsterdam, the Netherlands). The resulting protein concentration was determined by UV absorbance measurements at λ = 280 nm with an extinction coefficient ($E_{0.1\%}^{1cm}$) of 1.5. This value was calculated from the molar mass of rhIFNβ-1a (20,027.78 Da) without carbohydrate chain and its molar extinction coefficient (29,990 L mol^{-1} cm^{-1}) based on amino acid (Trp, Tyr, Phe and disulfide) composition [19]. Biogen Idec Inc. supplied stressed rhIFNβ-1a produced by incubating 1.4 mg/ml monomeric rhIFNβ-1a (< 1% of aggregates) for one hour at pH 2.1 in the presence of 1 M sodium chloride. After incubation, the solution had been neutralized to pH 7.1 and run over a Superose 12 size-exclusion column to isolate soluble aggregates. This resulted in 0.109 mg/ml stressed rhIFNβ-1a in a buffer of 8 mM dibasic sodium phosphate, 1.5 mM monobasic potassium phosphate, 137 mM sodium chloride and 2.7 mM potassium chloride at pH 7.1. Betaferon® (Schering, Berlin, Germany) was obtained from local hospitals and contained 0.25 mg/ml rhIFNβ-1b with human serum albumin (HSA), mannitol and sodium chloride after reconstitution of the lyophilized powder according to the manufacturer's instructions.

Characterization of rhIFNβ-1a structural variants

Visual inspection

Samples were inspected visually at the lab bench against a black background and compared with water as a control.

UV spectroscopy

UV spectra (λ = 190 – 1100 nm) of the samples were recorded at 25°C on an Agilent 8453 UV/VIS spectrophotometer in quartz cuvettes with a path length of 1 cm. Samples were diluted with the corresponding buffer to a concentration of 100 µg/ml rhIFNβ-1a and measured in the presence or absence of 0.01% (w/v) sodium dodecyl sulfate (SDS). The corresponding sample buffer was used as a blank.

Dynamic light scattering (DLS)

Samples were analyzed with dynamic light scattering (DLS) to obtain an average diameter of the particles (Z-ave) and their polydispersity index (PDI). A Malvern Zetasizer Nano ZS apparatus equipped with a red laser (λ = 633 nm), a detector at 173° and Dispersion Technology Software version 4.20 was used. Samples were diluted with the corresponding buffer to a concentration of 100 µg/ml rhIFNβ-1a and measured in the presence or absence of 0.01% (w/v) SDS.

Flow microscopy

Particulate matter in 50 µg/ml rhIFNβ-1a samples and the corresponding buffers was measured with a Micro-Flow Imaging instrument type DPA4100 (Brightwell Technologies, Inc., Canada). A high magnification setting allowed the detection of particles in the range of 0.75 to 70 µm with an analysis field depth of 100 µm. Prior to each run, Milli-Q water filtered through a 0.22 µm filter was flushed through the system to provide a clean background and to

optimize illumination. To equilibrate the system, 0.2 ml of sample was dispensed before analysis. Samples were drawn from a 1 ml pipette tip at a flow rate of 100 µL/min using a peristaltic pump and analyzed for 5 minutes. Particle size was measured as the equivalent circular diameter (ECD) representing the diameter of a circle occupying the same projection area as the particle. Aspect ratios (the ratio of the longest dimension to the perpendicular dimension at the midpoint) were derived from 200 images stored during one run.

Fluorescence spectroscopy

Fluorescence emission spectra of 50 µg/ml samples were measured at 25 °C from 310 to 410 nm with 1 nm steps in quartz cuvettes with a path length of 1 cm while stirring. An Edinburgh Instruments Steady State FS 920 fluorimeter was used. Samples were excited at 295 nm and slits were set at 3 nm. Dwell time per data point was 0.5 s and the sum of three scans was taken. The corresponding buffer spectra were subtracted. Emission maxima were determined with the FS 900 fluorescence spectrometer software.

High performance size exclusion chromatography (HP-SEC)

Samples (100 µg/ml) were analyzed with a TSKgel Super SW2000 column and Super SW guard column (Sigma Aldrich) and chromatograms were recorded with a Shimadzu SPD-6AV UV detector. A Waters 515 HPLC pump and 717 Plus autosampler were operated at a flow rate of 0.35 ml/min. The mobile phase consisted of 100 mM sodium phosphate buffer, 200 mM sodium chloride, 0.05% (w/v) sodium azide and 0.1% (w/v) SDS at a pH of 7.2 and was filtered through a 0.2 µm filter prior to use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Pre-cast gels (Ready Gels, Tris-HCl, linear gradient 4-20%, Biorad, Veenendaal, the Netherlands) were run under non-reducing and reducing (sample buffer containing 5% (v/v) β-mercaptoethanol) conditions at 200 V at room temperature. Samples analyzed under reducing conditions were

heated at 99°C for five minutes before applying to the gel. A volume of 10 µl of undiluted sample with 10 µl of sample buffer was applied to each well. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Biorad Mini-Protean 3 module. The electrophoresis buffer was 25 mM tris (hydroxymethyl) aminomethane, 192 mM glycine and 0.1% (w/v) SDS. Pre-stained broad range molecular weight markers (Biorad) were included for molecular weight determination and a Silver Stain Plus kit (Biorad) was used to visualize the protein bands. The gels were scanned with a Biorad GS-800 densitometer and Quantity One software.

Western blotting

SDS-PAGE gels were blotted onto a nitrocellulose sheet (VWR International, Amsterdam, the Netherlands) with a Biorad Mini Trans-Blot electrophoretic transfer cell and a transfer buffer containing 10 mM sodium hydrogen carbonate, 3 mM sodium carbonate, 20% (v/v) methanol and 0.1% SDS (w/v) at pH 10.0. Blots were blocked overnight at 4°C with 8% (w/v) non-fat milk powder (ELK, Campina Melkunie, Eindhoven, the Netherlands) in 0.005% (w/v) Tween 20 in phosphate-buffered saline (PBS, consisting of 3.6 mM KH_2PO_4, 6.4 mM Na_2HPO_4 and 145 mM NaCl at pH 7.2) with constant orbital shaking. After washing with 0.005% (w/v) Tween 20 in PBS, the blots were incubated with 0.2 µg/ml polyclonal rabbit anti-rhIFNβ antibody (Acris Antibodies, Hiddenhausen, Germany) in 0.1% (w/v) non-fat milk powder and 0.005% (w/v) Tween 20 in PBS for one hour at room temperature with constant orbital shaking. Blots were washed with 0.005% (w/v) Tween 20 in PBS. Blots were incubated with peroxidase labeled goat anti-rabbit immunoglobulin G (IgG) (Sigma Aldrich), diluted 1000-fold in PBS containing 0.1% (w/v) non-fat milk powder and 0.005% (w/v) Tween 20, for one hour at room temperature with constant orbital shaking. Blots were washed with 0.005% (w/v) Tween 20 in PBS and incubated in a solution of 0.05% (w/v) 4-chloro-1-naphtol (Sigma-Aldrich) in 17% (v/v) methanol and 0.0125% (v/v) H_2O_2. After color development the blots were stored overnight in the dark in water to increase the intensity of the bands.

Immunogenicity study

Mouse breeding

Heterozygous C57Bl/6 transgenic mice immune tolerant for hIFNβ developed by Hermeling et al. [20] were bred at the Central Laboratory Animal Institute (Utrecht University, the Netherlands). The strain was maintained by crossing the transgenics with wildtype C57Bl/6 mice obtained from Janvier (Bioservices, Uden, the Netherlands). The genotype of the offspring was determined by PCR showing the presence or absence of the hIFNβ gene in chromosomal DNA isolated from ear tissue. Transgenic C57Bl/6 mice were crossed with wildtype FVB/N mice obtained from Janvier (BioServices) and their C57Bl/6 x FVB/N hybrid offspring were genotyped using PCR. Both transgenic C57Bl/6 x FVB/N hybrid mice and their non-transgenic (wildtype) littermates, evaluated previously as a mouse model for human interferon beta [17], were used.

Animal experiment

The animal experiments were approved by the Institutional Ethical Committee. Food (Hope Farms, Woerden, the Netherlands) and water (acidified) were available *ad libitum*. Blood was drawn from the cheek pouches (submandibularly) of 32 wildtype and 32 transgenic mice before starting the treatment [17, 20]. Eight mice per group were injected intraperitoneally (i.p.) with 5 μg of bulk, reformulated or stressed rhIFNβ-1a, or 5 μg of Betaferon®-rhIFNβ-1b on days 0 to 4, days 7 to 11 and days 14 to 18. After an injection-free period of 6 weeks, all mice were rechallenged with 5 μg of reformulated rhIFNβ-1a i.p. on days 63 and 64. Blood was collected submandibularly from two out of eight mice per group per time point, just before treatment with rhIFNβ, on days 4, 7, 11, 14, 18, 21, 28, 43, 53, 56, 58, 60, 64, 65, 66 and 67. If administered rhIFNβ is not completely cleared from the circulation before blood drawing, remaining rhIFNβ levels could interfere with the BAB assay. Therefore, we performed an additional study following rhIFNβ blood levels in time with ELISA after a single i.p. injection of 5 μg of Betaferon®-rhIFNβ-1b or bulk rhIFNβ-1a in wildtype hybrid mice. From this study we estimated that the half-lives of rhIFNβ-1b and rhIFNβ-1a in the

mice were 2.5 and 4.5 hours, respectively. Since the time interval between the previous dose and the blood sampling on days 4, 11, 18, 64 and 65 was relatively short (i.e. 20 to 24 hours), low levels of rhIFNβ-1a may have remained in the plasma samples of these days. For the other blood sampling days and for rhIFNβ-1b, most likely sufficient time passed after the previous dose to allow for clearance of the protein from the circulation. On day 77, all mice were sacrificed by bleeding through cardiac punction under isofluran anesthesia. Blood samples were collected in lithium heparin gel tubes and centrifuged for 10 min at 3000 g, and the obtained plasma was stored at –80 °C until analysis.

Binding antibody assay

Titers of BABs against rhIFNβ were measured in the plasma by direct ELISA according to the protocol described in detail by Hermeling et al. [20] with minor changes. Plates were coated with bulk rhIFNβ-1a and blocked with 4% milk powder and 0.1% Tween 20 in PBS at room temperature for two hours. Plasma samples and the secondary antibody (horseradish peroxidase coupled anti-mouse IgG from Invitrogen, Zymed) were diluted in the blocking buffer described above in a ratio of 1:100 and 1:4000, respectively. Color conversion was initiated by adding 100 µl of 3,3',5,5'-tetramethylbenzidine (Roche) and stopped by adding 100 µl of 0.18 M sulfuric acid. Absorbance values were measured with an immuno plate reader (Novopath, Biorad) at a wavelength of 450 nm. The 100-fold diluted plasma samples were screened and defined positive if their mean absorbance values were at least three times higher than the 95th percentile value of negative control plasma. The titer of anti-hIFN□ IgG in positive plasma was determined by plotting the absorbance values of a 2-fold serial dilution against log dilution. The plots were fitted to a sigmoidal dose-response curve using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego CA, USA). The reciprocal of the dilution of the EC50 value was defined as the BAB titer.

Neutralizing antibody assay

NAB levels in the plasma samples of day 77 were assessed in a bioassay based on inhibition of induction of myxovirus resistance protein A (MxA) gene

expression in an A549 cell line as previously described [21]. The type of rhIFNβ (-1a or -1b) used in the assay was the same as the type used for injecting the animal. Both MxA and a control household gene derived mRNA (eukaryotic 18S rRNA) were detected with a real time RT-PCR multiplex assay. Neutralizing activity was expressed in ten-fold reduction units per ml (TRU/ml). Plasma samples with a neutralizing activity below 130 TRU/ml were considered negative. Positive samples showed neutralizing activities ranging from 1143 TRU/ml to more than 5120 TRU/ml.

Results and Summary

Characterization of rhIFNβ-1a structural variants

Visual inspection

The three rhIFNβ-1a solutions were colorless and transparent, and visual inspection did no reveal visible aggregation or precipitation.

UV spectroscopy

All UV spectra showed broad absorbance peaks around the wavelength of 280 nm indicating tryptophan, tyrosine or phenylalanine residues or disulfide bonds in the rhIFNβ-1a samples (Figure 1A) [22-23]. Protein aggregates cause scattering of light that can be observed as an increase in optical density (OD) [23]. The wavelength-dependent light scattering intensity is influenced by several factors such as aggregate size, shape and amount. As a measure for aggregation the OD at 350 nm (OD350) and the ratio between the OD at 280 nm and 260 nm (OD280/OD260) were used (Table I). As compared with reformulated rhIFNβ-1a (OD350 = 0.02; OD280/OD260 = 1.67), bulk and stressed rhIFNβ-1a showed a high OD350 (0.11 and 0.08, respectively) and a low OD280/OD260 (1.12 and 1.01), indicating the presence of aggregates (Table I). Light scattering at high wavelengths was most pronounced in bulk rhIFNβ-1a, which suggests the presence of large aggregates. Stressed rhIFNβ-1a showed the lowest OD280/OD260, most likely caused by absorption flattening due to extensive aggregation of the sample [23].
Aggregates of rhIFNβ formed by non-covalent protein bonds can be disassembled by SDS [24]. Adding 0.01% SDS changed the UV spectrum of bulk rhIFNβ-1a drastically (Figure 1A). Its OD350 and A280/A260 became similar to those of reformulated rhIFNβ-1a with SDS (Table I), indicating non-covalently bound aggregates. In contrast, the UV spectrum of stressed rhIFNβ-1a changed only slightly after adding SDS, suggesting the presence of a considerable amount of covalent aggregates. The initial OD350 and OD280/OD260 values, together with the marginal decrease in OD350 and

increase in OD280/OD260 following the addition of SDS suggest a low level of non-covalent aggregates in reformulated rhIFNβ-1a.

Dynamic light scattering (DLS)

According to studies on rhIFNβ-1a crystals performed by Karpusas et al., the protein has a cylindrical shape of roughly 2 x 3 x 4 nm [25]. Bulk rhIFNβ-1a showed a large Z-ave (2300 nm) and a large PDI (0.9), indicating that the sample contained aggregates heterogeneous in size (Table I). As the light scattering intensity is proportional to the sixth power of the particle radius, the size average of the protein sample is overestimated [26-27]. The strong light scattering of the aggregates inhibits the detection of the rhIFNβ-1a monomer, which may represent a much larger fraction by weight than the aggregated material [27]. As the size distributions varied considerably between repeated measurements, only Z-ave and PDI results are presented. The Z-ave and PDI of reformulated rhIFNβ-1a could not be determined due to the small size of the monomer, the low protein concentration and light-scattering components in the formulation buffer (i.e. arginine and Tween 20). Stressed rhIFNβ-1a also contained aggregates, which were smaller (95 nm) and more homogeneous (PDI = 0.3) in size than the aggregates in bulk rhIFNβ-1a. In concordance with our observations from UV spectroscopy, the addition of 0.01% SDS resulted in a large decrease in Z-ave (from 2300 to 27 nm) and PDI (from 0.9 to 0.3) of bulk rhIFNβ-1a, reflecting the dissociation of non-covalent protein complexes, whereas the mainly covalently bound aggregates in stressed rhIFNβ-1a stayed intact.

Flow microscopy

Flow microscopy enables the detection of protein particulates larger than 0.75 μm that are difficult to study with more conventional techniques such as UV, DLS, HP-SEC and SDS-PAGE. Reformulated rhIFNβ-1a showed a low particle count that was slightly higher than the particle count of the buffer control, which was 0.32 x 10^3 particles/ml, whereas the particle counts of bulk and stressed rhIFNβ-1a were two orders of magnitude higher (Figure 1B and Table I). Particle contents of bulk and stressed rhIFNβ-1a were in the

same range, and both samples had similar size distributions, mean sizes and mean aspect ratios. Thus, the aggregates larger than 0.75 μm in bulk and stressed rhIFNβ-1a were comparable in size, quantity and shape, while such aggregates were practically absent in reformulated rhIFNβ-1a.

Fluorescence spectroscopy

At a wavelength of 295 nm the tryptophans of rhIFNβ-1a were excited and a typical emission maximum around 350 nm was observed (Figure 1C). The wavelength of the fluorescence peak and its intensity provide information on the environment of the tryptophan at position 22 (Trp22), which is close to the receptor binding site and relatively exposed to the solvent [28-29], and of the tryptophans at positions 79 and 143, which are both inside the hydrophobic core of the protein that is stabilized through several hydrogen bonds and one disulfide bridge [25, 28-29]. The maximum fluorescence intensity of bulk rhIFNβ-1a (at 349 nm) was arbitrarily set at 1 and the fluorescence intensities of the other samples were calculated relative to this value (Table I). The fluorescence emission peak of reformulated rhIFNβ-1a showed a 3 nm red-shift and 14% increase in intensity in comparison with bulk rhIFNβ-1a, which may be attributed to the lower degree of aggregation and more exposed tryptophans [30-31]. For comparison, the spectrum of rhIFNβ-1a unfolded in 6 M guanidine hydrochloride showed a considerably larger red-shift of 8 nm (Figure 1C). Stressed rhIFNβ-1a showed a 4 nm blue-shift and an 8% intensity decrease in the fluorescence emission maximum, indicating a higher degree of aggregation. The same effect was observed previously by Fan et al. during heat induced aggregation of rhIFNβ-1a [32]. Nevertheless, Trp22 in stressed rhIFNβ-1a seems to be more accessible than a rhIFNβ-1a sample that was denatured by heating for 10 min beyond its transition temperature (Figure 1C). Contrary to the heated sample, stressed rhIFNβ-1a probably retained most of its native tertiary structure.

High-performance size-exclusion chromatography (HP-SEC)

Runkel et al. described the use of a TSKgel SW2000 HP-SEC column with a mobile phase of 100 mM sodium phosphate and 200 mM NaCl at pH 7.2 to analyze rhIFNβ-1a and rhIFNβ-1b products with large amounts of human

serum albumin (HSA) [7]. HSA is commonly used in formulations to prevent adsorption of hydrophobic protein products [7, 33-34]. The absence of HSA in our rhIFNβ-1a formulations may explain the low protein recovery of our samples in this HP-SEC procedure, i.e. 42% for bulk, 81% for reformulated and 61% for stressed rhIFNβ-1a (data not shown). Therefore, to inhibit adsorption of the protein to the solid phase and increase resolution, we added 0.1% SDS to our mobile phase [35-36]. This resulted in improved recoveries of 87% for bulk, 93% for reformulated and 71% for stressed rhIFNβ-1a. The SDS in our elution buffer disrupts non-covalently bound protein complexes and it hinders molecular weight calibration with standard proteins, so we numbered the different peaks as Peak 1-4 in order of elution (Figure 1D). Most bulk rhIFNβ-1a eluted in Peak 3 (50%), which is most likely the rhIFNβ-1a monomer (Table I). Another significant amount eluted in Peak 4 (19%), probably consisting of monomeric proteins of which the hydrophobic cores are slightly exposed, causing interactions with the column material. Peak 1 (8%) of the bulk sample eluted in the void volume of the column corresponding to soluble protein aggregates with a molecular weight higher than 150 kDa. The protein fraction in Peak 2 (10%) is probably a dimer, which has been reported before [7, 25]. The non-recovered fraction comprised 13% of the bulk sample and probably contained covalent aggregates too large to enter the column or unfolded protein irreversibly attached to the column material. The reformulated sample contained 83% monomers and its total sample recovery was high (93%). The majority of the stressed sample eluted in the higher molecular weight regions (31% in Peak 1 and 21% in Peak 2) and contained a non-recovered fraction of 29%.

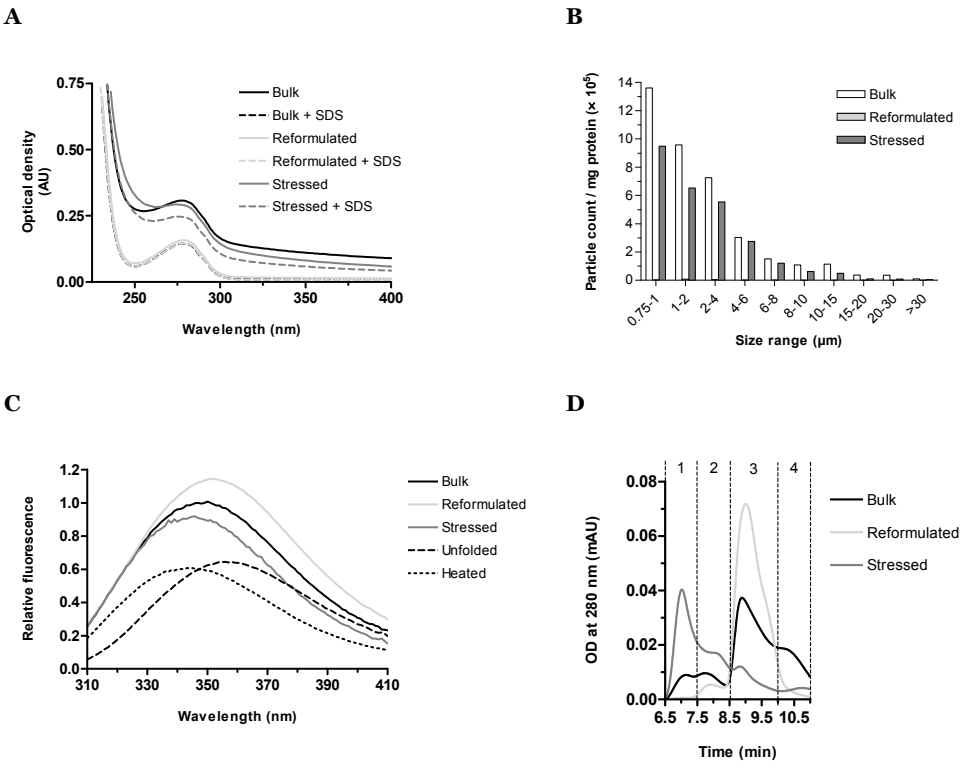


Figure 1. Graphs showing (A) UV spectra in the absence and presence of 0.01% (w/v) SDS, (B) particle distributions in specified size ranges based on flow microscopy analysis (with hardly any particles detected in reformulated rhIFNβ-1a), (C) fluorescence emission spectra (with rhIFNβ-1a unfolded in 6 M guanidine hydrochloride and rhIFNβ-1a heated at 90 °C for 10 min, for comparison), and (D) size-exclusion chromatograms, of bulk, reformulated and stressed rhIFNβ-1a. The HP-SEC peaks (D) are numbered from 1 to 4 (see text for details), with the vertical dashed lines showing the range of each peak.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

We applied denaturing polyacrylamide gel electrophoresis to assess the relative molecular masses of rhIFNβ-1a monomer, fragments, and covalent aggregates [37]. Under non-reducing conditions, bulk rhIFNβ-1a contained

monomer, dimer, trimer and larger aggregates (Figure 2A, lane B). The additional band at approximately 19.5 kDa most likely corresponds with a deglycosylated, monomeric form of rhIFNβ-1a [7, 38]. Under reducing conditions, the monomer and dimer showed a slight increase in apparent mass, which is probably due to the breaking of the intramolecular disulfide bridge of rhIFNβ-1a (Figure 2B, lane B). The decreased intensity of the dimer band observed under these conditions indicates that at least some of the dimers in bulk rhIFNβ-1a were formed through disulfide bonds. The covalent trimers and larger aggregates were non-disulfide mediated.
For reformulated rhIFNβ-1a, non-reducing SDS-PAGE showed monomers and dimers without any trimers or larger aggregates, similar to what was seen by HP-SEC (cf. Figure 1D and Figure 2A, lane R). Reducing SDS-PAGE showed that the dimers in reformulated rhIFNβ-1a were mainly formed through disulfide bridges (Figure 2B, lane R).
Under non-reducing conditions, stressed rhIFNβ-1a was hardly able to enter the gel due to its high percentage of covalent aggregates (Figure 2A, lane S). Reducing SDS-PAGE showed that the aggregates contained reducible bonds, as is clear from the monomers, dimers and trimers observed (Figure 2B, lane S). Non-reducing SDS-PAGE (Figure 2A) confirms the proposed nature of the various peaks observed during HP-SEC (Figure 1D).

Western blotting

The polyclonal anti-rhIFNβ-1a antibodies used for Western blotting reacted with the monomeric bands in all three rhIFNβ-1a products under both non-reducing and reducing conditions, including the barely visible monomeric band of stressed rhIFNβ-1a under non-reducing conditions (Figures 2C and 2D).

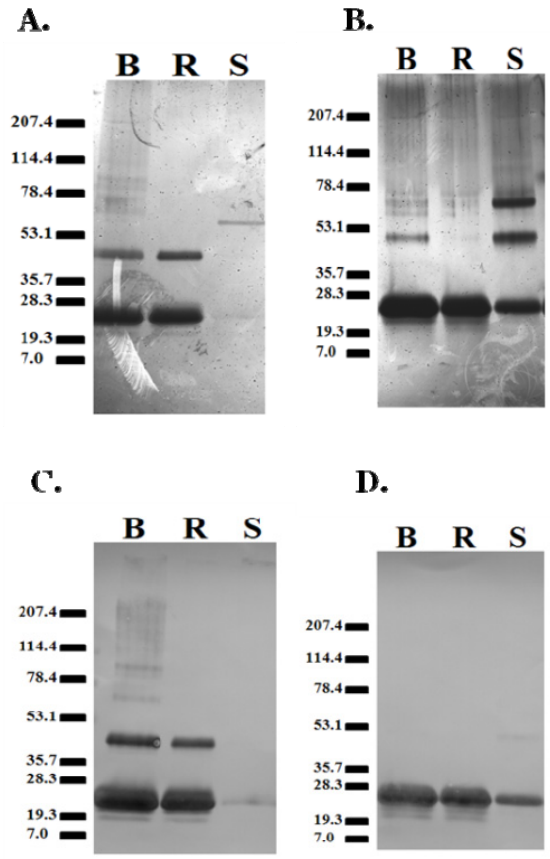


Figure 2. SDS-PAGE gels under (A) non-reducing and (B) reducing conditions and the corresponding Western blots of the gels under (C) non-reducing and (D) reducing conditions of the three structural variants of rhIFNβ-1a. Numbers on the left represent band positions (in kDa) of the molecular weight markers. Lane B = bulk rhIFNβ-1a; R = reformulated rhIFNβ-1a; and S = stressed rhIFNβ-1a.

Also dimers, trimers and larger aggregates in bulk rhIFNβ-1a and dimers in reformulated rhIFNβ-1a were recognized by the antibodies under non-reducing conditions (Figure 2C). In contrast, none of the non-reducible covalent protein
complexes in bulk, reformulated or stressed rhIFNβ-1a were recognized by the antibodies (Figure 2D), suggesting the destruction of specific epitopes upon formation of covalent links between monomers.

Table I. Overview of the physicochemical characteristics of bulk, reformulated and stressed rhIFNβ-1a.

Method	Parameter	Bulk	Reformulated	Stressed
UV	OD280/OD260	1.12	1.67	1.01
	OD280/OD260 with SDS[1]	1.74	1.76	1.06
	OD350 nm	0.11	0.02	0.08
	OD350 nm with SDS[1]	0.01	0.01	0.06
DLS	Z-ave (nm)	2300	ND[2]	95
	Z-ave with SDS[1] (nm)	27	ND[2]	120
	PDI	0.9	ND[2]	0.3
	PDI with SDS[1]	0.3	ND[2]	0.3
Flow microscopy	Mean size (ECD[3]; μm)	3.0	3.3	2.7
	Total particle count (× 10^3 / ml)	190	0.82	134
	Mean aspect ratio (0 - 1)	0.67	0.70	0.68
Fluorescence	Emission maximum (nm)	349	352	345
	Relative peak intensity	1.00	1.14	0.92
HP-SEC[4]	Fraction peak 1 (%)	8	0	31
	Fraction peak 2 (%)	10	6	21
	Fraction peak 3 (%)	50	83	14
	Fraction peak 4 (%)	19	4	5
	Unrecovered fraction (%)	13	7	29

[1] 0.01% (w/v) SDS was added to the rhIFNβ-1a preparations before analysis.

[2] ND: not detectable (see text for details).

[3] ECD: equivalent circular diameter.

[4] Fractions were calculated from the area under the curve (AUC) for each peak and an extinction coefficient ($E_{0.1\%}^{1cm}$) of 1.5 for rhIFNβ-1a (19). Peak numbers correspond with the numbers shown in Figure 1D.

Summary

Bulk rhIFNβ-1a was shown to contain monomeric protein and a low amount of heterodisperse rhIFNβ-1a aggregates with sizes ranging from dimers to aggregates of several micrometers. The aggregates were mainly formed through non-covalent bonds and disulfide linkages, and contained intact epitopes. In addition, some covalent, non-reducible aggregates were present

that were not recognized by the polyclonal antibody used for Western blotting.
Reformulated rhIFNβ-1a showed low aggregate levels, mainly dimers containing intact epitopes.
Stressed rhIFNβ-1a contained a high percentage of rather homogeneously sized covalent aggregates with a size of approximately 100 nm. Importantly, the covalent non-reducible aggregates did not contain detectable native epitopes.

Immunogenicity

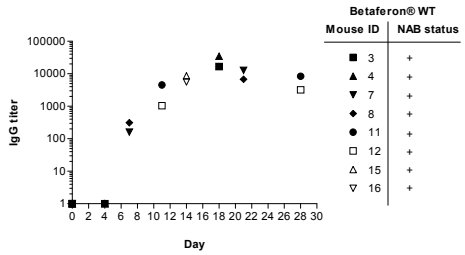
In order to test the immunogenicity of the three rhIFNβ-1a samples, the same schedule of injections was used as before [9-10, 17, 20]. Figure 3 shows the levels of IgG antibodies in wildtype and transgenic mice injected with the three different products. Please note that the presented BAB titers on days 4, 11 and 18 in the mice treated with bulk, reformulated and stressed rhIFNβ-1a might be underestimated due to low levels of rhIFNβ-1a in these samples. In general, the wildtype mice started to produce BABs between day 4 and day 11 and developed high IgG titers against all products. These high BAB levels persisted in the wildtypes. With the exception of two mice that did not respond to reformulated rhIFNβ-1a, the wildtype mice also developed NABs against the products (Figure 3A, C, E, and G). The formation of both BABs and NABs in these animals indicated that the native protein conformation in all preparations was at least partly intact.
Transgenic mice, on the other hand, did not develop NABs against any of the products (Figure 3B, D, F, H). Betaferon®, used as a positive control, broke the immune tolerance of the transgenics but did not induce NAB formation (Figure 3B), in correspondence with previous observations [17, 20]. Three out of seven transgenic mice treated with bulk rhIFNβ-1a showed low IgG titers at a single time point (Figure 3D), indicating a low immunogenicity of this material in transgenic mice. Reformulated rhIFNβ-1a did not break the immune tolerance of any of the transgenic mice (Figure 3F). From the low, transient IgG titers in only two out of six transgenic mice treated with stressed rhIFNβ-1a, it followed that the stressed product was hardly immunogenic (Figure 3H).
Some of the transgenic mice showed a wildtype-like immune response, including NABs and high, persistent IgG titers. Although they possessed the

transgene (as detected by PCR), they were not expressing hIFNβ and lacked immune tolerance, as was discussed in our previous publication describing the hybrid transgenic mouse model [17]. The hIFNβ gene of the transgenic mice is situated behind the promoter of murine IFNβ and its expression can be stimulated upon injection with polyICLC [20]. Without polyICLC injection, hIFNβ expression levels are not measurably different between wildtype and transgenic mice. The mice need to be naïve for immunogenicity testing and therefore cannot be treated with polyICLC beforehand. Instead, we analyzed the results of individual transgenic mice for the presence of both (i) NABs, and (ii) IgG titers exceeding 2,000 up to 8 weeks after the first injection. Based on these two selection criteria, the data of five transgenic mice (with ID numbers 13, 29, 42, 50 and 54) that showed a wildtype-like immune response were left out, as they would distort our conclusions on the ability of the rhIFNβ-1a samples to break immune tolerance. Generally, we observe about 10-20% hybrid hIFNβ transgenic mice in our studies exhibiting a wildtype-like immune response independent of the type of treatment. In the current study, we identified five outliers out of 32 transgenic mice (16%), i.e. 1/8 rhIFNβ-1b, 1/8 bulk rhIFNβ-1a, 1/8 reformulated rhIFNβ-1a and 2/8 stressed rhIFNβ-1a treated transgenic mice. All outliers showed high persistent levels of BABs, and they produced NABs while the other transgenics did not. The equal distribution of the outliers among the treatment groups clearly indicates that the effect is not product-related.

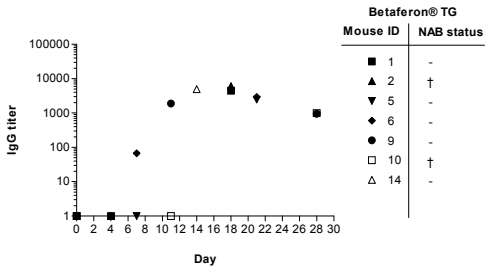
Immunological memory

Before the rechallenge with reformulated rhIFNβ-1a at day 63, wildtype mice showed high IgG titers (Figure 4). After the rechallenge, the wildtype mice showed IgG titers at day 70 that were higher than before the rechallenge, independent of the treatment group (Figure 4A-D). Such an enhanced secondary immune response is characteristic for a T cell dependent immune response typically observed after vaccination with foreign protein [14, 39].

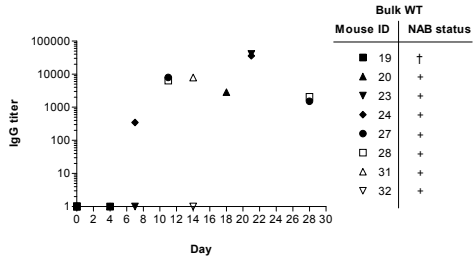
A


Betaferon® WT
Mouse ID
NAB status
3 +
4 +
7 +
8 +
11 +
12 +
15 +
16 +
IgG titer
Day

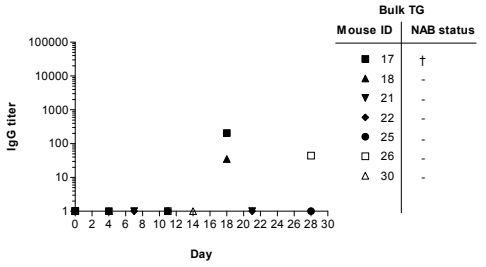
B


Betaferon® TG
Mouse ID
NAB status
1 -
2 †
5 -
6 -
9 -
10 †
14 -
IgG titer
Day

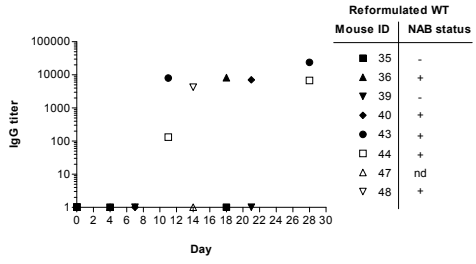
C


Bulk WT
Mouse ID
NAB status
19 †
20 +
23 +
24 +
27 +
28 +
31 +
32 +
IgG titer
Day

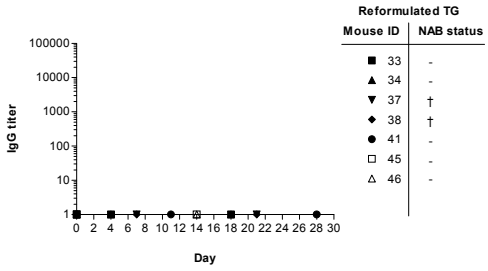
D


Bulk TG
Mouse ID
NAB status
17 †
18 -
21 -
22 -
25 -
26 -
30 -
IgG titer
Day

E


Reformulated WT
Mouse ID
NAB status
35 -
36 +
39 -
40 +
43 +
44 +
47 nd
48 +
IgG titer
Day

F


Reformulated TG
Mouse ID
NAB status
33 -
34 -
37 †
38 †
41 -
45 -
46 -
IgG titer
Day

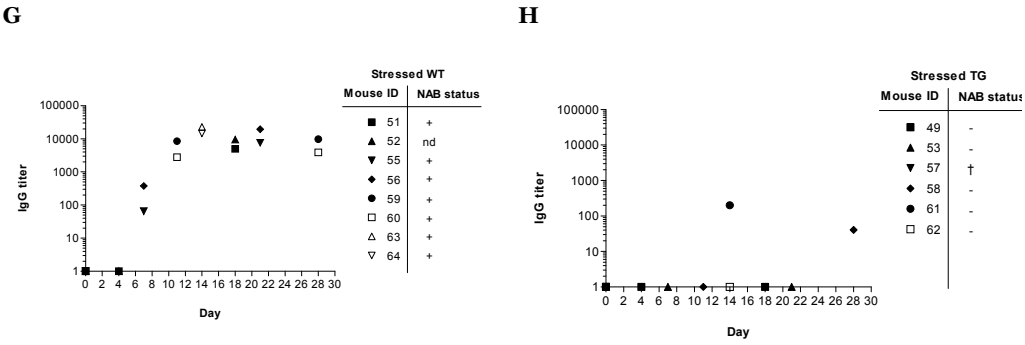


Figure 3. Immunogenicity of (A, B) Betaferon®, (C, D) bulk rhIFNβ-1a, (E, F) reformulated rhIFNβ-1a, and (G, H) stressed rhIFNβ-1a. Titers of total IgG against rhIFNβ in plasma of (A, C, E and G) wildtype (WT) and (B, D, F and H) transgenic (TG) mice injected daily, from Monday to Friday, i.p. with Betaferon® or one of the rhIFNβ-1a preparations for three weeks starting at day 0. Non-responders were given an arbitrary titer of 1. The legend shows the NAB status of the individual mice in the treatment group at day 77, with (+) positive for NABs, (-) negative for NABs, (†) died before day 77, and (nd) not determined. Seven out of 64 mice died during the study due to handling or other reasons, such as liver or heart problems. There were no signs of anaphylactic responses.

Transgenic mice treated with Betaferon®, however, showed a slight decrease in BAB level after the rechallenge with reformulated rhIFNβ-1a, indicating that they had not developed immunological memory for rhIFNβ (Figure 4A). Also bulk, reformulated and stressed rhIFNβ-1a treated transgenics did not show any BABs after the rechallenge, indicating the absence of memory (Figure 4B-D).

These results comply with the lack of antibody response observed in patients who, after a wash-out period, switched to Avonex®-rhIFNβ-1a treatment after having developed high levels of anti-rhIFNβ-1b antibodies following Betaferon® treatment [16]. Despite the cross-reactivity of anti-rhIFNβ antibodies, levels of pre-occurring BABs or NABs in patients did not increase after switching the treatment from Betaferon® to Avonex® [16, 40], from rhIFNβ-1a to high-dose intravenous rhIFNβ-1b [41], and from 1.6 to 8 million international units of rhIFNβ-1b [42], without a wash-out period. Especially patients with low titers may even reconvert to antibody negativity while

treatment continues, independent of the type of rhIFNβ that is administered [40, 43-46]. The
observed lack of immunological memory in immune tolerant mice as well as in RR-MS patients may be characteristic for the breakage of B cell tolerance for recombinant human therapeutic proteins.

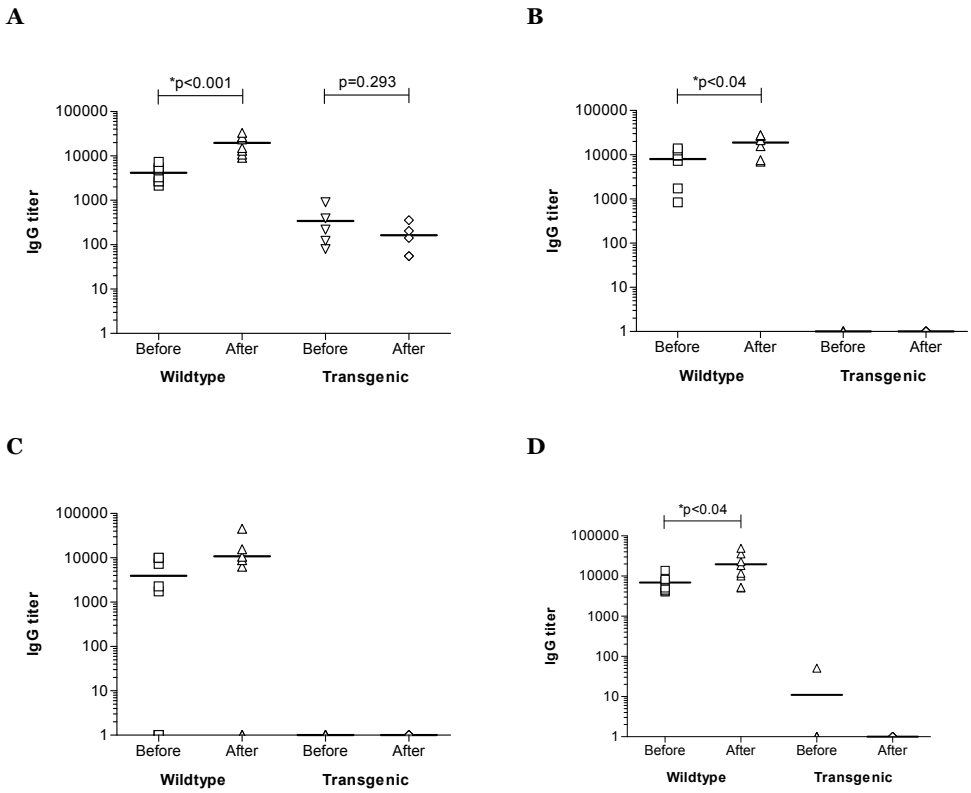


Figure 4. Titers of total IgG against rhIFNβ in individual mice on day 53, 56, 58 or 60 before the rechallenge (before) and at day 77 after the rechallenge (after) with reformulated rhIFNβ-1a in plasma of wildtype (left) and transgenic (right) mice treated with (A) Betaferon®, (B) bulk rhIFNβ-1a, (C) reformulated rhIFNβ-1a, and (D) stressed rhIFNβ-1a. Mean titers and titers of individual mice are shown, and non-responders were given an arbitrary titer of 1. Statistical analyses (unpaired t test, two-tailed) were performed between groups with 100% responders on $\log_{10}$ converted titers. Asterisks indicate that titers are significantly ($p < 0.04$) higher after the rechallenge than before.

Final remarks and conclusions

Bulk rhIFNβ-1a, which contained mainly non-covalently bound aggregates, induced a transient immune response in approximately 40% of the transgenic mice. Filtration of the bulk product reduced the aggregation level and reformulation in another buffer prevented the formation of new aggregates, thereby completely abolishing its potency to break immune tolerance. Despite the high percentage of aggregates in stressed rhIFNβ-1a, only about 30% of the transgenic mice receiving this product showed antibodies against rhIFNβ-1a. This is possibly explained by the absence of native epitopes in the covalent non-reducible aggregates as shown by Western blotting. Preservation of the native structure of the protein is a prerequisite for aggregates to break the tolerance of transgenic, immune tolerant mice (8). In addition to BABs, the wildtype mice formed NABs and immunological memory for the protein after 3-week administration of any of the rhIFNβ-1a samples or Betaferon®. This study confirms that wildtype animals cannot be used to study the immunogenicity of human therapeutic proteins and immune tolerant animal models are needed [47]. In this paper, transgenic mouse models showed that protein aggregates are able to break the immune tolerance for rhIFNβ. The potency of the aggregates to break tolerance not only depends on aggregate percentage but also largely on their physical properties such as degree of denaturation, molecular orientation and size. Moreover, we demonstrated that the breaking of immune tolerance for rhIFNβ in transgenic mice is characterized by the absence of NABs and immunological memory and thereby differs substantially from a classical T cell dependent immune response.

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Chapter 3

Immunological mechanism of antibody induction by human interferon beta aggregates in an immune tolerant mouse model

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Abstract

There are still controversial views on the immunological process leading to formation of anti-drug antibodies. Using a mouse model immune tolerant for human interferon beta, we previously demonstrated that interferon beta aggregates are the major cause of immunogenicity of interferon beta but do not induce an immunological memory upon rechallenge with non-aggregated human interferon beta. Thus, we deduced that the immune response against recombinant human interferon beta differs from a classical T cell dependent immune response. Most foreign antigens, such as viruses, depend upon the presence of CD4+ T cells to induce a proper immune reaction. Depletion of the CD4+ T cells in the transgenic mouse model immune tolerant for human interferon beta revealed that CD4+ T cells are necessary for the generation of anti-interferon beta antibodies, whereas rechallenge experiments with Betaferon®, a recombinant human interferon beta product containing highly levels of aggregates, enforced the previous made observations that B cell memory is not induced. Additionally, the immune response against recombinant human interferon beta was accompanied by the secretion of a slightly different set of cytokines distinct from the set of cytokines induced by the T cell dependent antigen ovalbumin. We conclude that induction of anti-drug antibodies by protein aggregates is facilitated by CD4+ T cells and a distinct cytokine profile but does not give rise to a memory B cell derived antibody response.

Introduction

Therapeutic proteins such as growth factors, hormones and monoclonal antibodies are increasingly being used for treatment of a variety of diseases. Although a considerable number of these drugs are homologues of human proteins, formation of anti-drug antibodies (ADAs) is still frequently observed in patients [1-2]. ADAs can lead to faster drug clearance from the blood, lowering drug efficacy, and formation of drug-immune complexes, which may result in anaphylactic reactions, serum sickness or other drug hypersensitivity reactions [3]. Severe side-effects of ADAs may be experienced by patients when ADAs cross-react with and neutralize the endogenous protein, as seen in the case of recombinant human (rh) epoetin [4-5]. The factors influencing immunogenicity can be divided into patient-dependent (e.g., HLA allele, genetic defects), treatment-dependent (e.g., dose, route of administration, frequency) and protein-product-dependent (e.g., glycosylation, primary sequence, production, purification methods, formulation) [6-7]. During the last years, animal models suggested that aggregation plays a pivotal role in shaping the immunogenic potential of drug proteins [8-9].

In general, immunogenicity of rh therapeutics can be classified by the patients' tolerance level: (1) low or non-existing tolerance due to e.g. genetic defects and (2) existing tolerance for the rh protein. In the first case the administered rh protein is considered foreign and a classical T-cell dependent (TD) B cell activation is initiated [10]. In the latter, tolerance is temporarily overcome, in which way remains unclear. It is believed that protein aggregates behave like T-cell independent type 2 (TI-2) antigens in that they are able to directly activate B cells [11]. Over the least years, the immunological dogma that the immune system primarily relies on the differentiation between self and non-self has been challenged. The fate of an antigen is not solely determined by being self or non-self, since potential danger signals of an antigen, e.g. lipopolysaccharide, also seem to be important [12]. This so-called "danger hypothesis" was introduced to explain why dendritic cells may become activated after cell injury and exposure to damaged self-proteins [13-14]. Regardless of the existing theories, the immunological mechanism underlying the immune response leading to formation of ADAs has not been sufficiently investigated and characterized. Recombinant human interferon beta is widely used to treat relapsing-remitting multiple sclerosis and production of ADAs is a well known

phenomenon [15]. IFNβ-1b (Betaferon®) contains about 60% non-covalent aggregates and it could be shown in a mouse model immune tolerant for human interferon beta that Betaferon® induces formation of ADAs [9] but after rechallenge with reformulated IFNβ-1a no immunological memory could be detected [16]. We used that model to study the immunological processes underlying the formation of ADAs and the absence of immunological memory in more detail. By administration of highly aggregated rhIFNβ (Betaferon®) we induced formation of ADAs, but after rechallenge with either highly aggregated or non-aggregated (reformulated IFNβ-1a) rhIFNβ no immunological memory was observed, which is reminiscent of T cell independent antigens. Depletion of CD4+ T cells led to complete abrogation of ADA formation, indicating a T cell dependent mechanism, thereby contradicting the classification of interferon beta a TI-2 antigen. Cytokine profiling added to the overall picture of an unconventional immune reaction against IFN beta aggregates.

Methods

Mouse breeding

Heterozygous C57Bl/6 tg mice immune tolerant for hIFNβ developed by Hermeling et al. [17] were bred at the Central Laboratory Animal Institute (Utrecht University, the Netherlands). The genotype of the offspring was determined by PCR showing the presence or absence of the hIFNβ gene in chromosomal DNA isolated from ear tissue. Tg C57Bl/6 mice were crossed with wt FVB/N mice obtained from Janvier (France) and their tg and wt C57Bl/6 x FVB/N hybrid offspring were used for in vivo experiments [9].

Immunization studies

Memory experiment

All animal experiments described were approved by the Institutional Ethical Committee. Food (Hope Farms, Woerden, the Netherlands) and water (acidified) were available ad libitum.
Bulk rhIFNβ-1a was obtained from Biogen Idec Inc. (Cambridge, MA, USA). Dialysis and subsequent filtration of bulk rhIFNβ-1a resulted in the reformulated product, as described in more detail by van Beers et al [16]. Protein concentration was determined based on the technique published by Gill et al [18]. Betaferon® was obtained from Schering (Berlin, Germany) and the lyophilized powder was reconstituted with phosphate buffered saline (PBS; Germany).
Blood samples were drawn from the cheek pouches (submandibular) of the mice before starting the injections. Injections were carried out as described previously [16-17, 19]. Briefly, mice were injected intraperitoneally (i.p.) with 5 μg of bulk IFNβ-1a or Betaferon® on days 0 to 4, days 7 to 11 and days 14 to 18. After an injection-free period of 6 weeks, 32 (transgenic (tg) = 16; wildtype (wt) = 16) animals were rechallenged with 5 μg of formulated rhIFNβ-1a i.p. on days 63 and 64 and 48 (tg = 24; wt = 24) animals were rechallenged with Betaferon® on days 64 and 65. Blood was collected submandibularly from two out of eight mice per group in the case of

rechallenge with reformulated IFNβ-1a and from 4 out of twelve mice per group in the case of rechallenge with Betaferon®, before any injection that day. On day 77, all mice were sacrificed. Plasma was stored at -20°C until further analysis. In addition, spleens were collected for Elispot analysis.

Immunization with TD and TI antigens within the T cell depletion study

Albumin from chicken egg white (OVA; Sigma, the Netherlands) was absorbed to aluminum hydroxide gel (Sigma, the Netherlands) according to manufacturer's instructions. Mice (tg = 25; wt = 25) were immunized with 5 µg i.p. of OVA absorbed to aluminum hydroxide gel in 100 µl PBS five times a week from Monday until Friday for three consecutive weeks. Pneumovax® (Sanofi Pasteur) was obtained from a local pharmacy and includes plain polysaccharide antigens from 23 different pneumococcal serotypes. Primary immunization with 1 µg i.p. of Pneumovx® in 100 µl PBS per mouse (tg = 12; wt = 12) was done on day 0, followed by a boost injection with another 1 µg of Pneumovax® on day 11 as suggested by T. Scheikl [20]. Plasma from all animals was collected prior to immunization, and additionally on day 7, 11, and 14 blood from four animals per group was collected. Spleens were removed from all animals on day 21, processed to a single cell suspension and analyzed by flow cytometry.

Immunization with Betaferon® within the T cell depletion study

Mice (tg = 25; wt = 25) were immunized i.p. with 5 µg of Betaferon® five times a week, Monday until Friday for three consecutive weeks. Prior to immunization all animals were bled and additionally on day 7, 11 and 14 blood from the cheek pouch was drawn from four animals per group. Animals were sacrificed on day 21 and spleens were removed and prepared for flow cytometry.

In vivo depletion of CD4+ T cells

Mice (tg = 38; wt = 38) were depleted of $CD4^+$ cells by three i.p. injections of 150 µg of the rat anti-CD4 antibody GK1.5 [21] in 100µl PBS before

immunization. Depletion was maintained by administration of 150 µg GK1.5 every 3-4 days of immunization until spleens were removed on day 21. Depletion of CD4+ T cells was confirmed and monitored weekly by flow cytometry of single-cell suspensions of spleens using a non-competing anti-CD4 monoclonal RM4-4 antibody (Pharmingen). Measurements were taken using a FACSCanto II® (BD Bioscience, The Netherlands) and analysis was performed with the FACSDiva software v6.1.1 (BD Bioscience, the Netherlands). Depletion efficiency was on average >98% (n=24) throughout the experiment (data not shown). A control group was injected with PBS (vehicle of GK1.5). Isotype-matched rat Ig was not considered a good control, since injection of rat Ig would eventually lead to a mouse anti-rat response and result in inflammation and false interpretation of data [22].

Cytokine profiling

For cytokine profiling, 12 animals (tg = 6; wt = 6) were injected i.p. with either 5 µg of Betaferon® in 100µl PBS, 5 µg of OVA absorbed to aluminum hydroxide gel in 100µl PBS or 100 µl of PBS five times a week, Monday until Friday for three consecutive weeks. 36 animals were sacrificed on day 9 and 30 animals were sacrificed on day 18. Expression of 22 cytokines on a protein level was measured in spleen lysates with a microarray (RayBiotech, GA, USA) according to the manufacturer's protocol. Visualization was achieved by x-ray exposure. Arrays were scanned and analyzed with the TotalLab TL100 software, version2006 (TotalLab Limited, Great Britain). Each membrane carried a biotinylated antibody directly printed on the membrane as a positive control which was used for normalization of the signal.

Binding antibody assay

Plasma was analyzed by direct ELISA, as described in detail by Hermeling et al. with adjustments [17].
Briefly, microtiter plates were coated with antigen (Supp. Table 1). Samples were added 100 times diluted in blocking buffer and incubated for one to two hours. In case of Pneumo23 samples needed to be preadsorbed to pneumococcal cell wall polysaccharide (CWPS) antigens to capture non-specific antibodies against CWPS, a known contaminant of the Pneumovax®

vaccine [20]. In brief, mouse serum was mixed with a 2 μg/ml CWPS and incubated for 30 min at room temperature. Adsorbed samples were then added 100 fold diluted in blocking buffer and incubated for two hours. After adding secondary antibodies (Supp. Table 1) color reaction was initiated by adding 3,3',5,5'-tetramethylbenzidine (TMB; Roche, the Netherlands) and stopped by 0.18 M sulfuric acid. Optical density values were measured at 450 nm wavelength on a microplate reader (Novopath; Biorad, The Netherlands) and data analysis was performed with GraphPad Prism 4.03 software (San Diego, CA, USA). Samples were considered positive when the mean was at least three fold higher than the mean of the negative plus the typical 95th percentile of the standard deviation. Titers were measured following the same ELISA protocol. The plots were fitted to a sigmoidal dose-response curve and the reciprocal of the dilution of the EC50 value was considered the titer of the plasma

Neutralizing antibody assay

Plasma collected at day 77 was subjected to a neutralizing antibody (NAB) assay. The assay is based on inhibition of myxovirus resistant protein A (MxA) gene expression in an A549 (human lung carcinoma cell line) cell line as previously described [19]. Neutralizing activity was defined as the dilution of tested sample reducing rhIFNβ activity and expressed in ten-fold reduction units per mL (TRU/mL), as described by Kawade [23]. Plasma samples below 130 TRU/mL were considered negative. Positive samples showed neutralizing activity ranging from 648 TRU/mL to more than 5120 TRU/mL.

Mouse memory B cell limiting dilution assay (LDA)

Direct presence of memory B cells (MBCs) was measured by a B cell limiting dilution assay, as described by Crotty et al [24]. In brief, a single cell suspension from spleen was seeded into a 96-well plate in a two fold serial dilution starting at 1x10^6 cells/well. Splenoctyes were re-stimulated with a mitogen and antigen mix (Lipopolysaccharide [Enzo Life Sciences, The Netherlands]; Pokeweed mitogen [kind gift of Crotty]; IFNβ-1a [Biogen, USA]) and incubated for 5 days at 37°C in a humid chamber containing 5% CO_2. After incubation memory B cells were measured by ELISPOT.

Multiscreen HTS 96-well plates (Milipore) were coated overnight at 4°C with 5 μg/ml of IFNβ-1a. Stimulated cells were transferred to the coated ELISPOT plates and incubated 5 hrs and immunoglobulin secreting spots were detected as described [24]. A well was scored positive for MBCs if the detected antibody-secreting cells (ASCs) were higher than the mean of pre-existing long living plasma cells plus 3* standard deviation and if the well contained more than 6 ASCs. In order to determine pre-existing long living plasma cells a direct ex-vixo ELISPOT was performed on day one [25].

Statistics

Where appropriate, a nonparametic Mann-Whitney test was applied. For the limiting dilution assay a chi-square analysis was performed. Differences between frequencies of memory B cells in tg versus wt animals were analyzed with a one-way repeated measures ANOVA. *P* values are presented in figure where a statistically significant difference was found.

Results

Interferon beta fails to trigger a memory B cell response

Patients switching from Betaferon® (rhIFNβ-1b) to Avonex® (rhIFNβ-1a) treatment do not display a sustained antibody response against interferon beta [26], suggesting that Betaferon® is not able to induce memory B cells against interferon beta. To investigate this phenomenon in our mouse model, immune tolerant for human IFNβ, we injected both transgenic (tg) and wildtype (wt) animals with bulk rhIFNβ-1a (slightly aggregated) and Betaferon® (highly aggregated; see Table 1) for three consecutive weeks to initiate an immune response.

Table 1. Differences in rhIFNβ products.

a

Product	host cells	glycosilated	homology natural IFNβ
Betaferon® (IFNβ-1b)	E.coli	no	slightly different
Bulk (IFNβ-1a)	CHO	yes	100%
Reformulated (IFNβ-1a)	CHO	yes	100%

b

Product	aggregates?	non-covalent bonds	native epitopes
Betaferon® (IFNβ-1b)	yes, large amount heterodisperes Aggregates	yes	yes
Bulk (IFNβ-1a)	yes, low amount heterodisperse aggregates	mostly, some covalent bonds	yes
Reformulated (IFNβ-1a)	no	-	yes

Animals were then kept injection-free for six weeks to wash out the human therapeutic and thus leading to a decrease in titers. The wash-out phase was followed by a rechallenge with reformulated IFNβ-1a (non-aggregated) [16] and Betaferon®. As expected, Betaferon® elicited an immune response in the tg animals, whereas slightly aggregated material hardly did. Wildtype animals developed a typical immune response with high IgG titers against both IFNβ products comparable to IgG titers seen after immunization with a foreign antigen (Fig. 1). After rechallenge with reformulated rhIFNβ-1a or Betaferon® tg animals continued to show decreasing antibody production. In contrast, the second immune response of wt animals was stronger than the first response during the three week immunization (Fig. 1).
The same trend in antibody response could also be measured in the IgG1 and IgG2a subtypes, both being present in the tg and wt animals (Supp. Fig. 1 and 2).
The induction of memory B cells was studied using a limiting dilution assay from spleens taken on day 77, i.e. two weeks after rechallenge. Splenocytes were isolated and after five days of stimulation, splenocytes were subjected to an ELISPOT assay to identify immunoglobulin-secreting cells. Wt animals treated and rechallenged with both Betaferon® and non-aggregated IFNβ-1a product showed typical immunoglobulin spots, representing memory B cells (MBCs) (Fig.2; only shown for Betaferon® treated and rechallenged animals). Though the tg animals did not show an increase in antibody titers after rechallenge, the limiting dilution assay revealed small and faint immunoglobulin spots, which were significantly lower in number than the spots detected in the wt animals (Fig. 2).
Both tg and wt groups had so called outliers, meaning that one or two animals per group showed an atypical immune response, e.g. tg animals behaving like wt animals with high IgG titers and intense immunoglobulin spots and wt animals behaving like tg animals with low or even absent IgG titers and faint or absent immunoglobulin spots. In case of the wt animals acting like tg animals, false interpretation of the PCR used for genotyping could be reasoned. In the presence of the transgene it could be reasoned that the mouse carried a dysfunctional human gene, subsequently leading to a dysfunctional expression of the human protein and lack of tolerance. The heterozygous animals are bred with wt in-house animals or animals ordered at the Centre d'Elevage Janvier (France), both inbred strains in which genetic drift is a known phenomena [27] and which could explain the outliers. More detailed elaboration of the model can be found elsewhere [9, 16]. Since

outliers did not occur group-specific but were evenly distributed among the groups, we considered them to be true outliers. Nevertheless, they did not affect the outcome of the statistical analysis and were included.

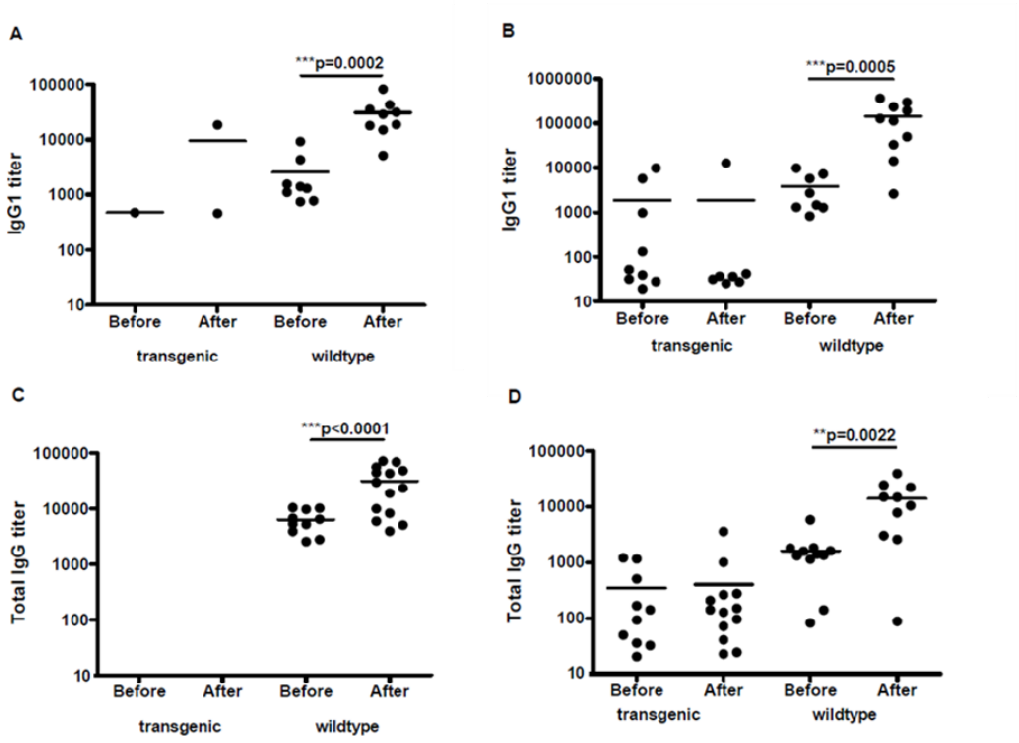

Figure 1 No memory formation in tg animals (A) IgG1 titers of bulk rhIFNβ-1a treated animals (tg n=8; wt n=8) before and after rechallenge with reformulated rhIFNβ-1a; (B) IgG1 titers of Betaferon® treated animals (tg n=8; wt n=8) before and after rechallenge with reformulated rhIFNβ-1a; (C) total IgG titers of bulk rhIFNβ-1a treated animals (tg n=12; wt n=12) before and after rechallenge with Betaferon®; (D) total IgG titers of Betaferon® treated animals (tg n=12; wt n=12) before and after rechallenge with Betaferon®. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. Before rechallenge covers day 53, 56, 58 and 60; after rechallenge covers day 67 and 77. Outliers within treatment groups have been included in the figure and statistical analysis. P values indicate a significant increase in IgG titers after rechallenge. * p< 0,05; ** p< 0,01; *** p<0,001. Due to handling, liver or heart problems, six out of 80 mice died during the study.

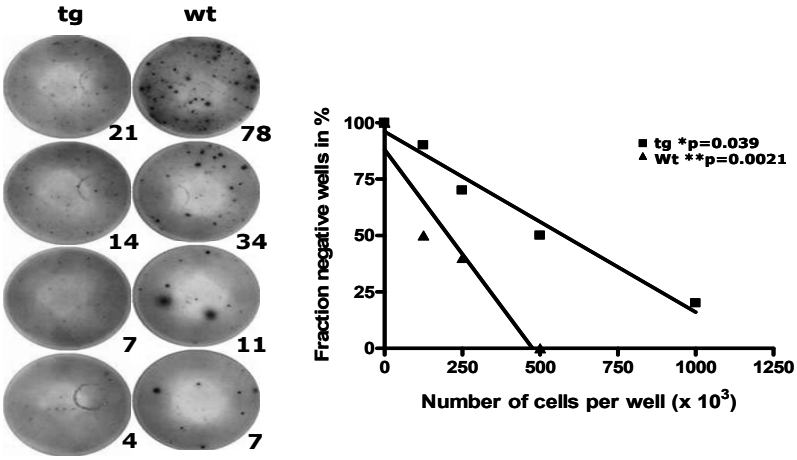

Figure 2 Limiting dilution analysis of memory B cells from Betaferon® treated and rechallenged animals (n=6). Splenocytes were stimulated for 5 days with mitogen and IFNβ conditioned medium. After ELISPOT analysis, MBCp cell frequency was determined by a linear regression model with a goodness–of-fit measure. Calculated MBCp frequency of the tg animals was 1/7.4 x 10^5, whereas for the wt animals it was 1/2.75 x 10^5. A repeated measured one-way ANOVA was performed to test for significant differences between tg and wt MBCp frequency with a p value <0.0001. Far left, ELISPOT quantification of the secretion of anti-IFNβ IgG. Numbers in bottom right corners indicate spots counted in wells.

Lack of neutralizing anti-interferon beta antibodies

The observation that rechallenegd tg animals lacked formation of memory, but had abundant isotypes of antibodies triggered our interest in further characterizing the ADAs formed against aggregated rh therapeutic. Therefore we measured neutralizing antibodies (NABs) against human IFNβ in our mouse model after rechallenge on day 77. Tg animals treated with aggregated IFNβ-1b gave rise to binding antibodies (BABs) but no NABs, in contrast to the wildtype animals producing both BABs and NABs (Table 2).

	tg		wt	
	Rechallenge			
	Bulk	Betaferon®	Bulk	Betaferon®
Treatment				
Bulk	0/6	0/12	8/8	10/12
Betaferon®	1/7	1/11	8/8	7/10

Table 2 Tg animals do not develop neutralizing antibodies. Neutralizing antibody (NA) assay performed with plasma samples on day 77 of tg and wt animals treated with bulk IFNβ-1a and Betaferon® and rechallenged with reformulated IFNβ-1a (n=8) on day 63/64 and Betaferon® (n=12) on day 64/65. Numbers indicate the NA responders out of animals per group tested.

Distinct cytokine profile after treatment with aggregated IFN beta

Cytokines are key regulators of the immune system by delivering agonistic or antagonistic signals, thereby giving indications about the underlying immune mechanism. Therefore, we studied the cytokine profiles of tg and wt animals upon treatment with Betaferon® or OVA to investigate possible differences in cytokine production. Expression levels were normalized on the array itself with internal controls and levels were presented as the percentage compared to saline treated animals (Fig. 3). On day 9, the early onset of the immune response, pro-inflammatory cytokines such as TNF alpha and IL-12 were upregulated in all treated animals, though IL-17, known to be a potent pro-inflammatory cytokine, was only upregulated in tg animals exposed to Betaferon®. We also observed a considerable upsurge of anti-inflammatory cytokines, though Betaferon® treated animals showed a less prominent expression of IL-4 and IL-13. On day 18, the last day of injection, the pattern of released cytokines had changed (Fig. 3). In general cytokine levels decreases, especially pro-inflammatory cytokines. Total IgG titers on day 9 and 18 were also measured to show that Betaferon® were indeed able to breach the tolerance (Supp. Fig. 3). Animals exposed to ovalbumin had a solid immune response regardless of their genotype, indicating yet again that the formation of ADAs in the tg animals follow a different immunological pathway as upon challenge with a cognate foreign antigen, such as ovalbumin.

Though the major differences in cytokine production were found between treatment groups (Betaferon® and OVA), also considerable differences could be seen between tg and wt animals treated with Betaferon®. These observations support the concept of an atypical immune response towards aggregated interferon beta.

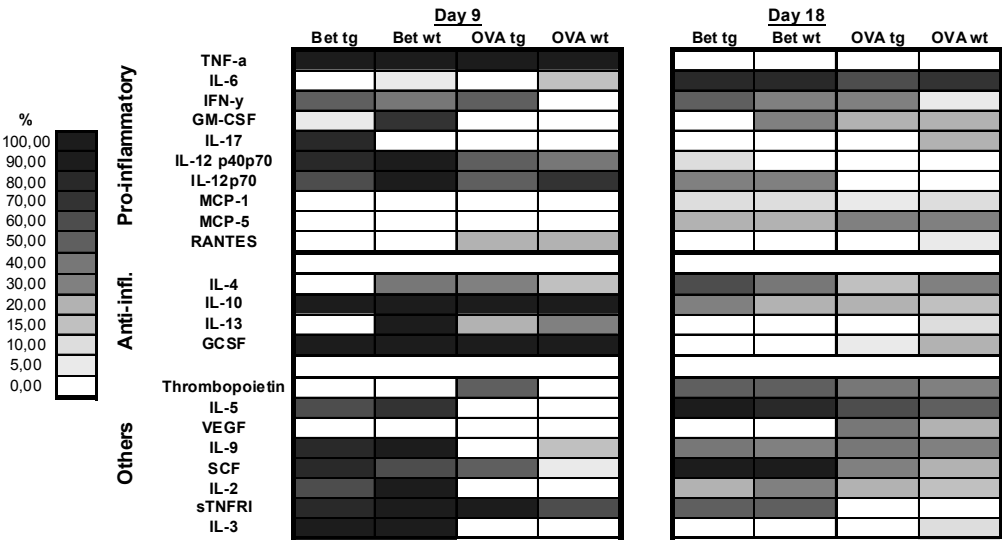

Figure 3 Betaferon® treated tg animals show distinct cytokine profile. Microarray analysis of 22 cytokines of mice being sacrificed 5 hours after the last injection on day 9 (n=6) and 18 (n=5). Scale on far left indicates the increase of cytokine signal in percentage compared to saline treated groups.

The role of CD4+ T cells

Antigens can be categorized into two groups defined by their dependency on CD4+ helper T cells: (a) T cell dependent (TD) or (b) T cell independent (TI) [28]. The majority of immune reactions involve TD antigens, leading to an antigen specific immune response with memory formation to provide instant protection against pathogens invading the body a second time. Immune reactions against typical TI-2 antigens such as polysaccharides, mostly present on bacteria, do not need help by CD4+ T cells but may directly interact with B cells by cross linking their B cell receptors (BCRs), However,

this mechanism does not lead to the generation of memory B cells [29]. Based on our observation of lack of memory formation after injection of Betaferon® but apparent isotype switching, we investigated the dependency of IFNβ antibody formation on the presence of CD4+ T cells. Animals were divided into two groups, CD4+ T cell depleted (-) and CD4+ T cell undepleted (+). Each of the two groups received the same treatment: (1) a well known TD antigen, ovalbumin, (2) TI-2 antigen Pneumo23 (polysaccharide vaccine), and (3) Betaferon®. CD4+ T cell depleted animals treated with ovalbumin, regardless of their genotype, had a diminished immune response, whereas the undepleted animals treated with ovalbumin showed IgG titers comparable between tg and wt groups (Fig. 4 B), confirming a successful depletion. Animals, regardless of their genotype and their depletion status, displayed a typical total IgG response when Pneumo23 was administered (Fig. 4 C), proving the selective depletion of CD4+ T cells. IgG1 and IgG2a titers of undepleted animals treated with ovalbumin followed the same trend as the total IgG titers (Supp. Fig. 4 C and D). Interestingly, tg animals depleted of their CD4+ T cells did not trigger an IgG response against Betaferon®, whereas the undepleted control group showed considerable IgG, IgG1 and IgG2a titers (Fig. 4 A, Supp. Fig. 4 A and B). Collectively these data indicate that formation of ADAs against aggregated interferon beta in IFNβ tg mice is CD4+ T cell dependent, but does not lead to an immunological memory.

Significantly lower titers in tg animals compared to wt animals

Throughout all animal studies were observed lower IgG titers in the tg animals than in the wt animals immunized with Betaferon®. Comparing the titers of the two animal groups being treated with Betaferon® it was found that in every *in vivo* study the IgG titers were significantly lower in the tg animals (Figure 5).

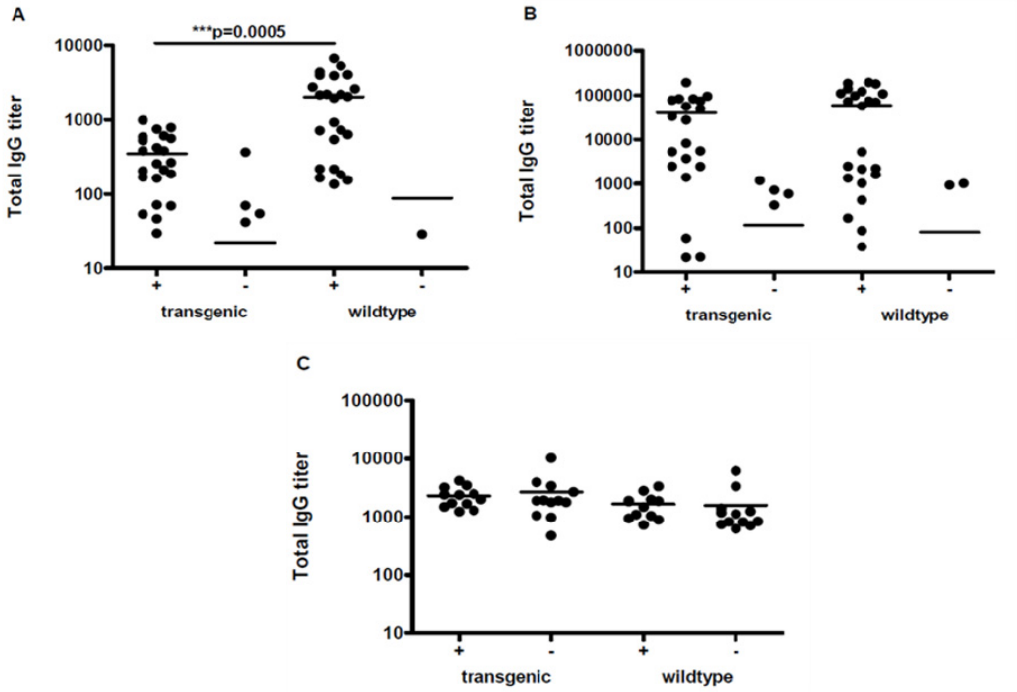

Figure 4. Depletion of CD4+ T cells abolishes any immune response against aggregated rhIFNβ-1b and OVA in both tg (n=12) and wt (n=12) animals but does not influence the immune response against polysaccharide vaccine Pneumovax23® (tg n=8; wt n=8). + represents the undepleted animals, - the depleted animals. (A) Total IgG titers of aggregated rhIFNβ-1b treated animals; (B) total IgG titers of OVA treated animals; (C) total IgG titers of Pneumovax23® treated animals. P values indicate significant lower IgG titers in tg animals compared to wt animals. * p< 0,05; ** p< 0,01; *** p<0,001. Three out of 104 mice died during the study due to handling.

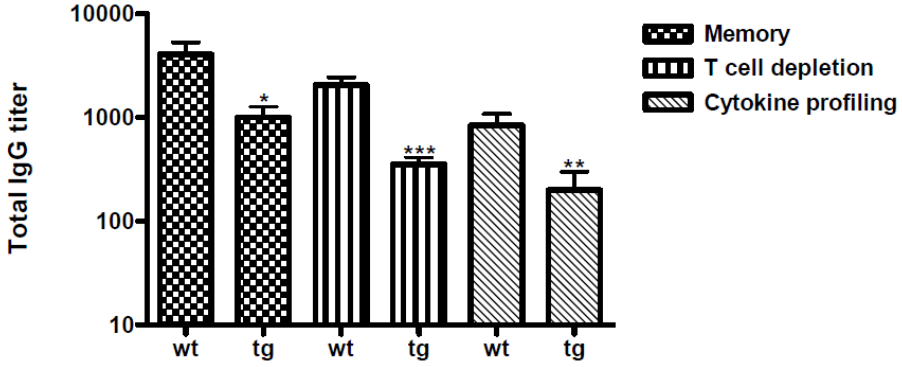

Figure 5. Total anti-IFNβ IgG titers of tg and wt animals treated with Betaferon® measured during the memory, the T cell depletion (undepleted animals) on day 7, 11, 14, 18 and 21 and cytokine profiling (only day 7, 9, 14 and 18). Error bars represent averaged titers of at least 10 animals plus SEM. P values indicate significantly lower titers in the tg animals compared to the wt animals. *p<0.05, **p<0.01, ***p<0.001.

Discussion

We used mice which are tolerant towards human IFNβ to characterize the immune response after a challenge with Betaferon®, a product containing high amounts of aggregates. In this unique model we could show that administration of aggregated material elicited a humoral immune response which turned out to rely on the help by CD4+ T cells. But one should keep in mind that all CD4+ T cells were depleted throughout the experiment. A transient depletion of specific CD4+ T subsets, such as regulatory T cells or Th17 T cells, could shed more light on the involvement of T cells.
The immune response against aggregated material could be due to special features of aggregated versus non-aggregated interferon beta, such as repetitive epitopes on the surface of the aggregates, as reported with other structured antigens such as virus-like particles [30-31]. An alternative explanation may be the exposure of hydrophobic moieties [32], a danger signal, or a combination of both the former and the latter aspect. After rechallenge with either non-aggregated or aggregated material, tg animals did not show an immunological memory to the injected therapeutic,

suggesting that IFN beta aggregates may mimic TI-2 antigens. By direct cross linkage of the B cell receptor, TI-2 antigens can trigger formation of antibodies. Important factors influencing immunogenicity potential of TI-2 antigens are the spacing of the epitopes, their number and rigidity [33-35], which could also explain the diverse response against aggregated therapeutics seen in mouse models, where immunogenicity was linked to aggregation levels [36-37]. The presence of antibody-secreting cells in the limiting diluting memory B cell assay of the tg animals was surprising, but could indeed hint to another B cell population being responsible for formation of ADAs, such as marginal zone (MZ) B cells which have a different survival mechanism than follicular B cells [38-39]. MZ B cells are the main effector cells directed against TI-2 antigens, though lately evidence occurred that they also participate in TD immune responses and bridge innate and the adaptive immune system [40].

Despite the fact that wt and tg animals treated with interferon beta aggregates shared similar cytokine profiles, the cytokine levels revealed slight differences between tg and wt animals, thus further reinforcing the complex interplay between the innate and adaptive branch of the immune system upon exposure to protein drugs [41]. Nevertheless, more in- depth studies are needed to reveal if the differences are significant.

Although interferon beta aggregates did not induce B cell memory, we found that T cells were crucially contributing to B cell activation and induction of anti-interferon beta antibodies. During a classical T-cell/B-cell interaction initiated by TD antigens, T cells provide co-stimulatory signals to initiate the maturation of the B cells in plasma cells and later on into memory B cells. Thus the question rises why aggregated rh IFNβ was capable of stimulating the maturation process leading to plasma cells but did not give rise to memory B cells. One could speculate that aggregated rh therapeutics trigger B cell activation due to the repetitive epitopes they carry, but no other stimulatory signals are present to support further maturation of the B cells into memory B cells. One should keep in mind that rh therapeutics are still self-proteins. The possible lack of further stimulating signals and thus leading to an incomplete or insufficient activation of T and B cells may also be supported by the overall lower IgG titers of tg animals compared to wt animals.

In conclusion, this model has shown that Betaferon® leads to the formation of ADAs, but lacks the formation of B cell memory, very much in agreement with clinical observations in multiple sclerosis patients. In addition,

Betaferon® initiated a slightly different release of cytokines compared to wt animals. After depletion of CD4+ T cells, the immune response against aggregated therapeutic was completely gone, suggesting that breakage of tolerance against human interferon beta relies on help of CD4+ T cells. Finally, it can be concluded that tg mice which are tolerant against human IFNβ are useful tools to investigate the immunological mechanism underlying the formation of ADAs after administration of human interferon beta aggregates. This model may set the basic approaches for characterizing breakage of tolerance and/ or induction of immunogenicity by other protein therapeutics in appropriate mouse models prior to entry into man.

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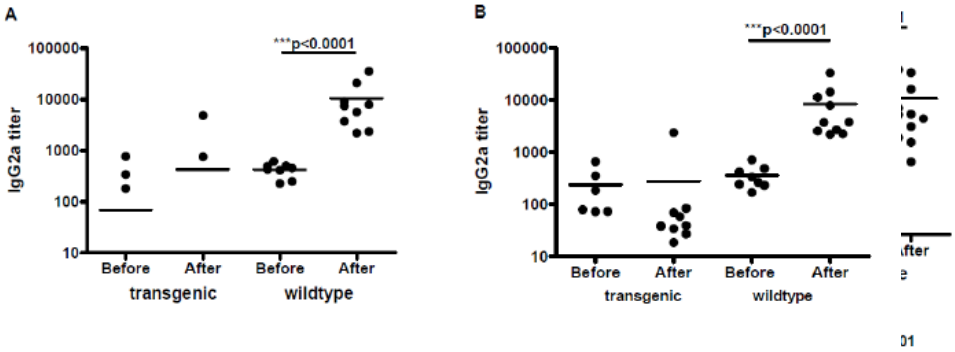
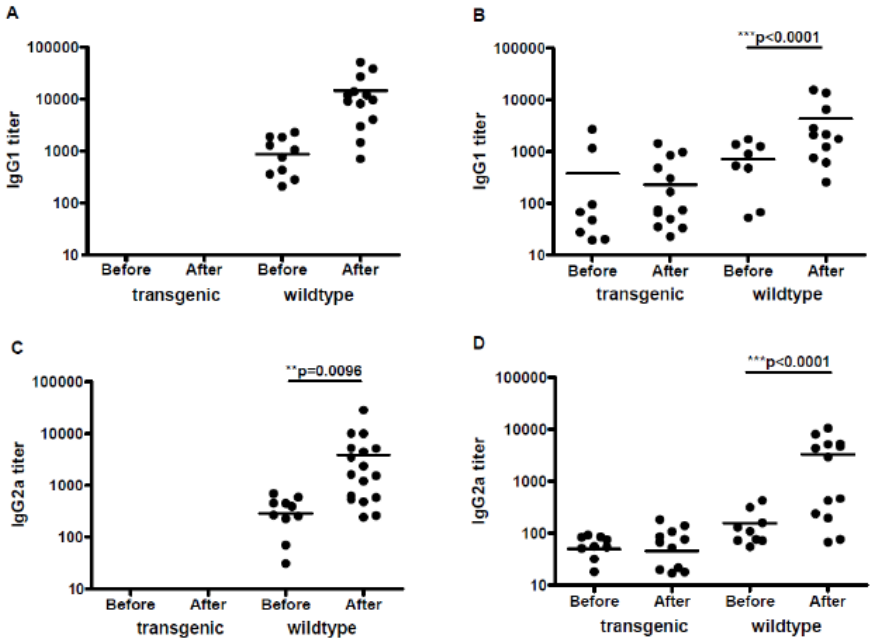
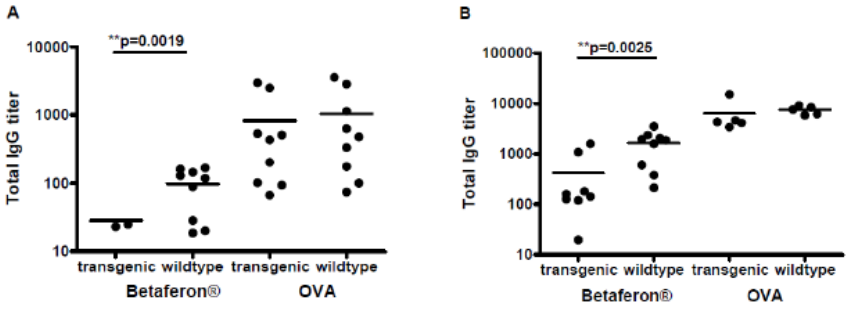
Supplementary Material



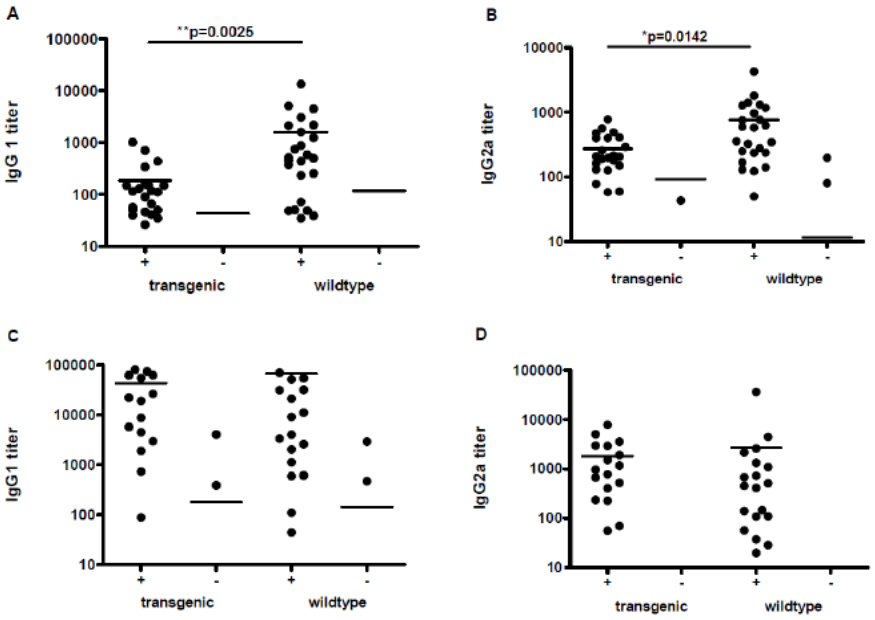
Supplementary Figure 1. IgG2a subtypes confirm absence of memory after rechallenge with reformulated IFNβ-1a. (A) IgG2a titers of bulk rhIFNβ-1a treated animals (tg n=8; wt n=8) before and after rechallenge; (B) IgG1 titers of Betaferon® treated animals (tg n=8; wt n=8) before and after rechallenge. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. Before rechallenge covers day 53, 56, 58 and 60; after rechallenge covers day 67 and 77. P values indicate a significant increase in IgG titers after rechallenge. * p< 0,05; ** p< 0,01; *** p<0,001. Due to handling, liver or heart problems, six out of 80 mice died during the study.



Supplementary Figure 2. IgG1 and IgG2a titers confirm absence of memory formation in tg animals rechallenged with Betaferon® (A) IgG1 titers of bulk rhIFNβ-1a treated animals (tg n=12; wt n=12) before and after rechallenge; (B) IgG1 titers of Betaferon® treated animals (tg n=12; wt n=12) before and after; (C) IgG2a titers of bulk rhIFNβ-1a treated animals (tg n=12; wt n=12) before and after rechallenge; (D) IgG2a titers of Betaferon® treated animals (tg n=12; wt n=12) before and after rechallenge with Betaferon®. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. Before rechallenge covers day 53, 56, 58 and 60; after rechallenge covers day 67 and 77. P values indicate a significant increase in IgG titers after rechallenge. * p< 0,05; ** p< 0,01; *** p<0,001. Due to handling, liver or heart problems, six out of 80 mice died during the study.



Supplementary Figure 3. Total IgG titers in animals treated with Betaferon® and OVA show typical development. (A) Total IgG titers after 9 days of immunization of Betaferon® and OVA treated tg and wt animals (n=6) (B) Total IgG titers after 18 days of immunization of Betaferon® and OVA treated tg and wt animals (n=5). Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. P values indicate significant lower IgG titers of tg animals compared to wt animals treated with Betaferon®. * p< 0,05; ** p< 0,01; *** p<0,001.



Supplementary Figure 4. T cell depletion also abrogated secretion of IgG1 & IgG2a antibodies against Betaferon® and OVA in both tg and wt (n=12) animals being undepleted (+) and depleted (-) (A) IgG1 titers of Betaferon® treated animals; (B) IgG2a titers of Betaferon® treated animals; (C) IgG1 titers of OVA treated animals; (D) IgG2a titers of OVA treated animals .P values indicate significant lower IgG titers in tg animals compared to wt animals. * p< 0,05; ** p< 0,01; *** p<0,001. Three out of 104 mice died during the study due to handling.

	IFNbeta	OVA	TNP	Pneumo23
Coating concentration	2 µl/ml	5 µl/ml	0.5 µl/ml	0.05 µl/ml
Incubation temperature	RT	RT	37°C	37°C
Blocking reagent	4%milk/PBS/ 0.05% Tween 20	4%milk/PBS/ 0.05% Tween 20	1%BSA/PBS/ 0.05% Tween 20	1%BSA/PBS/ 0.05% Tween 20
2nd antibody dilutions	Polyclonal Peroxidase-Rabbit Anti-Mouse IgG 1:4000, Monoclonal Peroxidase Goat Anti-Mouse IgG1 1:2000 (all Invitrogen, Breda, The Netherlands) Monoclonal Peroxidase-Rat Anti-Mouse IgG2a 1:2000 (Abcam, Cambridge, UK)			

Supplementary Table 1. ELISA conditions detecting anti- IFNbeta, anti-OVA, anti-TNP and anti-Pneumo23 antibodies. Listed are concentrations of coating reagents, incubation temperature, blocking reagent and dilution of 2nd antibodies.

RT = room temperature; BSA = bovine serum albumin

Chapter 4

Immunological aspects of the formation of anti-drug antibodies against aggregated recombinant human alpha in an immune tolerant mouse model

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Manuscript submitted

Abstract

Formation of anti-drug antibodies (ADAs) against recombinant human (rh) therapeutics is still a subject of controversial discussions, though it is commonly accepted that aggregation is a major cause leading to immunogenicity. In a previous study we showed in an mouse model immune tolerant for human interferon beta that the antibody response against aggregated recombinant human interferon beta is not characterized by memory, though still dependent on the presence of CD4+ T cells. Following up on those findings we challenged mice immune tolerant for human interferon alpha with aggregated rh interferon alpha (rhIFNα2a) and characterized the antibody response. Aggregated rhIFNα2a was prepared by metal catalyzed oxidation, and characterized by Size Exclusion Chromatography, Far-UV SD, Intrinsic Steady State Fluorescence and Western blotting. Oxidized rhIFNα2a had a substantial loss of monomers and showed higher molecular weight species with intact epitopes. As seen with aggregated interferon beta, after rechallenge with both native and aggregated rhIFNα2a no memory response was observed. Depletion of CD4+ T cells abolished the antibody response against aggregated material. Additionally, depletion of the marginal zone B cells led to an increase in the level of ADAs. Mixing native rhIFNα2a with another aggregated protein (Betaferon®) did not lead to an immune response against native rhIFNα2a. Collectively, these data provide evidence that the antibody response in immune tolerant animals initiated by administration of aggregated rh protein therapeutic is dependent upon CD4+ T cells and is influenced by the presence of marginal zone B cells, but does not result in formation of neutralizing antibodies or memory B cells.

Introduction

The class of rh therapeutics, including e.g. growth hormones, cytokines and monoclonal antibodies, is constantly growing. Although these drugs are human protein homologues, the formation of ADAs is a frequently observed side-effect [1-2]. Consequences of ADAs can be a faster clearance of the drug from the blood, reduction of drug efficacy, and formation of drug-immune complexes, which may initiate anaphylactic reactions, serum sickness or other related drug hypersensitivity reactions [3]. Life-threatening side-effects of ADAs may occur when ADAs cross-react with and neutralize the endogenous protein, as reported in the case of rh eopoetin [4-5]. Immunogenicity can be influenced by many factors, such as patient-dependent (e.g., HLA allele, genetic defects), treatment-dependent (e.g., dose, route of administration, frequency) and protein-product-dependent (e.g., glycosylation, primary sequence, production, purification methods, formulation) [6-7]. Animals models immune tolerant for the human protein drug of interest increasingly indicate that aggregation has an important influence on the immunogenic potential of rh therapeutic proteins [8-10]. There are two type of antigens based on their need for T cell help: (1) T cell-dependent antigens (TD), and (2) T-cell independent antigens (TI). TI antigens are also subdivided into TI- type 1 (TI-1) and TI-type 2 (TI-2), categorized by their capability to induce a specific immune response. TI-1 antigens, such as bacterial lipopolysaccharide induce a non-specific B cell proliferation, whereas TI-2 antigens, such as polysacharides, induce a specific B cell activation and proliferation, leading to antigen-specific antibody production [11-13]. Due to the absence of T helper cells, TI-2 antigens fail to induce a memory response.
In general, the patients' state of tolerance towards the therapeutic drug can be divided in two categories: (a) low or non-existing tolerance due to e.g. genetic defects and (b) existing tolerance for the rh protein. Low or non-existing tolerance leads to recognition of the rh protein as foreign and a classical T cell - B cell activation is initiated as seen with TD antigens [14]. In the case of an existing tolerance, it is temporarily overcome upon administration of the rh therapeutic and the reason behind it remains a matter of intense discussion. Due to the repetitive epitopes of aggregates it is argued that they behave like TI-2 antigens in that they are able to directly activate B cells [15]. In mice, marginal zone (MZ) B cells are strategically located at the marginal sinuses of the spleen and thus display the first line of

defense against blood-borne pathogens, such as TI-2 antigens [16]. After a first round of antibody production by MZ B cells, they initiate the adaptive arm of the immune system and conventional B-2 B cells (also named follicular B cells) start to produce antigen-specific antibodies, thus MZ B cells are also thought to bridge the innate and the adaptive immune system [17]. Though theories about immunological mechanism are more and more explored, production of ADAs and the underlying immunological actions remain unclear.

Recombinant human interferon alpha (rhIFNα) is widely used to treat viral infections and cancer and production of ADAs is a well known phenomenon [18]. It could be shown in a mouse model immune tolerant for human IFNα2a that aggregated rhIFNα2a induced formation of ADAs [8, 10]. In the present study we used that model to investigate the immunological processes underlying the formation of ADAs in more detail.

Methods

Materials

Recombinant human IFNα2a was provided by Hoffmann-La Roche (Basel, Switzerland). Betaferon® was obtained from Schering (Berlin, Germany) and the lyophilized powder was reconstituted with phosphate buffered saline (PBS; Germany).
Tris, glycine, SDS, L-ascorbic acid, copper chloride, disodium hydrogen phosphate, EDTA, were purchased from Sigma–Aldrich, Germany. Glacial acetic acid and acetonitrile were purchased from Boom, The Netherlands. Slide-A-Lyzer dialysis cassettes, molecular weight cut-off 10 kDa, were purchased from BioRad. All chemicals were of analytical grade and used without further purification. Deionized water was purified through a Purelab Ultra System (ELGA LabWater Global Operations, UK) prior to use.

Methods

Preparation of Native and Oxidized recombinant human IFNα2a Formulations

A solution of 1.5 mg/mL rhIFNα2a in 25 mM ammonium acetate, 120 mM NaCl, acetic acid, pH 5, was dialyzed against sodium phosphate buffer 10 mM pH 7.4 to obtain starting material, referred to as native rhIFNα2a. Aggregated rhIFNα2a was prepared by metal catalyzed oxidation, essentially as described earlier by Hermeling et al. [10]. After dilution to 0.3 mg/mL, native rh IFNα2a was incubated with 0.04 mM $CuCl_2$ for 10 minutes. Subsequently, L-ascorbic acid 40 mM was added to a final concentration of 4 mM. After three hours the reaction was quenched by adding 100 mM EDTA to a final concentration 1 mM [19]. The oxidized samples were dialyzed against 10 mM phosphate buffer pH 7.4 for 24 hours before use.

Preparation of the mixture rhIFNα2a/Betaferon® and Betaferon® solution.

Betaferon® solution was obtained by dissolving the lyophilized powder with a solution of 0.54% NaCl, as reported in the medication guide provided by Roche. The mixture of native rhIFNα2a with Betaferon®, was obtained by mixing 0.66 mL of IFNα2a 0.15 mg/mL in sodium phosphate buffer 10 mM with 0.33 mL of Betaferon® 0.15 mg/mL, in order to have a theoretical final protein concentration of 0.05 mg/mL IFNβ1b and 0.1 mg/mL of IFNα2a .

UV spectroscopy

UV/VIS measurements were performed with an Agilent 8453 UV/VIS spectrophotometer (Waldbronn, Germany), which included a Peltier element for temperature control and a magnetic stirrer. The Peltier element was steered by the Agilent 89090A controller. Quartz cells with a pathlenght of 1 cm were used for all measurements. Scans were taken from 200-900 nm with 1 nm intervals. The protein concentration was determined by measuring the absorbance at 278.5 nm (extinction coefficient (0.1 %; 1 cm; 278.5 nm) = 1.06).

Far-UV circular dichroism (Far-UV CD) spectroscopy

Far UV CD spectra were recorded from 190 to 250 nm using a Jasco J-815 CD spectrometer (Jasco International, Tokyo, Japan). Analyses were performed in a 1-mm path length quartz cuvette at 20 °C using a scan rate of 100 nm/min, a response time of 2 s, and a bandwidth of 1 nm. Each spectrum was the result of an averaging of 8 repeated scans, background corrected with the corresponding buffer spectrum. The CD signals were converted to molar differential extinction coefficient, Δε.

Intrinsic steady state fluorescence

Intrinsic fluorescence was measured in 96-well plates using the plate reader unit of the FS920 fluorescence spectrometer (Edinburgh Instruments). For the measurement, all the formulations were diluted to a concentration of 0.1 mg/ml to avoid inner filter effects. The concentration after dilution was confirmed with a BCA assay. The protein was selectively excited at 295 nm.

The emission spectra were recorded from 305 to 500 nm using a step size of 2 nm, gain of 115, Z-position of 20 x 10^3 um, number of flashes 100 with a frequency of 400 Hz. Triplicates for each sample were analyzed.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Acrylamide gradient gels (4-20%, tris-HCl), were run under reducing (sample buffer containing 5% (v/v) β-mercaptoethanol) and nonreducing (sample buffer without β-mercaptoethanol) conditions at 200 V at room temperature. The electrophoresis buffer was 25 mM tris(hydroxymethyl)aminomethane, 192 mM glycine, and 0.1% (w/v) SDS. Gel electrophoresis was performed with a Biorad Protean III system (Biorad, Veenendaal, The Netherlands). Samples analyzed under reducing conditions were boiled for 2 min before application to the gel. A low-range molecular weight standard (Biorad) was included on the gel for determination of molecular weight.

Western Blotting

Protein bands in SDS-PAGE gels were blotted onto a polyvinylidene difluoride (PVDF) immuno blotting membranes with a mini trans-blot electrophoretic transfer cell, one bio-ice cooling unit, two gel holder cassettes, four fiber pads, four filter papers. Blots were blocked for 2 hours at room temperature with 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) with constant orbital shaking. After washing with 0.1% (v/v) Tween 20 in PBS and with water, the blots were incubated with Rabbit Anti-Human Interferon Alpha (PBL Biomedical Laboratories) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Blots were washed with 0.1% (v/v) Tween 20 in PBS and with water. Blots were incubated with peroxidase labeled anti-rabbit IgG (Sigma-Aldrich) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at +4°C with constant orbital shaking. Blots were washed with 0.1% (v/v) Tween 20 in PBS and with water and incubated in a solution of 4-chloro-1-naphtol (Sigma- Aldrich) in methanol (20% (v/v)), water, and H_2O_2 (0.015% (v/v)). After color development the blots were stored overnight in the dark in water to increase the intensity of the bands.

Gel Permeation Chromatography

Samples (100 ug/mL) were analyzed with a TSK-GEL 3000 column (Stuttgart, Germany), using a mobile phase of 50 mM sodium phosphate and 200 mM sodium chloride, pH 7.2, filtered through a 0.2-mm filter prior to use, at a flow rate of 0.50 mL/min by a Waters 2695 controller equipped with an autosampler. Chromatograms were recorded with a photodiode array detector (model 2996, Waters, Milford, MA, USA). The column was calibrated by analyzing protein standards obtained from Sigma-Aldrich with known molecular weights (i.e., thyroglobulin, BSA, ovalbumin, α-chymotrypsin, and myoglobin).

Immunization studies

Mouse breeding

Heterozygous FVB/N transgenic (tg) mice immune tolerant for hIFNα2a, described before by Braun et al. [20], were bred at the Central Laboratory Animal Institute (Utrecht University, the Netherlands). Wildtype (wt) FVB/N animals were purchased at the Centre d'Elevage Janvier (France). Food (Hope Farms, Woerden, the Netherlands) and water (acidified) were available ad libitum. All animal experiments described were approved by the Institutional Ethical Committee.
The genotype of the offspring was determined by PCR showing the presence or absence of the hIFNα gene in chromosomal DNA isolated from ear tissue.

In vivo depletion of CD4+ T cells

Mice (tg = 27; wt = 27) were depleted of $CD4^+$ cells by three i.p. injections of 150 μg of the rat anti-CD4 antibody GK1.5 [21] in 100 μl PBS before immunization. Depletion was maintained by administration of 150 μg GK1.5 every 3-4 days of immunization until spleens were removed on day 23. Depletion of CD4+ T cells was confirmed and monitored weekly by flow cytometry of single-cell suspensions of spleens using a non-competing anti-CD4 monoclonal RM4-4 antibody (Pharmingen). Measurements were taken

using a FACSCanto II® (BD Bioscience, The Netherlands) and analysis was performed with the FACSDiva software v6.1.1 (BD Bioscience, the Netherlands). Depletion efficiency was on average >98% (n=22) throughout the experiment. A control group was injected with PBS (vehicle of GK1.5). Isotype-matched rat Ig was not considered a good control, since injection of rat Ig would eventually lead to a mouse anti-rat response and result in inflammation and false interpretation of data [22].

In vivo depletion of marginal zone B cells

Mice (tg = 24; wt = 24) were depleted of MZ B cells by one i.p. injection of 100 μg anti-LFA ($\alpha L\beta_2$) and 100 μg anti-CD49d (α_4) in 200μl PBS before immunization [23]. Depletion was maintained by a second injection on day 11. Efficiency of depletion of MZ B cells was confirmed and monitored weekly by flow cytometry of single-cell suspensions of spleens using the MZ B cell markers CD 21, CD 23, B220 and IgM (Pharmingen). Measurements were taken using a FACSCanto II® (BD Bioscience, The Netherlands) and analysis was performed with the FACSDiva software v6.1.1 (BD Bioscience, the Netherlands). MZ B cells were identified by being IgM^+, $B220^+$, $CD21^{high}$ and $CD23^{low}$ positive [24]. A control group was injected with PBS.

Immunization with rh therapeutics

Memory experiment

Blood samples were drawn from the cheek pouches (submandibular) of the mice before starting the injections and on day 7, 11, 14, 21, 28, 42, 53, 56, 58, 60, 64, 65, 66 and day 67. Injections were carried out as described previously [25-27]. Briefly, mice were injected intraperitoneally (i.p.) with 10 μg of native or aggregated rhIFNα2a on days 0 to 4, days 7 to 11 and days 14 to 18. After an injection-free period of six weeks, 21 (transgenic (tg) = 11; wildtype (wt) = 10) animals were rechallenged with 10 μg of native or aggregated rhIFNα2a i.p. on days 63 and 64. Blood was collected submandibularly from seven mice per group and time point, before any injection that day. On day 77, all mice were sacrificed. Plasma was stored at -20°C until further analysis.

T cell and marginal zone B cell depletion experiments

Mice (T cell depletion: tg = 9/wt = 9; MZ B cell depletion: tg = 8/wt = 7) were immunized i.p. with 10 μg of aggregated rhIFNα2a five times a week, Monday until Friday for three consecutive weeks. Prior to immunization all animals were bled and additionally on day 9 and 16 blood from the cheek pouch was drawn from five animals per group. Animals were sacrificed on day 23 and spleens were removed, processed to a single cell solution and prepared for flow cytometry analysis.

Mixed native rhIFNα2a/aggregated Betaferon® study

Mice (tg = 6; wt = 6) were injected i.p. with 10 μg of native or aggregated rhIFNα2a or a mixture of 10 μg native rhIFNα2a and 5 μg Betaferon® on days 0 to 4, days 7 to 11 and days 14 to 18. Prior to immunization all animals were bled and additionally on day 9 and 14 blood from the cheek pouch was drawn from six animals per group. Animals were sacrificed on day 21.

Immunization with TD and TI antigens

T cell depletion and marginal zone B cell depletion

Albumin from chicken egg white (OVA; Sigma, the Netherlands) was adsorbed to aluminum hydroxide gel (Sigma, the Netherlands) according to manufacturer's instructions. Mice (T cell depletion: tg = 9/wt = 9; MZ B cell depletion: tg = 8/wt = 7) were immunized with 5 μg i.p. of OVA adsorbed to aluminum hydroxide gel in 100 μl PBS five times a week from Monday until Friday for three consecutive weeks. Pneumovax® (Sanofi Pasteur) was obtained from a local pharmacy and includes plain polysaccharide antigens from 23 different pneumococcal serotypes. Primary immunization with 1 μg i.p. of Pneumovax® in 100 μl PBS per mouse (T cell depletion: tg = 9/wt = 9; MZ B cell depletion: tg = 8/wt = 7) was done on day 0, followed by a boost injection with another 1 μg of Pneumovax® on day 11 as suggested by T.

Scheikl [28]. Plasma from all animals was collected prior to immunization, and during T cell depletion additionally on day 9 and 16 blood was collected from five animals per group. During the course of the MZ B cell depletion blood was collected on day 7, 11 and 14 from seven mice per group. Spleens were removed from all animals on day 21, processed to a single cell solution and analyzed by flow cytometry.

Binding antibody assays

Plasma was analyzed by direct ELISA, as described in detail by Hermeling et al. with adjustments [26].
Briefly, microtiter plates were coated with antigen (Supp. Table 1). Samples were added 100 fold diluted in blocking buffer and incubated for one to two hours. In case of Pneumo23 samples needed to be preadsorbed to pneumococcal cell wall polysaccharide (CWPS) antigens to capture non-specific antibodies against CWPS, a known contaminant of the Pneumovax® vaccine [28]. In brief, mouse serum was mixed with a 2 µg/ml CWPS and incubated for 30 min at room temperature. Adsorbed samples were then added 100 fold diluted in blocking buffer and incubated for two hours. After adding secondary antibodies (Supp. Table 1) color reaction was initiated by adding 3,3',5,5'-tetramethylbenzidine (TMB; Roche, the Netherlands) and stopped by 0.18 M sulfuric acid. Optical density values were measured at 450 nm wavelength on a microplate reader (Novopath, Biorad, The Netherlands) and data analysis was performed with GraphPad Prism 4.03 software (San Diego, CA, USA). Samples were considered positive when the mean was at least three fold higher than the mean of the negative plus the typical 95th percentile of the standard deviation. Titers were measured by applying serial 2-fold diluitons and following the same ELISA protocol. The plots were fitted to a sigmoidal dose-response curve and the reciprocal of the dilution of the EC50 value was considered the titer of the plasma. Each plate contained negative serum from untreated wildtype animals and positive serum from treated wildtype animals.

Neutralizing antibody assay

Plasma collected at day 77 was subjected to a neutralizing antibody (NAB) assay. The assay is based on inhibition of myxovirus resistant protein A (MxA) gene expression in an A549 (human lung carcinoma cell line) cell line as previously described [25]. Neutralizing activity was defined as the dilution of tested sample reducing rhIFNα activity and expressed in ten-fold reduction units per mL (TRU/mL), as described by Kawade [29]. Plasma samples below 270 TRU/mL were considered negative. Positive samples showed neutralizing activity ranging from 648 TRU/mL to more than 5120 TRU/mL.

Statistics

Since a Gaussian distribution was not assumed, where appropriate, a nonparametric Mann-Whitney test was applied. *P* values are presented in figures where a statistically significant difference was found.

Results

Physicochemical characterization of native and aggregated rhIFNα2a

Two batches of each sample were prepared. Reported analytical data represent the average ± lower/upper value for the two batches.

UV spectroscopy

UV spectroscopy was used for obtaining information regarding the presence of aggregates in the solution and to determine the concentration of native rhIFNα2a. A decrease in A280/A260 ratio was observed for the oxidized sample as compared to the unstressed sample, parallel to an increase in the optical density at 350 nm, indicating the presence of aggregates [30].

Size Exclusion Chromatography (SEC) studies

To confirm the presence of soluble aggregates, size exclusion chromatography was employed. The results are summarized in Table 1. Native rhIFNα2a showed a main peak of monomeric protein which represented about 99% of the protein. A small percentage of dimer was detected in both batches. The oxidized sample contained a substantial reduction in monomer content and a large fraction of higher molecular weight species, characterized by broad peaks which did not allow an exact estimation of the molecular weight.
The total peak area of the stressed sample was comparable to that of the native protein, indicating that the recovery was practically 100%, i.e. the amount of insoluble aggregates was negligible.
To confirm the presence and to purify rhIFNα2a from the mixture with Betaferon®, SEC was employed. The overlay of the chromatograms clearly shows that rhIFNα2a is in the monomeric state (Supp. Fig.2a). This technique was furthermore used to detect possible changes in Betaferon®, after the addition of the alpha protein.

No changes in the soluble fraction of Betaferon® can be seen before and after the addition of rhIFNα2a (Supp. Fig. 2b). These findings highlight that the mixing does not lead to measurable changes in the content and aggregation status of the two proteins (and HSA) according to SEC.

Table 1. Summary of SEC results of rhIFNα2a and its oxidized form.

Samples	Soluble fraction[1]		Insoluble fraction[3]
	Monomer	Larger aggregates[2]	
Native rhIFNα2a	99.0 ± 0.6	1.1 ± 0.6	0
Oxidized rhIFNα2a by metal catalysis	55.3 ± 8.5	44.7 ± 8.5	0

[1] Percentages were calculated based on SEC peak areas relative to the total peak area in SEC of native rhIFNα2a: $AUC_{peak}/AUC_{native,\ total}$ x 100%

[2] For the oxidized protein due to the broad peaks (indicating a heterogeneous size population), an exact estimation of the molecular weight of the multimers was not achievable.

[3] The insoluble fraction was defined as the fraction that was not recovered by SEC; percentages were calculated from the total peak area in SEC and the total peak area in SEC of native rh IFNα2a: $(AUC_{native,\ total}-AUC_{sample,\ total})/AUC$x 100%

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to investigate the presence of covalent aggregates, SDS-PAGE gels under nonreducing and reducing conditions were run (Fig. 1).

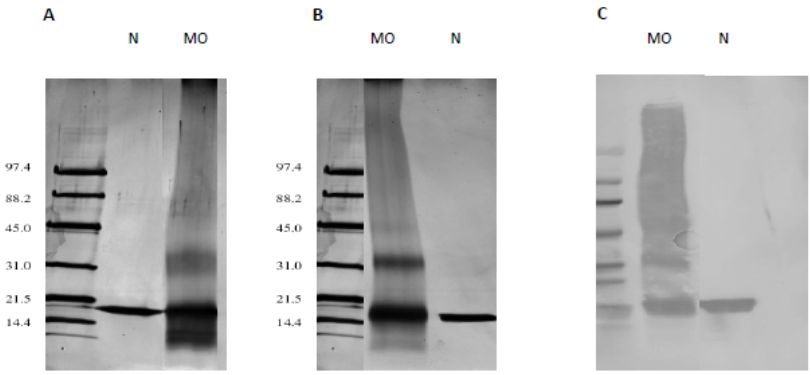

Figure 1. SDS-PAGE of native (N) and oxidized (MO) rhIFNα2a under reducing conditions (A), nonreducing conditions (B). Western Blotting obtained from gel run under nonreducing conditions (C)

Native rhIFNα2a showed a monomer band at about 16.9 kDa under both conditions. For the metal oxidized, only small differences were detected between gels run under nonreducing and reducing conditions, indicating that the formation of the higher molecular weight species was not completely mediated by disulfide bonds. These findings are in contrast with our previous results, when reducible covalent aggregates were found for rhIFNα2b oxidized following the same procedure [10], which may be due to slight differences in experimental procedures and/or the different protein source. For the mixed aggregate study a band with the same molecular weight as native rhIFNα2a was found for the rhIFNα2a recovered using the SEC system (Supp Fig. 3). The analysis of Betaferon® alone and when mixed with rhIFNα2a shows almost identical bands, since the molecular weight of the alpha and beta IFNs are similar and therefore could not be resolved from each other.

Western Blotting

The presence of immunodominant epitopes was tested with rabbit polyclonal antibody against human interferon alpha. The monomer as well as the multimeric species present in metal catalyzed oxidized IFNα2a reacted with the antiserum as the untreated protein, pointing that the stress applied did not destroy the epitopes which are recognized by the antiserum (Fig. 1).

Another analysis was performed to investigate the presence of immunodominant epitopes in the rhIFNα2a recovered from the mixture study. The native protein and the one recovered from the HP-SEC system both reacted with the antiserum, so rhIFNα2a in the mixture with Betaferon® had maintained native epitopes.

FAR-UV CD

This spectroscopic technique provides valuable information about the secondary structure of a protein [31]. Native IFNα2a presents five α-helices [32], as reflected by a spectrum characterized by two minima at 208 and 222 nm, respectively (Supp. Fig. 1). Oxidized rhIFNα2a showed a small reduction of the intensity of the entire spectrum, indicating a decrease in α-helical content but not major changes towards different secondary structures.

Intrinsic fluorescence spectroscopy

Fluorescence spectroscopy was employed to investigate the environment of the tryptophans. Native rhIFNα2a showed an emission maximum at 326 ± 1 nm. The emission spectrum of the stressed sample was red shifted to 330 ± 1 nm and showed a considerable decrease in intensity, indicating a substantial change in the tertiary structure (Supp. Table 2).

Immunization studies

Interferon alpha fails to initiate production of memory B cells

In our mouse model immune tolerant for human interferon beta, rechallenge with either non aggregated or aggregated material failed to induce a memory response [27]. To asses if this phenomenon also occurs with other recombinant human therapeutics, we immunized both transgenic (tg) and wildtype (wt) animals with native and aggregated rhIFNα2a for three consecutive weeks, followed by a six-week injection-free period to allow the body to clear all drug from the blood, thereby initiating termination of

antibody production. After a total of 9 weeks after start of initial immunization, animals were rechallenged with either native or aggregated rhIFNα2a.

Native rhIFNα2a did not elicit an immune reaction in most of the tg animals, whereas robust IgG titers could be measured in the wt animals (Fig. 2 A & C). Aggregated rhIFNα2a was capable of inducing ADA production in the tg animals and the wt animals reacted as expected with high IgG titers (Fig. 2 B & D). Tg animals being immunized with native rhIFNα2a still had no measurable IgG titers after rechallenge with native rhIFNα2a, whereas the wt animals showed high IgG titers, even higher than after the initial injections, hence indicating memory effect (Fig. 2 A). Comparable results could also be observed in the native rhIFNα2a immunized and with aggregated rhIFNα2a rechallenged animals (Fig. 2 C). Tg animals being immunized with aggregated rhIFNα2a and rechallenged with native rhIFNα2a showed no significantly higher titers after the rechallenge (Fig. 2 C), whereas the wt animals showed high IgG titers, even higher than after the initial injection (Fig. 2 B). The same was true for animals being immunized and rechallenged with aggregated rhIFNα2a (Fig. 2 D). The same trend in titer development could be observed with the isotype IgG1 present in both tg and wt mice (Supp. Fig. 4).

In figure 2 A and C one tg animal reacted as if it was a wt animals with high and persistent titers, a phenomenon already observed in our immune tolerant mouse model for human interferon beta [9, 27]. The same was true for titers measured in tg animals immunized with aggregated rhIFNα2a (Fig. 2 B & D), which could explain why titers were slightly but not significantly increased after rechallenge. We also saw the opposite effect in the wt animals, i.e. outliers with lack of both binding and neutralizing antibodies. In case of the wt animals acting like tg animals, false interpretation of the PCR used for genotyping could be reasoned. For the transgenic outliers it could be argued that these mice carried a dysfunctional human gene, subsequently leading to a dysfunctional expression of the human protein and lack of tolerance. The heterozygous animals were bred with wt in-house animals or animals ordered at the Centre d'Elevage Janvier (France), both inbred strains in which genetic drift is a known phenomena [33] and which could explain the outliers. Since outliers did not occur group-specific but were evenly distributed among the groups, we considered them to be true outliers. Nevertheless, they did not affect the outcome of the statistical analysis and were included.

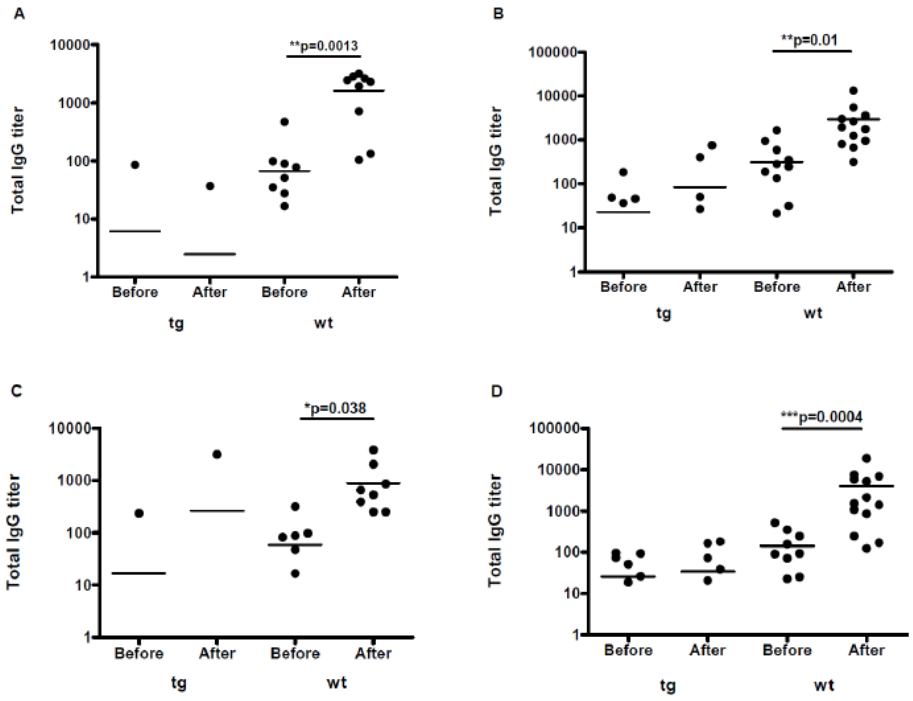

Figure 2. No memory formation in tg animals (tg n=11; wt n=10). (A) Total IgG titers of animals being immunized and rechallenged with native rhIFNα2a; (B) total IgG titers of animals being immunized with aggregated rhIFNα and rechallenged with native rhIFNα2a; (C) total IgG titers of animals being immunized with native rhIFNα2a and rechallenged with aggregated rhIFNα2a; (D) total IgG titers of animals being immunized and rechallenged with aggregated rhIFNα2a. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. Before rechallenge covers day 53, 56, 58 and 60; after rechallenge covers day 67 and 77. P values indicate a significant increase in IgG titers after rechallenge. * p< 0.05; ** p< 0.01; *** p<0.001. Due to handling, liver or heart problems, four out of 84 mice died during the study.

Lack of neutralizing anti-interferon alpha antibodies

During a TD immune response, activated B cells can undergo two developments: (1) maturation into short-lived plasma cells, or (2) formation of a germinal center in which hypermutation is initiated and antigen-specific B cells become high-affinity long-lived memory B cells [34]. These processes are normally T-cell dependent and include isotype switching and affinity maturation to ensure survival of the host by production of high-affinity neutralizing antibodies and memory B cells.

The lack of memory in the tg animals but the presence of isotype switched B cells raised the question if neutralizing antibodies were present. Therefore we measured neutralizing antibodies (NABs) against rhIFNα2a in our mouse model after rechallenge on day 77. Wt animals showed production of binding antibodies and neutralizing antibodies, whereas the tg animals treated with aggregated rhIFNα2a gave only rise to binding antibodies (Fig. 3). Nevertheless, some tg animals showed neutralizing antibodies but they could be related to the outliers identified in the section described above.

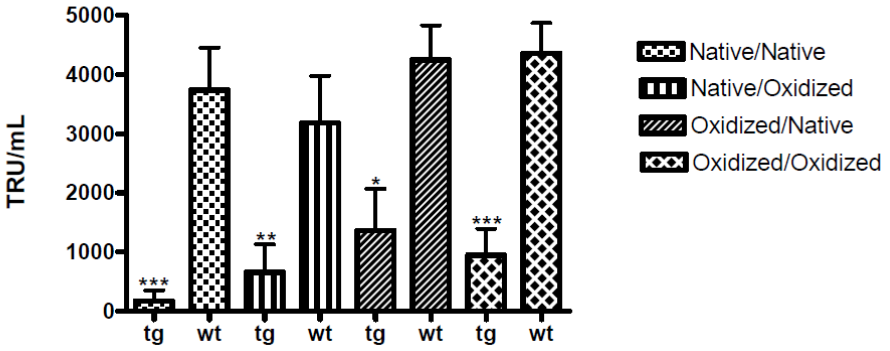

Figure 3. Neutralizing antibody titers from day 77 expressed in ten-fold reduction units per milliliter (TRU/mL). Legend indicates immunization/rechallenge regime. Each bar represents the mean of at least 10 mice per group and the SEM. P values indicate a significant difference in neutralizing antibody titers. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The role of CD4+ T cells

CD+ T cells are helper cells, supporting the B cells by presenting TD antigens to them and providing cytokines for maturation to plasma and memory B cells [35]. Immune processes involving TD antigens lead to antigen specific antibody production and formation of memory to provide instant protection against pathogens invading the body a second time. TI-2 antigens follow different immunological pathways and do not involve CD4+ T cells but may directly interact with B cells by cross linking their B cell receptors (BCRs). However, TI-2 immune processes do not lead to the generation of memory B cells [12], thus making us wonder if CD4+ T cells were necessary to induce the formation of ADAs in our model. To investigate the role of CD4+ T cells

in our model, animals were divided into two groups, (a) CD4+ T cell depleted (-) and (b) CD4+ T cell undepleted (+). Both depleted and undepleted groups received the same treatment: (1) a TD antigen, ovalbumin, (2) a TI antigen, Pneumo23 (polysaccharide vaccine), and (3) aggregated rhIFNα2a. The TD antigen ovalbumin initiated a robust IgG response in undepleted animals, regardless of their genotype (Fig. 4 B). Pneumo23 as a typical TI-2 antigen showed no differences in IgG titers between all animals, in spite of their genotype and depletion status (Fig 4 C). Undepleted tg animals treated with aggregated rhIFNα2a developed measurable IgG titers (Fig. 4 A). Upon CD4+ T cell depletion, both tg and wt did not form anti-IFNα antibodies anymore. Subtyping of the immune response into IgG1 and IgG2a titers followed the same trend as the total IgG titers of undepleted tg and wt animals treated with aggregated rhIFNα2a (Supp. Fig. 5).

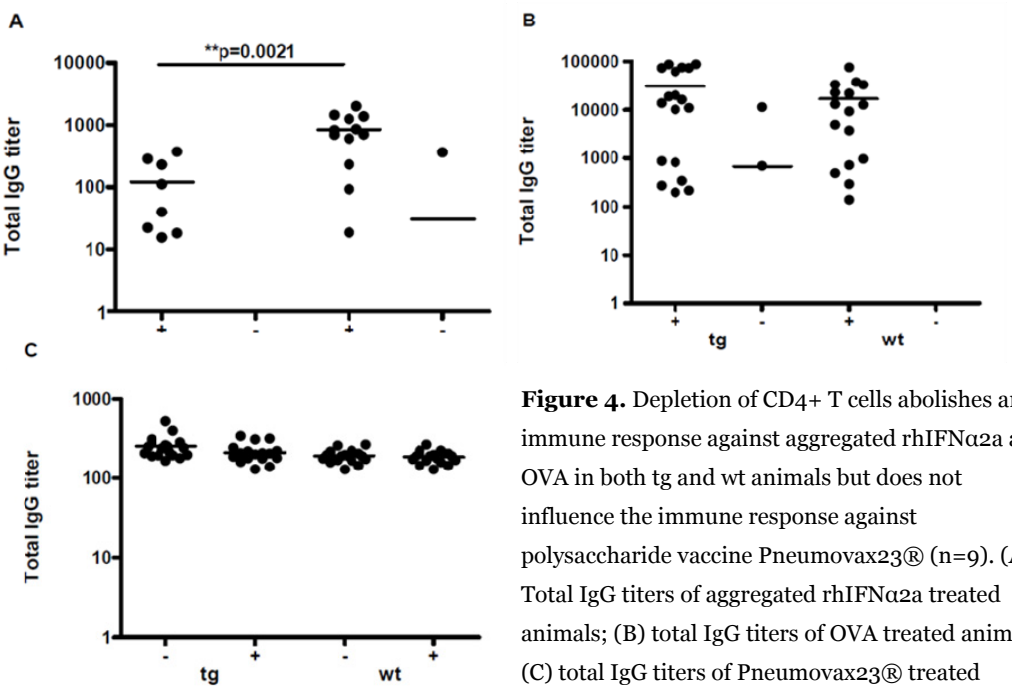

Figure 4. Depletion of CD4+ T cells abolishes any immune response against aggregated rhIFNα2a and OVA in both tg and wt animals but does not influence the immune response against polysaccharide vaccine Pneumovax23® (n=9). (A) Total IgG titers of aggregated rhIFNα2a treated animals; (B) total IgG titers of OVA treated animals; (C) total IgG titers of Pneumovax23® treated animals. (-) reflects titers from depleted animals, (+) from undepleted animals. Each symbol represents an individual plasma sample; small horizontal lines indicate mean. Four out of 122 mice died during the study due to handling.

The role of marginal zone B cells

To examine the role of MZ B cells in the formation of ADAs we treated mice with monoclonal antibodies against LFA and α_a integrins, two integrins which direct the MZ B cells to the sinuses of the spleen. This treatment results in selective depletion of the MZ B cells without affecting other immune cells [36]. The depletion regime resulted in MZ B cell depletion for about one week before newly generated MZ B cells repopulated the spleen, but depletion could be renewed after a second round of monoclonal antibody treatment. At the time treatment with aggregated rhIFNα2a, ovalbumin and Pneumo23 started, MZ B cell numbers had dropped to about 20% (Supp. Fig 6).

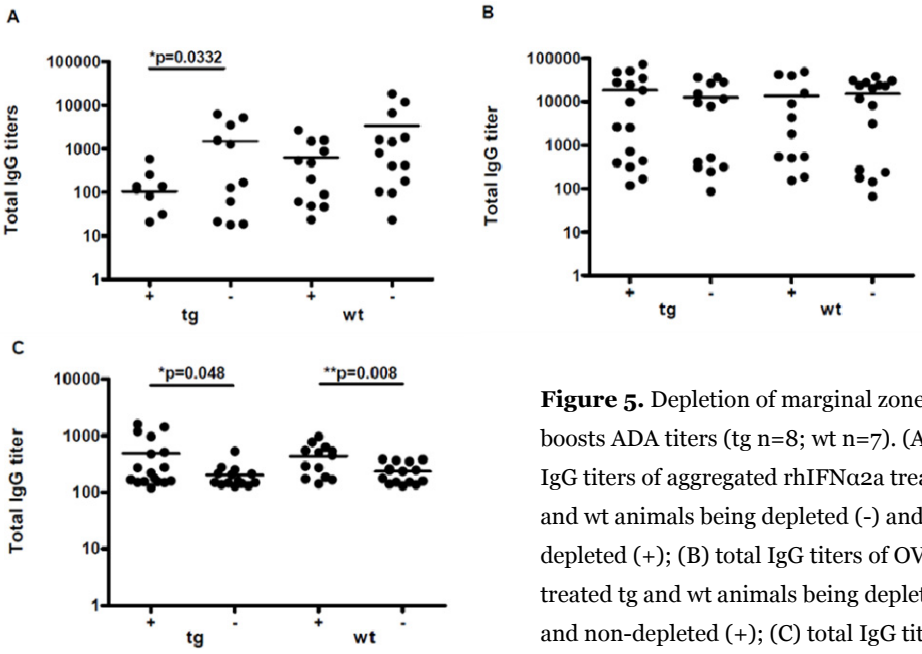

Figure 5. Depletion of marginal zone B cells boosts ADA titers (tg n=8; wt n=7). (A) Total IgG titers of aggregated rhIFNα2a treated tg and wt animals being depleted (-) and non-depleted (+); (B) total IgG titers of OVA treated tg and wt animals being depleted (-) and non-depleted (+); (C) total IgG titers of Pneumovax23® treated tg and wt animals being depleted (-) and non-depleted (+). Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. P values indicate significant differences in IgG titers in depleted compared to non-depleted animals. * p< 0.05; ** p< 0.01; *** p<0.001.

Ovalbumin treated tg and wt animals showed no difference between IgG titers whether depleted or undepleted (Fig. 5 B), whereas the Pneumo23 treated animals showed reduced IgG titers in both wt and tg animals after depletion (Fig. 5 C). In contrast, tg animals being treated with aggregated rhIFNα2a and depleted of their MZ B cells showed significantly increased IgG titers with respect to undepleted tg animals. Anti-IFNα IgG titers of the depleted tg animals were now comparable to IgG titers of wt animals, whether depleted or undepleted (Fig. 5 A), a phenomena which could also be observed by measurements of isotypes IgG1 and IgG2a (Supp. Fig.7).

Protein aggregates fail to induce production of ADAs against a co-administered native therapeutic protein

Protein aggregates are thought to induce formation of ADAs due to their repetitive epitopes [15]. To investigate if the presence of repetitive epitopes alone triggers an immune response against a nearby native protein, we mixed native rhIFNα2a with Betaferon®, a highly aggregated IFNβ product [9, 27]. As described above, we ensured that the recovery of the two proteins after mixing did not induce aggregation of the native rhIFNα2a (Suppl. Fig. 2). All native material could be recovered. We then immunized tg and wt animals with each therapeutic alone and the mixture. Injection of plain native rhIFNα2a only evoked an immune response in the wt animals, whereas the aggregated material induced anti-IFNα2a antibodies in both tg and wt animals (Fig. 6 A). In the case of the mixture of native rhIFNα2a with Betaferon®, no induction of anti-IFNα2a antibodies in the tg animals could be seen, in contrast to a robust response in the wt animals (Fig. 6 A). Additionally, the total IgG titers of the wt animals treated with either native, aggregated rhIFNα2a or the mixture native rhIFNα2a/Betaferon® did not differ from each other. Both tg and wt animals developed a robust and comparable IgG response against Betaferon® (Fig. 6 B).

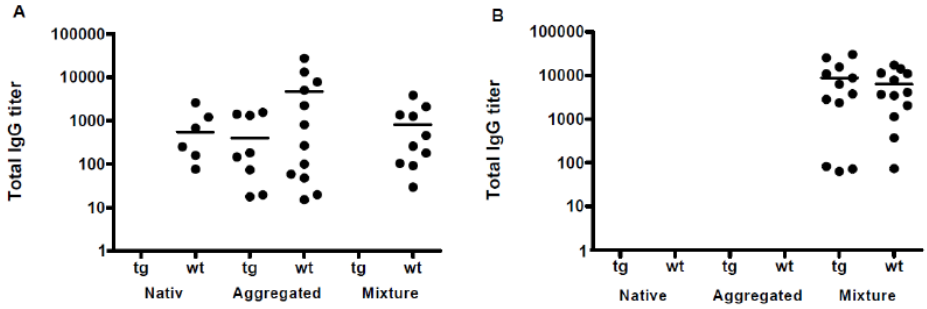

Figure 6. Interferon beta aggregates do not induce immune response when mixed and co-administered with native rhIFNα2a (n=6). (A) total IgG titers against rhIFNα2a in both wt and tg animals being treated with either native rhIFNα2a, aggregated rhIFNα2a or a mixture of native rhIFNα2a and interferon beta aggregates (Betaferon®) (B) total IgG anti-IFNβ antibodies. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean.

Tg IgG titers were significantly lower compared to wt IgG titers throughout all studies

Throughout all experiments we observed significantly lower titers in the tg animals compared to the wt animals. Figure 7 summarizes these observations, indicating the incapability of the rh therapeutic to efficiently activate and sustain B and T cell activation, leading to low titers.

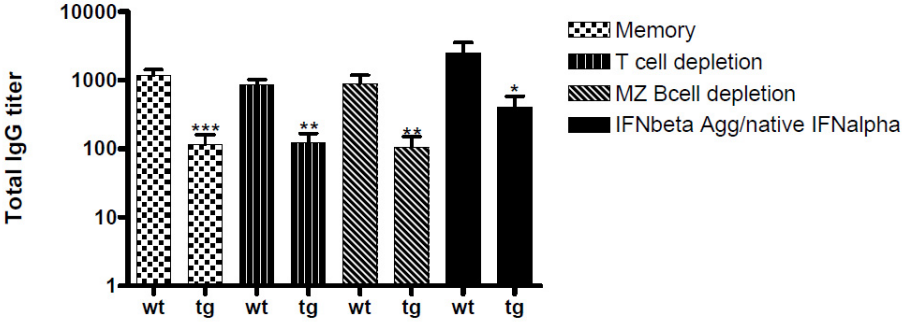

Figure 7. Significant differences between total IgG titers from aggregated rhIFNα2a treated wt and tg animals in all experiments at all time points presented in this study. Legend indicates the *in vivo* study. Each bar represents the mean of total IgG titers of aggregated rhIFNα2a treated wt or tg mice and the SEM. P values indicate significantly lower IgG titers in tg versus wt animals. p< 0,05; ** p< 0,01; *** p<0,001

Discussion

Here, we explored the immunological processes and immune cells taking part in forming the response against aggregated rh therapeutics, in specific IFNα2a. We used mice being immune tolerant for human IFNα to investigate the occurrence of memory upon rechallenge with native and aggregated material and could not find formation of an immunological memory. Though the absence of memory B cells suggested a T cell independent mechanism, we found that CD4+ T cells were crucial for the formation of ADAs. Nevertheless, it should be considered that all CD4+ bearing cells were depleted throughout the experiment and more fine-tuned experiments such as transient depletion or specific depletion of T cell subset like regulatory T cells or Th_{17}. A matter of interest remains if activated B cells recruit their own CD4+ T cells by taking the role of antigen presenting cells, or if other mechanisms and immune cells activate T cells to support maturation of B cells into plasma B cells.
The immune response triggered by aggregation is possibly due to the repetitive epitopes being displayed on their surface. Repetitive structures as seen with virus-like particles have been reported to induce production of antibodies [37-38]. Hydrophobic moieties may also influence the immunogenic potential of a protein [39], such as functioning as a danger signal to the immune system, or a combination of both the former and the latter aspect. Another possibility is the presence of a small number of foreign T cell epitopes in the rh therapeutic due to manufacturing processes. The immunogenic potential of rh IFNβ-1b (Betaferon®) is believed to be mostly dependent on its high aggregation level, but also on the lack of glycosylation and difference in amino acid sequence compared to natural IFNβ [26, 40]. It is believed that the repetitive epitopes of aggregated rh therapeutics are capable of cross linking the B cell receptor, like TI-2 antigens. It was recently shown that both charge and number of presented epitopes influence the potential to trigger the B cell receptor, but that distance of the repetitive epitopes did not matter [41], explaining in part the diverse response seen against rh therapeutics in tg animal models where immunogenicity was linked to aggregation level [10, 42].
Although we found a lack of memory, but a dependency on CD4+ T cells and abundant presence of isotypes IgG1 and IgG2a, the question remains how the T-cell/B-cell interaction takes place. It is surprising that the B cells mature to plasma cells secreting different IgG subtypes, probably with the help of T

cells, but then retain in their maturation level and fail to become high affinity memory B cells. Though we compare rh protein drugs to TI-2 antigens due to their ability to cross-link the BCR, one should not forget that rh therapeutics still are self-proteins. TI-2 antigens also carry other foreign motifs which may activate innate cells to support B cell maturation in the absence of T cells. The rh therapeutics probably lack these additional motifs, thus providing too weak a signal to support a proper B cell development. It can also be speculated that the foreign T cell epitopes a rh protein drug may carry, are not immunodominant and thereby also fail to prolong the immune response. Other observations supporting that idea are the lower IgG titers in tg compared to wt animals. After the initial activation of the B cells by cross-linking the BCR, the lack of co-stimulatory signals probably led to a termination of the immune response against the aggregated rh therapeutic. It has been shown that without proper germinal center formation an induction of hypermutation in B cells rarely occurs and most plasma B cells undergo apoptosis without producing high-affinity antibodies or becoming memory B cells [43].

Another explanation for the absence of memory B cells could be the involvement of another set of B cells, such as MZ B cells. It was recently shown that haptens, which present repetitive epitopes, did produce plasma cells but were not involved in germinal center reactions, essential for development of memory B cells [44], thus presenting a possible link to our observed lack of memory. Therefore our interest was evoked to investigate the influence of MZ B cells, which have different functions and pathways than conventional B-2 B cells [17, 45]. To our surprise, depletion of MZ B cells resulted in a boost in ADA formation rather than an impairment. It could be argued that after MZ B cell depletion conventional B-2 B cells compensated for the loss, since it was recently shown that B-2 B cells can function like MZ B cells [46]. Another factor to keep in mind is the biological activity of rhIFNα in our mouse model, which may have influenced the MZ B cell and up until now the direct effect of interferons on MZ B cells is now not known.

Overall it became clear that the immune response against aggregated material cannot be explained by conventional immunological concepts and that the aggregates do play a role. When aggregated material was co-administered with another native protein, no antibodies where formed against the native protein, thus showing that the immune response against aggregated protein drugs is specific and not only a bystander effect caused by aggregations per se. But our experiment could have been biased, since both

aggregated and native proteins could have disassociated after injection, thereby neither internalized nor presented together by the same immune cell. For a proper evaluation of the precise influence of aggregated structure on native proteins, both should be internalized together by one immune cell or be in close proximity of a B cell, how preciously this can be done remains to be investigated.

In conclusion, this model has shown that aggregated rhIFNα2a leads to the formation of ADAs, but does not lead to B cell memory. In contrast, depletion of CD4+ T cells abolished the antibody response, indicating the formation of ADAs induced by aggregated therapeutic protein relies upon CD4+ T cells. Additionally, reduction of MZ B cells resulted in an increase in ADA titers, suggesting a role of MZ B cells in the formation of ADAs. Overall, it can be concluded that the antibody response against aggregated rh therapeutics is neither fully comparable to TI-2 nor classical TD antigen-induced responses probably due to being a self protein and that many open questions remain. Nevertheless, the mouse model presented here is a useful tool to investigate the immunological mechanism underlying the formation of ADAs. In accordance to our mouse model immune tolerant for human interferon beta [9, 26-27], these models may mark the first steps for characterizing the immune responses set in motion after administration of aggregated rh therapeutic proteins. The research presented in this paper may set the basics to evaluate immunogenic potential of other aggregated therapeutics in appropriate mouse models prior to entry into man.

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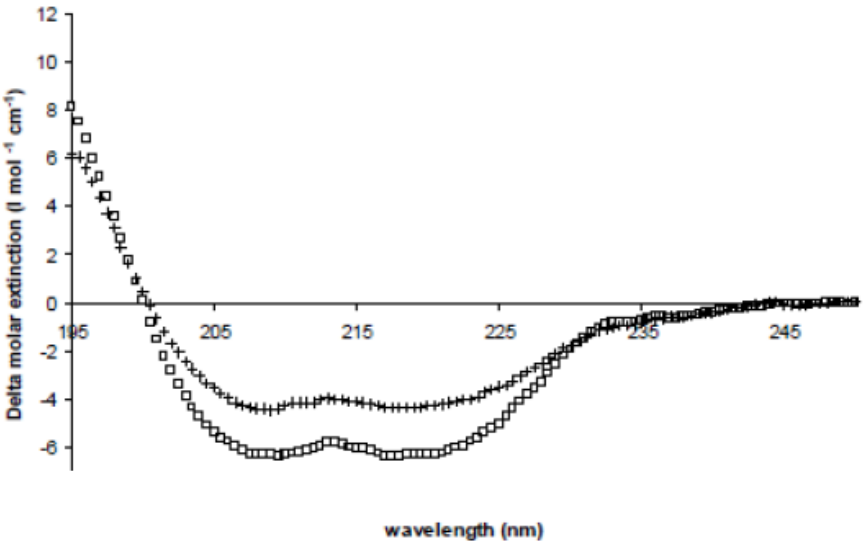
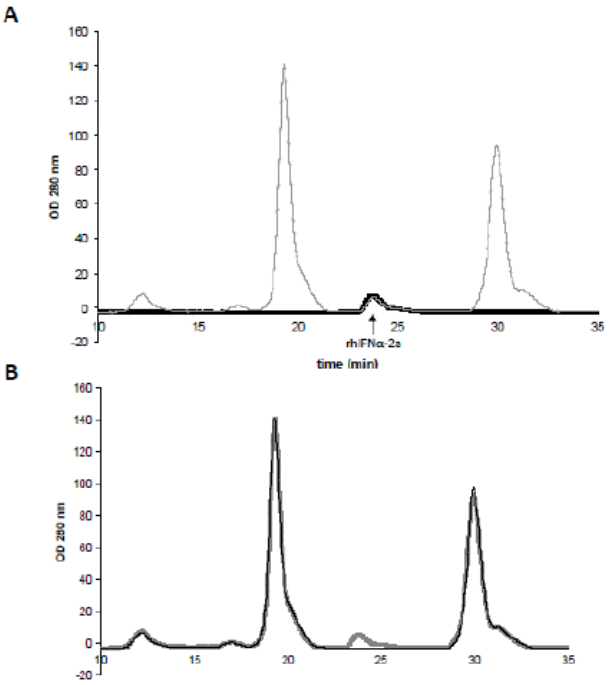
Supplementary Material

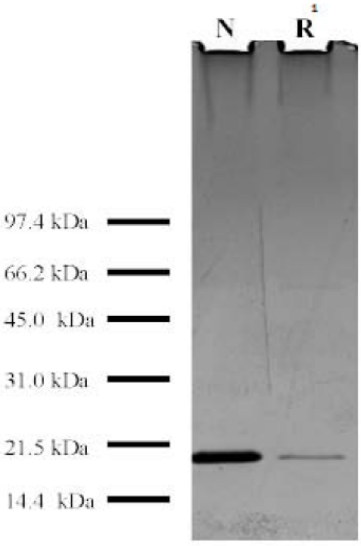


Supplementary Figure 1. Far-UV CD spectrum of native (▫▫▫) and oxidized (+++) IFNα-2a.

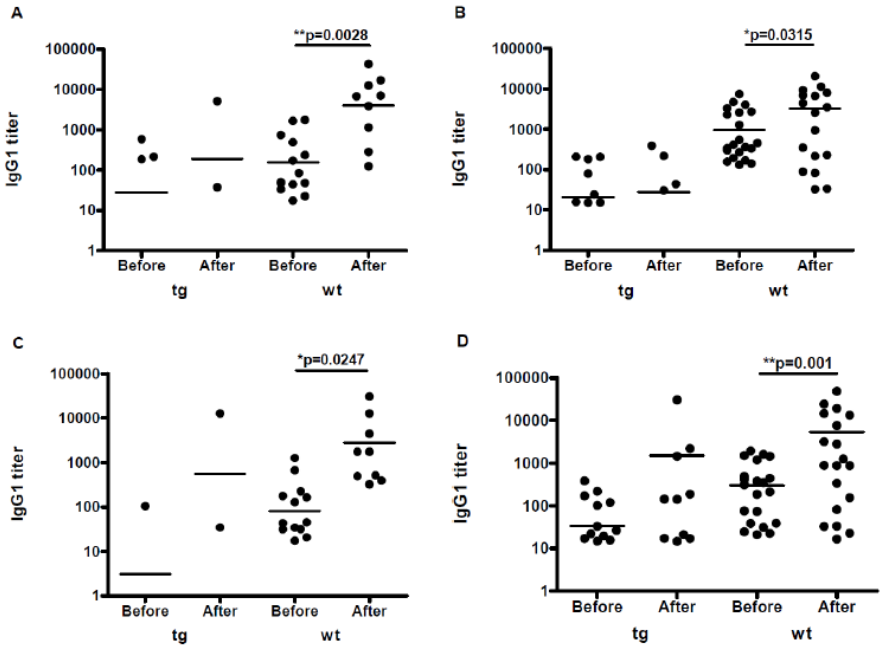


Supplementary Figure 2. (A). HP-SEC of native rhIFNα2a (black line) and the mixture Betaferon® /rhIFNα2a (grey line). The identity of the peaks from left to right is: Larger aggregates, HSA dimer, HSA monomer, rhIFNα2a, excipients used in the Betaferon® formulation[1] **(B)** HP-SEC of Betaferon® (black line) and the mixture Betaferon® /rhIFNα2a (grey line). The identity of the peaks from left to right is: Larger aggregates, HSA dimer, HSA monomer, rhIFNα2a, excipients used in the Betaferon®

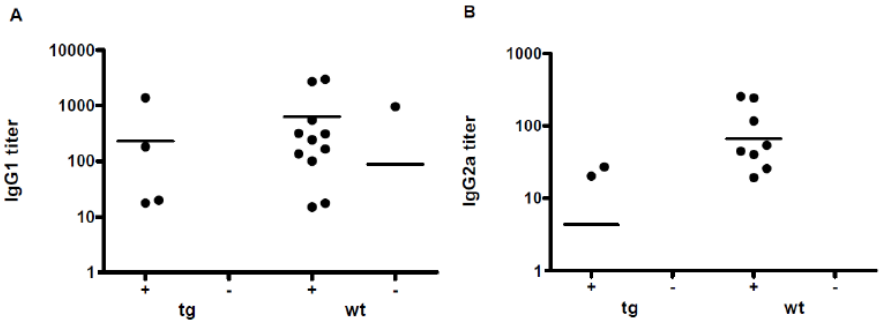
[1] The identity of the excipients has not been confirmed.



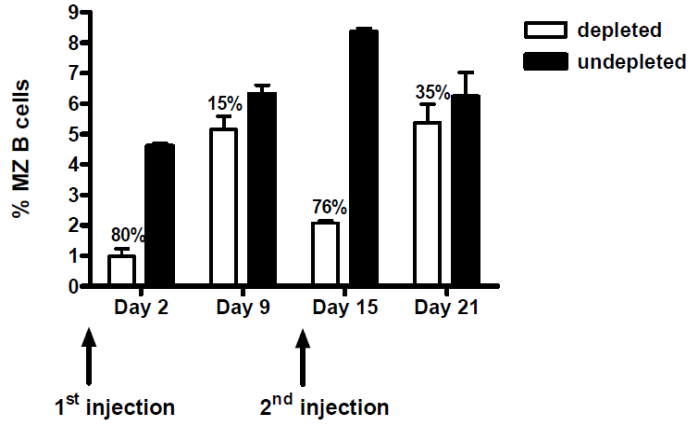
Supplementary Figure 3. SDS-PAGE of native rhIFNα2a (N) and rhIFNα2a recovered from the mixture with Betaferon®
[1] The sample has not been concentrated after being recovered from the HP-SEC



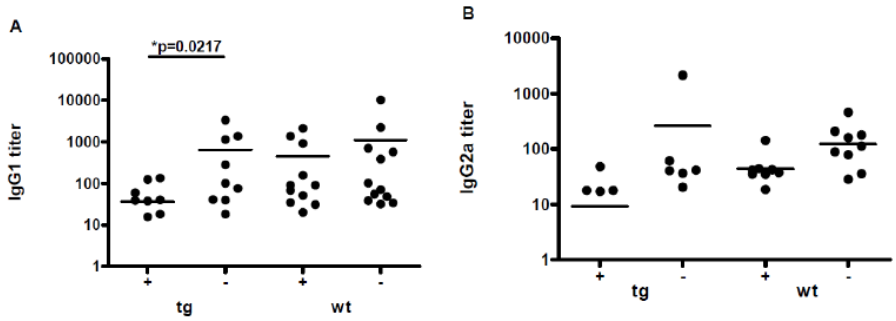
Supplementary Figure 4. IgG1 titers confirm the absence of memory formation in tg animals (tg n=11; wt n=10) (A) Total IgG titers of animals being immunized and rechallenged with native rhIFNα2a; (B) total IgG titers of animals being immunized with aggregated rhIFNα and rechallenged with native rhIFNα2a; (C) total IgG titers of animals being immunized with native rhIFNα2a and rechallenged with aggregated rhIFNα2a; (D) total IgG titers of animals being immunized and rechallenged with aggregated rhIFNα2a. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. Before rechallenge covers day 53, 56, 58 and 60; after rechallenge covers day 67 and 77. P values indicate a significant increase in IgG titers after rechallenge. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Due to handling, liver or heart problems, four out of 84 mice died during the study



Supplementary Figure 5. IgG1 and IgG2a titers are also absent in CD4+ T cells depleted (-) animals treated with aggregated rhIFNα2a in both tg and wt animals (n=9). (A) IgG1 titers (B) IgG2a titers. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. P value indicates a significant lower IgG2a titer in tg undepleted (+) animals compared to wt undepleted (+) animals. * p< 0,05; ** p< 0,01; *** p<0,001. Four out of 122 mice died during the study due to handling.



Supplementary Figure 6. IgG1 and IgG2a titers reinforce the observed boost in ADA formation in tg animals treated with oxidized IFNα and depleted of their marginal zone B cells (tg n=8; wt n=7). (A) IgG1 titers of oxidized IFNα treated tg and wt animals being depleted (-) and non-depleted (+) (B) IgG2a titers of oxidized IFNα treated tg and wt animals being depleted (-) and non-depleted. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. P values indicate significant lower titers in non-depleted tg animals compared to non-depleted wt animals. * p< 0,05; ** p< 0,01; *** p<0,001.



Supplementary Figure 7. IgG1 and IgG2a titers reinforce the observed boost in ADA formation in tg animals treated with aggregated rhIFNα2a and depleted of their MZ B cells (tg n=8; wt n=7). (A) IgG1 titers of aggregated rhIFNα2a treated tg and wt animals being depleted (-) and undepleted (+) (B) IgG2a titers of aggregated rhIFNα2a treated tg and wt animals being depleted (-) and undepleted. Each symbol represents an individual plasma sample; small horizontal lines indicate the mean. P value indicates significant higher titers in the depleted tg animals compared to undepleted tg animals. * p< 0,05; ** p< 0,01; *** p<0,001.

Supplementary Table 1. ELISA conditions detecting anti-IFNα, anti-IFNβ, anti-OVA, and anti-Pneumo23 antibodies[1]

	IFNα, IFNβ, OVA	Pneumo23
Coating concentration	2 µl/ml	0.05 µl/ml
Preadsorption plasma	-	30min, 2 µg/ml CWPS
Incubation temperature	RT	37°C
Blocking reagent	4%milk/PBS/ 0.05% Tween20	1%BSA/PBS/ 0.1% Sodium Acide/0.05% Tween20
2nd antibody dilution	total IgG (Invitrogen, Breda, the Netherlands) 1:4000; IgM (Invitrogen), IgG1 (Invitrogen), IgG2a (Abcam, Cambridge, UK) all 1:2000	

[1]Listed are concentrations of coating reagents, incubation temperature, blocking reagent and dilution of 2nd antibodies.

RT = room temperature; BSA = bovine serum albumin

Supplementary Table 2. Spectroscopic characteristics of native and oxidized rhIFNα2a.

Samples	UV Optical density		CD Δε			Fluorescence maximum	
	350 nm	280/260 nm	208 nm	222nm	208/222 nm	λ (nm)	Intensity (a.u)
Native IFN alpha 2a	6.0×10^{-3} $\pm 2 \times 10^{-3}$	1.85 ±0.03	-5.34 ±0.97	-5.03 ±0.96	-1.06 ±0.01	326 ±1	19.7 ±0.7
Oxidized by metal catalysis IFN alpha 2a	0.22 ±0.07	1.10 ±0.15	-3.12 ±1.28	-2.89 ±1.16	-1.08 ±0.01	330 ±1	7.9 ±1.3

Chapter 5

Marginal zone B cells influence magnitude of antibody response against Betaferon® in immune tolerant mice

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Manuscript in preparation

Abstract

Immunogenicity of recombinant human (rh) therapeutic drugs is a known problem. Little is understood about the immunological mechanism underlying the formation of anti-drug antibodies (ADAs). We have previously shown that Betaferon®, a rh interferon beta (IFNβ) 1b product containing high levels of aggregates, induces formation of ADAs in an immune tolerant mouse model. Additionally, though we could not detect presence of memory after rechallenge, we saw a dependency on the presence of CD4+ T cells. These observations on the immune response against Betfaeron® led us to believe that mechanisms of ADA formation are unconventional and differ from the classical T-cell/B-cell interactions. Given the absence of memory but dependency on CD4+ T cells, we hypothesized that marginal zone (MZ) B cells, a B cell subset, might be the source of ADAs. Here we investigated the effect of depletion of MZ B cells on the immune response against Betaferon® in an immune tolerant mouse model. Hybrid mice bred on a mixed C57BL/6 and FVB/N background immune tolerant for human IFNβ were depleted of their MZ B cells by using monoclonal antibodies directed against $\alpha L\beta_2$ and α_4 integrins. The absence of MZ B cells during the first phase of immunization led to a decrease of ADA titers. Once the spleen was repopulated with MZ B cells an apparent compensation mechanism led to comparable titers between depleted and undepleted tg animals in a later phase of antibody formation. Our data are the first to support the involvement of MZ B cells in the formation of ADAs to rh interferon beta.

Key words: Aggregated recombinant human therapeutics, marginal zone B cells, mouse model, anti-drug antibodies

Introduction

Recombinant human (rh) protein therapeutics are a major class of drugs and due to their homology to the native human protein have been considered nonimmunogenic. Nevertheless, it has become clear that immunogenicity of rh therapeutics is a frequently occurring phenomenon [1-2]. In cases where patients carry a genetic defect and therapeutic drug functions as a substitution n, as in the case of rh factor VII, the immune system lacks tolerance and the administered rh protein is considered foreign [3]. In cases where the patients are immune tolerant, it is believed that aggregation of the therapeutic drug causes immunogenicity, mainly due to aggregates presenting repetitive epitopes on their surface and thereby cross-linking and activating B cells to produce ADAs [4]. Temporal breach of tolerance by aggregated rh proteins has been demonstrated in animal models immune tolerant for the administered therapeutic protein [5-6].

It is currently under intense investigation how an antigen precisely triggers the B cell and more specific, its B-cell receptor (BCR) [7-8]. It was recently shown that the distance between presented epitopes is not as important as the valency to efficiently trigger the BCR [9]. Another interesting cell population in the context of aggregates are MZ B cells. MZ B cells are a subset of B cells, besides B-1 B cells and follicular B-2 cells [10-12], being involved in the first line of defense against blood-borne pathogens, especially those carrying T-cell independent antigens, and they have the capability to bridge the innate and the adaptive immune response [13].

In previous studies we investigated the potential of Betaferon® to initiate formation of ADAs in an immune tolerant mouse model [14-15] and we also showed that rechallenge with Betaferon® did not elicit a secondary immune response [6]. In addition, we found a pivotal role for CD4 T cells (manuscript submitted/chapter 3). Here we report the effect of depletion of MZ B cells on the antibody formation induced by Betaferon® in our immune tolerant model.

Results and Discussion

MZ B cell depletion was achieved by injection of two antibodies, anti-LFA ($\alpha L\beta_2$) and anti-CD49d (α_4). Depletion efficiency reached up to 97% after the first injection, dropped to 55% until the second injection on day 12 and raised again up to 80% on day 16 to finally drop to 59% at the day when animals were sacrificed (Figure 1).

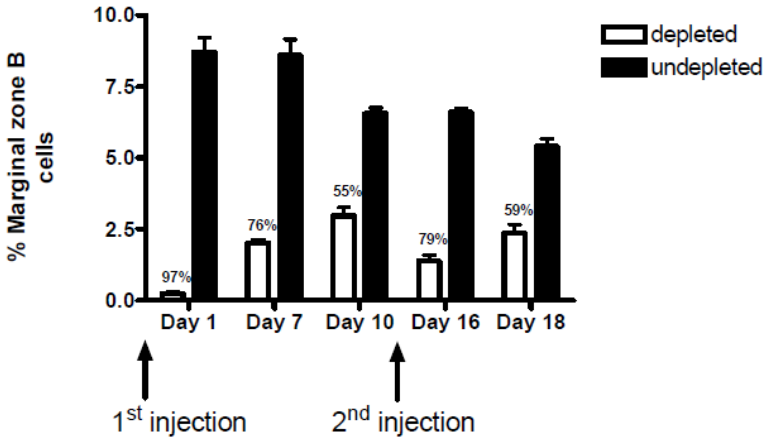

Figure 1. MZ B cell depletion efficiency rate as monitored by flow cytometry using general B cell ($B220^+$ and IgM^+) and MZ B cell specific surface markers ($CD21^{high}$, $CD23^{low}$). Four animals (depleted n=2, undepleted n=2) were sacrificed and spleens removed to be analyzed for depletion efficiency on day 1, 7, 10, and 16. On day 18, spleens from eight animals (depleted n=4, undepleted n=4) were taken and processed to be analyzed. Figure shows the averaged percentage of MZ B cells from whole B cell pool ($B220^+$ and IgM^+).

MZ B cell depletion affects first phase of immune response against Betaferon®

Figure 2 summarizes the total serum IgG titers obtained from the tg and wt animals treated with Betaferon® and either depleted of their MZ B cells or undepleted on 4 different blood collection days. By comparing IgG titers of

depleted and non-depleted tg animals, no ADAs could be measured at day 8 in the depleted tg animals, whereas significant difference could be found on day 10 and 14 (Fig. 2A). On day 18 the titers between the depleted and undepleted animals were comparable. The wt animals did not show any significant differences between depleted and undepleted animals (Fig. 2B). Depletion of the MZ B cells influenced the early response against Betaferon® in the tg animals and led to significantly decreased ADA titers, thus indicating the importance of MZ B cells in the formation of ADAs at the early onset of antibody production. It is likely that due to the aggregates in Betaferon® repetitive epitopes are present and exposed to the MZ B cells leading to cross-linking of the BCR and activation of the MZ B cells in the absence of T cells, such as seen with TI-2 antigens.

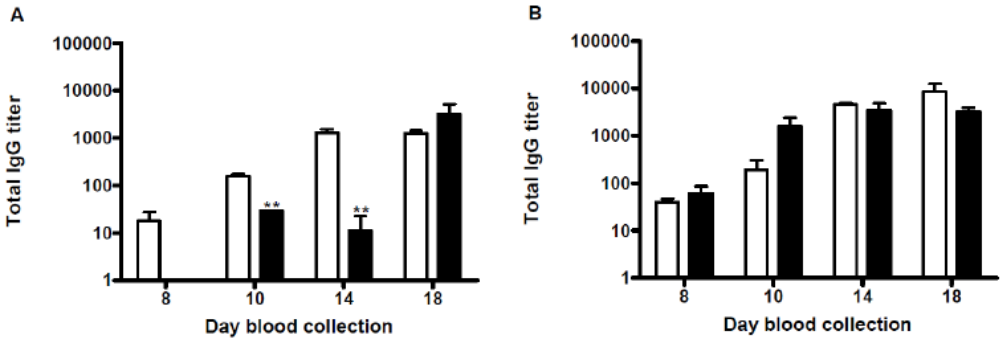

Figure 2. IgG titers against human IFNβ in depleted (black bars) and undepleted (white bars) (A) tg (n=10) and (B) wt (n=10) animals. Each bar shows the averaged IgG titers plus SEM. Statistical comparisons are between the IgG titers of depleted versus undepleted animals and p values indicate significantly lower IgG titers in depleted animals. * p< 0,05;**p<0,001; *** p<0,0001.

Re-population of the spleen by MZ B cells results in comparable titers between depleted and non-depleted tg animals

As seen in figure 1, efficiency of MZ B cell depletion was almost 100% and within the first ten days almost half of the MZ B cells had re-populated the spleen. The re-population influenced the late immune response against

Betaferon®. Once the MZ B cells re-entered the spleen the IgG titers raised until they were comparable to the undepleted tg animals on day 18 (Fig 2A). The second depletion on day 11 had no effect on the formation of ADAs, indicating that the MZ B cells are important at the early onset of the serum IgG response. As reported by others, once MZ B cells get activated they can migrate to the B-2 B cells compartment and by presenting antigen activate B-2 B cells and thus support the initiation of an adaptive immune response [16-17]. This might be an explanation for the comparable titers between depleted and undepleted tg animals on day 18. During the re-population phase the MZ B cells initiated an adaptive immune response and thus the second round of depletion was ineffective to prevent formation of ADAs by B-2 B cells. The immune response of wt animals was not affected throughout the experiment (Fig. 2B).

Significant differences between tg and wt titers indicates lack of appropriate co-stimulating signals

An interesting point is the observation that the total IgG titers of the wt animals were significantly higher compared to the tg animals (Fig. 3). We have also observed this phenomenon in previous studies in both our interferon alpha and beta models [6] (chapter 3 and 4). One explanation is the lack of co-stimulatory signals since the aggregated therapeutic is still a self-protein. Though aggregation may activate B cells directly and induce antibody production, the B cells need help to proliferate and continue antibody production [18]. Without that help the B cell will not produce many B cell clones and discontinue antibody production, resulting in low IgG titers in the tg animals. Ovalbumin (OVA) absorbed to aluminum was used as T-cell dependent antigen and as seen in Figure 4 A and B, was not influenced by the absence of MZ B cells. Though during the first line of defense MZ B cells also contribute to the response against TD antigens, such as OVA, the strong adjuvant effect of aluminum triggered sufficient amount of B-2 B cells, resulting in a strong immune response regardless of the absence of the MZ B cells.

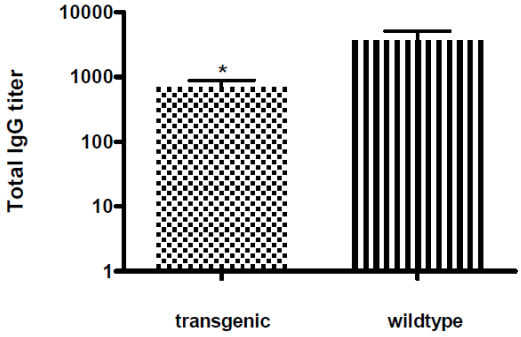

Figure 3. Significant differences between total IgG titers from Betaferon® treated wt and tg animals from all plasma samples presented in this study. Each bar represents the mean of total IgG titers of Betaferon® treated wt or tg mice and the SEM. P values indicate significantly lower IgG titers in tg versus wt

T-cell dependent and T-cell independent controls support the efficient depletion of the MZ B cells

Antibody production by the B-2 B cells is so efficient that the absence of antibodies produced during early response by the MZ B cells is not significant, in contrast to the TI-2 antigen, polysaccharide. TI-2 antigens depend very much on the presence of MZ B cells, though also B-2 B cells produce TI-2-specific antibodies. Nevertheless, during the immunization schedule the total IgG antibody titers of the depleted animals were significantly impaired by the absence of the MZ B cells compared to the undepleted groups, regardless of their genotype (Fig. 4 C and D). In conclusion, the depletion of the MZ B cells was specific.

Concluding remarks

Using a mouse model immune tolerant for human IFNβ we could demonstrate that depletion of MZ B cells influences the immune response against Betaferon®, how precisely remains a matter of further research.

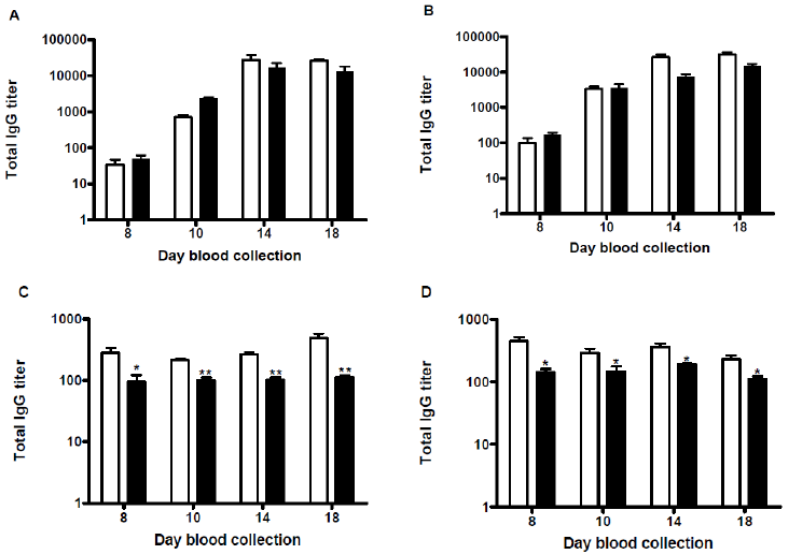

Figure 4. IgG titers against OVA in depleted (black bars) and undepleted (white bars) (A) tg (n=10) and (B) wt (n=10) animals; IgG titers against Pneumovax® in depleted and undepleted (C) tg (n=10) and (D) wt (n=10) animals. Statistical comparisons are between the IgG titers of depleted versus undepleted animals and p values indicate significantly lower IgG titers in the depleted animals. * p< 0,05; ** p< 0,01; ***p<0,0001.

Nevertheless, it could also be shown that MZ B cells are not the only source for ADAs. In conjunction with our pervious findings in the same model, it became clear that the immune response against aggregated human therapeutics cannot be simply compared to either a T-cell independent or a T-cell dependent mechanism initiated by foreign antigens. Though characteristics of both mechanisms can be found during the course of ADA formation, we still deal with a self-protein. This research adds some understanding on how the complex nature of the immune system deals with synthetically engineered proteins. It also underlines the usefulness of appropriate animal models in characterizing the mechanism leading to formation of ADAs.

Materials and Methods

Materials

Betaferon® was obtained from Schering (Berlin, Germany) and the lyophilized powder was reconstituted with phosphate buffered saline (PBS; Braun, Germany) according to manufacture's instructions.

Methods

Mouse Breeding

Heterozygous C57Bl/6 tg mice immune tolerant for hIFNβ were developed by Hermeling et al.[14]. Animals were bred and kept at the Central Laboratory Animal Institute (Utrecht University, the Netherlands). The genotype of the offspring was determined by PCR showing the presence or absence of the hIFNβ gene in chromosomal DNA isolated from ear tissue. Male tg C57Bl/6 mice were crossed with female wt FVB/N mice obtained from Janvier (France) and their hybrid offspring, both tg and wt, were used for *in vivo* experiments [15].

Immunization

Injections were carried out as described previously [14, 19-20]. Briefly, mice (tg = 22; wt = 22) were injected intraperitoneally (i.p.) with 5 μg of Betaferon® or 5 μg of ovalbumin (Sigma-Aldrich, the Netherlands) absorbed to alumn hydroxygel (Sigma-Aldrich, the Netherlands) on five consecutive

days for three weeks. Pneumovax® (Sanofi Pasteur) was obtained from a local pharmacy and includes plain polysaccharide antigens from 23 different pneumococcal serotypes. Primary immunization with 1 µg i.p. of Pneumovax® in 100 µl PBS per mouse (tg = 22; wt = 22) was done on day 0, followed by a boost injection with another 1 µg of Pneumovax® on day 11 as suggested by T. Scheikl [21]. Blood samples were drawn from the cheek pouches (submandibular) of the mice before starting the injections and additionally on day 4, 10 and 14. All animals were sacrificed on day 18 and their spleens were removed, processed to a single-cell suspension and analyzed by flow cytometry. Blood was centrifuged and the obtained plasma stored at -20˚C until further analysis.

In vivo depletion of marginal zone B cells

Mice (tg = 12; wt = 12) were depleted of MZ B cells by one i.p. injection of 100 µg anti-LFA (αLβ2) and 100 µg anti-CD49d (α_4) in 200µl PBS before immunization [22]. Depletion was maintained by a second injection on day 11.

Flow cytometry

Efficiency of depletion of MZ B cells was confirmed and monitored weekly by flow cytometry of single-cell suspensions of spleens using the MZ B cell markers CD 21, CD 23, B220 and IgM (Pharmingen). Measurements were taken using a FACSCanto II® (BD Bioscience, The Netherlands) and analysis was performed with the FACSDiva software v6.1.1 (BD Bioscience, the Netherlands). MZ B cells were identified by being IgM^+, $B220^+$, $CD21^{high}$ and $CD23^{low}$ positive [23].

Binding antibody assay

RhIFNβ & OVA as coating agents

Plasma was analyzed by direct ELISA, as described in detail by Hermeling et al. with adjustments [14]. Briefly, microtiter plates were coated with either 2 μg/mL rhIFNβ or OVA overnight at 4˚C. All subsequent incubation steps were done at room temperature. Plates were washed five times with PBS containing 0.05% Tween20 (Sigma-Aldrich, the Netherlands) and then blocked for two hours with PBS/4% (w/w) milk/0.05Tween20. After another washing cycle, samples were added 100 times diluted in blocking buffer and incubated for two hours. After adding the secondary antibody anti-mouse total IgG (Invitrogen, the Netherlands) color reaction was initiated by adding 3,3',5,5'-tetramethylbenzidine (TMB; Roche, the Netherlands) and stopped by 0.18 M sulfuric acid. Optical density values were measured at 450 nm wavelength on a microplate reader (Novopath; Biorad, The Netherlands) and data analysis was performed with GraphPad Prism 4.03 software (San Diego, CA, USA). Samples were considered positive when the mean was at least three fold higher than the mean of the negative plus the typical 95th percentile of the standard deviation. Titers were measured following the same ELISA protocol. The plots were fitted to a sigmoidal dose-response curve and the reciprocal of the dilution of the EC50 value was considered the titer of the plasma.

Pneumovax® as coating antigen

Microtiter plates were coated with 0,05 μg/ml Pneumovax® for 5 hours at 37˚C. All subsequent steps were carried out at 37˚C and as described above. The samples needed to be preadsorbed to pneumococcal cell wall polysaccharide (CWPS) antigens to capture non-specific antibodies against CWPS, a known contaminant of the Pneumovax® vaccine [21]. In brief, mouse serum was mixed with a 2 μg/ml CWPS and incubated for 30 min at

room temperature. Adsorbed samples were then added 100 fold diluted in blocking buffer and incubated for two hours and Elisa was proceed as in the case of rhIFNβ and OVA.

Statistical analysis

Where appropriate a nonparametric Mann-Whitney test was performed using GraphPad Prism 4.03 software (San Diego, CA, USA) to determine significance. P values are indicated in the graphs when a significant difference was found.

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Part II

Natural antibodies and their influence on immunogenicity

Chapter 6

B-1 cells and naturally occurring antibodies: Influencing the immunogenicity of recombinant human therapeutic proteins?

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Abstract

Recombinant human therapeutic proteins are increasingly being used to treat serious and life-threatening diseases like multiple sclerosis, diabetes mellitus, and cancer. An important side-effect of these proteins is the development of anti-drug antibodies, which can be neutralizing and thus interfere with efficacy and safety of the drug. Some biophysical properties, e.g., aggregation, also can initiate the immunogenic response to human therapeutics. Many other factors including patients' characteristics may influence this response. Besides induced antibodies, auto-antibodies (i.e., naturally occurring antibodies [NAs]) against therapeutic relevant proteins in naïve patients are increasingly being identified. The role of autoreactive B cells and their escape from deletion, production of naturally occurring antibodies and their pivotal function in the immune system, the dualistic role of B-1 cells in autoimmunity, and the influence of NAs on disease outcome and their possible impact on the efficacy of human therapeutics will be presented and discussed.

Introduction

Currently about 200 recombinant human (rh) proteins are used as therapeutics, for example interferon beta to treat multiple sclerosis (MS) and monoclonal antibodies (mAbs) to treat several types of cancer. Because of their similarity with endogenous proteins, human therapeutics are expected to be non-immunogenic. Nevertheless patients develop anti-drug antibodies (ADAs) to almost all protein drugs. Immunogenicity has become a significant issue in the use of protein therapeutics, since anti-drug antibodies (ADAs) due to cross-reactivity with the endogenous protein have led in some cases to life-threatening conditions [1]. Many factors have been identified which influence the immunogenicity of protein drugs like storage and handling, route of administration, age, sex, and genetic profile of the patient. In recent years, more and more publications have appeared investigating the structural aspects, e.g., aggregation, which might cause immunogenicity of a therapeutic protein. In most cases the immunological aspect has been neglected. Therefore, little is understood about the immunological mechanism behind the breakage of tolerance against self-proteins and the possible role of naturally occurring antibodies. Over the last few years more and more evidence accumulated about autoreactive B cells secreting low affinity antibodies that react with many self-structures. One might speculate that the presence of NAs against a therapeutic protein can be predictive for a breakage of tolerance. This review aims at introducing a more immunological approach to the immunogenicity of recombinant human therapeutics.

Autoreactive B cells – who are they and where do they come from?

Since the early days of immunology it was believed that discrimination between self and non-self of the body was pivotal to survival. Immunological tolerance was the most important basic feature of the immune system and autoimmunity was considered to result in autoimmune diseases. Due to these circumstances autoreactive B cells have exclusively been associated with pathogenic consequences [2, 3]. But during the past few years, more and more evidence has accumulated in favor of a physiological role for autoreactive B cells and autoantibodies, resulting in a profound paradigm

shift in immunology and the concept of tolerance to self-antigens [4, 5]. The acceptance of autoimmunity being pivotal for the maintenance of the immune system is increasing [5-8].

The major B cell population linked to support a normally functional immune system is the B-1 pool. Mature B cells are normally categorized into three subgroups, follicular B cells (FO, also called B-2), marginal zone (MZ) B cells, and B-1 cells which are further subdivided into CD5+ B-1a cells and CD5- B-1b cells [9]. B-1 cells have attracted a lot of attention during the past few years mainly because of their special characteristics.

B-1a and B-1b cells reside in the peritoneal and pleural cavities and participate in the first line of defense against invading pathogens [10]. They have important regulatory functions [11, 12], they participate in both innate-like and adaptive immune responses [13, 14], and last but not least they are the source of natural antibodies [14]. Though increasing insights have been gained in regards to their functions, their origin and development is still not entirely clear. Two theories are discussed: (a) B-1 B cells originate from a progenitor cell distinct from a B-2 precursor cell [15], or (b) environmental input drives transitional B-2 cells into the B-1 compartment [16]. The debate is still ongoing, though a recent finding suggests a shared ancestor for both B-1 and B-2 cells, the so called B1 restricted progenitor (B1P) [17••]. Nevertheless, B-1 cells are not a homogenous population, so indeed both theories may have validity, i.e., most of the mature B-1 B cells derive from a fetal progenitor cell, but later on in adulthood may also be generated by changing the fate of transitional B cells into B-1 cells (Figure 1). This hypothesis is also supported by the novel finding that mice lacking the TNF receptor-associated factor 6 (TRAF6) are depleted of their B-1a B cell compartment, showing an important role for receptor –mediated signaling for formation and/or survival for a subset of B-1 cells [18•].

The B cell receptor – or how do autoreactive B cells develop under the pressure of the immune system to delete autoreactive lymphocytes?

There are two major steps deciding upon the fate of a developing B cell: (a) signaling of the pre-BCR in the absence of antigen, and (b) signaling via the B cell receptor (BCR) in the presence of antigen.

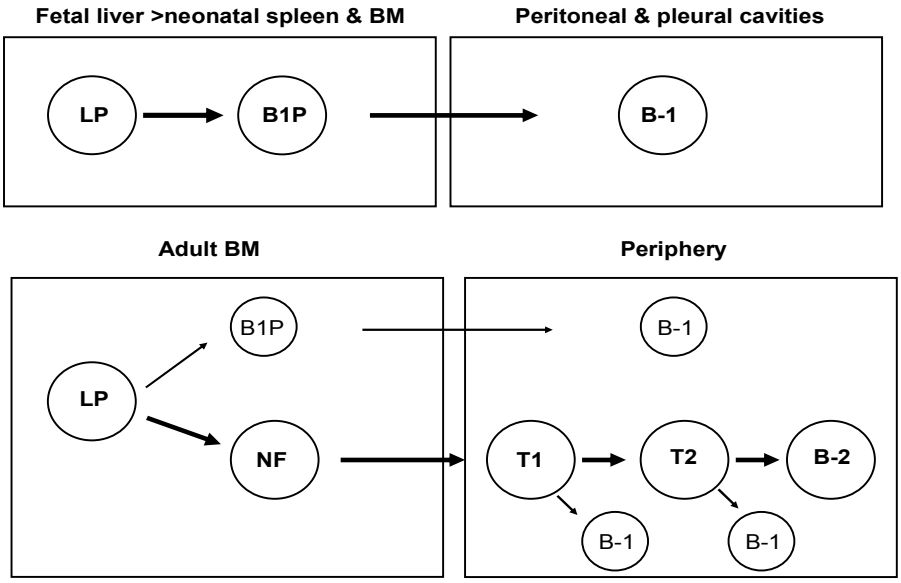

Figure 1. B-1 ontogeny. B cells origin from lymphoid progenitor cells. In the fetal liver, and to some extend in the neonatal spleen, BM and adult BM, lymphoid progenitor cells progress into B1 restricted progenitors, which give raise to most B-1 cells in adulthood. Besides that, lymphoid progenitor cells also give raise to newly formed B cells which develop via the stage of transitional T1 and T2 B cells to B-2 cells. Environmental input from BCR, cytokines or other unknown factor can change the fate of transitional B cells into B-1 cells.

Lymphoid progenitor (LP); newly formed (NF) B cells

The development of B cells requires rearrangement and expression of functional Ig heavy-chains and light-chain genes to build a proper BCR. The genes specifying the heavy and light chains are found in both the variable (V) and constant (C) regions of the chains. The variable region of the heavy chain consists of three gene segments, designated V-D-J. Successive rearrangement of the V-D-J segments ensures the high diversity of the immune system. The heavy chain is rearranged and expressed first. Then it is paired with a surrogate light chain (SLC) in order to form a pre-BCR with strong signaling properties. The surrogate light chains are then later replaced by light chains to form the BCR (IgM), a marker of immature B cells. At this stage up to 75% of the B cells are self-reactive since they are created via a random process of rearrangement. Selection is based on the strength of the pre-BCR signaling in the absence of antigen. After transition into immature, IgM-expressing B cells, the BCR is checked for self-reactivity in an antigen-

dependent matter. Three main mechanisms in the bone marrow are involved in detecting and eliminating self-reacting B cells: (a) deletion by induction of apoptosis if the BCR strongly binds self-antigen [19], (b) induction of anergy, which describes a state of unresponsiveness to BCR and Toll like receptor signaling [20], and (c) receptor editing by random light chain replacement if a weak interaction with the self-antigen is present [21]. Random light chain replacement can decrease binding to self-antigens and seems to be the most prominent mechanism to reduce the number of self-reactive B cells [22, 23]. After passing those central checkpoints, approximately 7% of autoreactive B cells are released into circulation. In the periphery, CD4+ T cells and the B cell activating factor (BAFF) are suspected to control and, if necessary, silence autoreactive B cells [24, 25], leading to a mature and naïve B cell population with less than 5% being autoreactive.

So the question is how self-reactive B-1 cells escape all these central and peripheral checkpoints aiming at preventing autoimmune reactions. As described before, there are two critical regulatory steps in the selection of developing B cells: (a) signaling of the pre-BCR in the absence of antigen, and (b) signaling via the BCR in the presence of antigen. It was recently shown that the selection process differs between fetal liver, where most of the B-1 cells originate, and the bone marrow (BM), where most of the B-2 cells develop. In the BM, the presence of the heavy chain gene segment V_H11 leads to inefficient paring with SLCs and a weak pre-BCR signaling. This severely hampers B cell development in the BM and the cells normally continue heavy chain assembly until a strong pre-BCR signal can be obtained [26]. In contrast, developing B cells in the liver are preferably selected for proliferation in the presence of the V_H11 heavy chain segment and the resulting weak pre-BCR signal. Taking into consideration that V_H11 inefficiently pairs with SLCs it was not surprising when it was shown that V_H11 can only associate with certain distinct light chains, thereby also explaining the restricted repertoire of the B-1 BCR [••27]. Indeed, there is a dominant presence of the V_H11 heavy chain among the B-1 population.

After selection at the pre-BCR levels, the immature B cells are exposed to self-antigens. On the one hand, strong signaling of the BCR after encounter with a self-antigen in the BM leads to apoptosis, anergy, or receptor editing whereas in the fetal liver it leads to positive selection into B-1 cells. The molecular mechanism behind this is still unclear. Besides the strength model, a density model has been established. There is a direct correlation between high BCR density on the surface of a developing B cell and the fate

decision to commit to the B-1 lineage [28]. It has also been speculated that cytokines, such as BAFF, favor the survival of low-affinity autoreactive B cells [29].
Summarizing this, autoreactive B-1 cells do not escape the checkpoints, but they are regulated differently. Due to their autoreactive character, they are actually positively selected to develop into mature and autoreactive B-1 cells.

Naturally occurring antibodies – critical for homeostasis of the immune system

Although autoimmunity is still considered mainly a pathological state, there are data indicating that autoreactive B cells and their antibodies help to maintain homeostasis of the immune system. As early as the 1980s, auto- and polyreactive antibodies in healthy individuals were reported. Because they were found in cord blood and in newborn babies [30] they were thought to arise in the absence of antigenic stimulus and hence were termed naturally occurring antibodies (NAs). NAs are mostly polyreactive (thus having the capability to bind to a variety of self- and non-self antigens), low-affinity antibodies circulating constantly in the body arising from germline variable region genes without excessive somatic mutations; NAs are secreted by the previously described B-1 cells.
NAs fulfill different functions in the first line of defense against invading pathogens [31]. By binding to pathogens, antigen-Ab complexes are formed which then can bind complement and induce lysis or neutralization of viruses [32]. Those complexes are also more efficiently filtered by the spleen, the major secondary lymphoid organ, thereby preventing the pathogen from infecting vital organs [33]. The NAs direct the pathogen to the marginal zone of the spleen, hence inducing production of early antibodies by MZ B cells [34].

Besides their pivotal role in defending the body against invading pathogens, NAs also have crucial influence on protecting the body against waste products. It was recently shown that NAs bind to apoptotic cells [35, 36] and thereby facilitate uptake by dendritic cells. NAs also prevent activation of the adaptive immune system by the ‘danger’ molecules released upon apoptosis like heat-shock proteins and other reactive species and actually prevent autoimmune events. On the one hand NAs fulfill an important role in

homeostasis of the immune system and preventing autoimmune diseases, while on the other hand NAs are still heavily considered to augment autoimmune diseases [37].

B-1 cells in autoimmunity - Promoter or preventer?

Due to their autoreactive nature, B-1 cells may cause (alternative =promote?) autoimmunity. To avoid such scenarios, B-1 cells have several regulatory mechanisms to keep a balance between homeostasis and becoming a threat. During a course of infection, B-2 cells are activated, migrate to germinal centers (GC), and become either long living IgG antibody-secreting plasma cells or memory cells by switch recombination and somatic hypermutation. B-1 cells are also activated by antigen-binding to their BCR and mature into IgM-producing, short living plasma cells. Somatic hypermutation into IgG-secreting memory cells of B-1 cells within GCs would result in catastrophic autoimmune events, given the nature of the self-reactive antibodies they produce. In order to avoid maturation of B-1 cells and subsequently production of high-affinity IgG antibodies towards many self-antigens, B-1 cells are excluded from entering the memory B cell compartment [•38], by which mechanism remains a subject of investigation.

Another factor being pivotal for maintaining an 'immune friendly' state of B-1 cells is the expression of the cell surface molecule CD5. CD5 is a regulatory molecule which interacts with signaling downstream of the BCR. It is associated with the BCR, thereby being capable of modulating and controlling the activation of B-1 cells via their BCR. CD5 is speculated to lower the strength of incoming signals and thus setting a high threshold for the activation of autoreactive B-1 cells. The more CD5 present on the cell surface, the stronger the signaling via the BCR needs to be in order to result in activation, which makes the regulation of CD5 important for maintaining anergy in autoreactive B-1 cells and prevents autoimmune events [39]. On the contrary, down regulation or dysregulation of CD5 expression allows weaker signals to activate autoreactive B cells and thereby might influence and promote autoimmunity [40].

Naturally occurring antibodies – Can they be 'bad guys' as well?

Another issue is the presence of autoantibodies in healthy individuals and their possible role in pathogenicity. A subset of mycobacterial-infected patients suffers from a very severe progression of the disease. It is speculated that autoantibodies against interferon-γ cause this elevated susceptibility [41, 42]. The interferon-γ NAs seem to neutralize endogenous interferon-γ produced in response to the infection leading to insufficient levels of physiological interferon-γ, thereby promoting spread of the mycobacterium. It seems that affinity maturation of these autoreactive antibodies must have taken place, since NAs normally lack neutralizing capacities. Several mechanisms have been proposed to trigger autoreactive B cells into somatic hypermutation and production of IgG-antibodies: (a) high levels of circulating interferon-γ may overcome T cell tolerance leading to presentation to autoreactive B cells, (b) molecular mimicry between pathogen and cytokine, and (c) pathogens might secret haptens interacting with host proteins leading to activation of the immune cells [43].

Antibodies against myelin basic protein have also recently been detected in healthy individuals. *In vitro* testing of sera from those healthy individuals revealed similar characteristics and effects on added myelin basic protein and mononuclear cells as sera from multiple sclerosis patients containing disease-associated myelin basic protein antibodies [44]. This indicates that the disease-associated myelin basic protein antibodies might in fact have developed from NAs during the development of autoimmunity.

In regards to treatment with recombinant human therapeutics, the following scenario can be speculated: If a patient has NAs against an endogenous protein, administration of the recombinant human protein will increase blood levels. This might lead to a saturation or even over-stimulation of both autoreactive T cell and B cell receptors, subsequently breaking the anergetic status/tolerance proceeding to activation and maturation of anti-drug antibodies.

Since the first reports of naturally occurring antibodies occurred, NAs have attracted a lot of interest in the pharmaceutical industry. Erythropoietin-associated antibody mediated pure red cell aplasia (PCRA) was a rare phenomenon among anemic patients until the year 1998. Previous to then, little research had been dedicated to its cause. There was only one case in which a woman with naturally occurring antibodies against erythropoietin (EPO) was described [••45], though the author suggested that a previous

viral infection had been the cause of producing the anti-EPO antibodies. Most reports suggest that patients treated with recombinant human EPO developed anti-EPO antibodies which do not only neutralize exogenous rh Epo but also naturally occurring EPO and lead to PCRA [46]. After 1998, indeed almost all cases of PCRA were correlated to administration of Eprex (rh EPO; Johnson & Johnson). Due to the replacement of human serum albumin with polysorbate 80 as a stabilizer in 1998, micelles were formed which associated with EPO and presented it in a repetitive pattern that can cause breakage of tolerance [1]. This led to a dramatic increase of PCRA between 1998 and 2002. Retroperspectively, the PCRA cases described before 1998 might also have been due to aggregates in the formulation and not to pre-existing NAs. No reports are present in which naturally occurring antibodies are related to a higher possibility to develop PCRA after starting rh EPO treatment.

In the case of interferons and cytokines, many healthy naïve patients have been identified to posses NAs against them [•47, 48]. Nevertheless, the relationship between NAs and higher incidences of therapy failure when treated with the self-antigen as a therapeutic remain weak [49].

One of the major drawbacks in measuring the presence of NAs and their influence is the lack of standardized assays. In most cases, there are conflicting publications about the presence of NAs and almost each group presents different assays [50, ••51]. Since NAs are mostly both low affinity and present in low titers, assays for their existence need to be very sensitive. Increased sensitivity, however, normally comes at the cost of specificity, and several combined assays are crucial to identify NAs. Standardization gets even more difficult, if not impossible, when looking at patients with autoimmune diseases like type I diabetes or hemophilia A. In both cases patients have inherited deficiencies resulting in truncated proteins (Factor VIII) or abnormally low levels of functional proteins (insulin). The immune system in hemophiliac patients has not been tolerized to the functional Factor VIII protein and considers the rh Factor VIII therefore as foreign, producing neutralizing antibodies (often called inhibitors) [52]. In diabetic patients there are autoantibodies against insulin (termed insulin autoantibodies, or IAA) before treatment caused by the autoimmune response of the body. During treatment, a high number of patients develop insulin antibodies (IA) against the exogenous administered insulin [53, 54]. No matter how clean the rh therapeutic will be, immunogenicity will occur in those patients due to absence of tolerance or activation of regulatory cells.

Looking at treatment with rh therapeutics where tolerance is given (IFN-α; IFN-β, etc.), the question of the influence of NAs on treatment outcome remains to be investigated. There are numerous assays available at the moment to measure autoantibodies against rh therapeutics including ELISA, RIP, BIAcore and bioassays being capable of differentiating between binding and neutralizing antibodies [55]. These assays can be optimized to be sensitive enough to measure NAs. Nevertheless, even here no consensus in choice of assay type to detect antibodies against protein therapeutics is found, so it is still a long way to standardized assays for detection of pre-existing antibodies, our NAs. Attemps have been made with limited success to link NAs with immunogenicity incidences of rh therapeutics [data not published yet], but one must be aware that science lacks any means of comparison in terms of detecting NAs and even so in detecting anti-drug antibodies. Without the necessary standardized tools to evaluate collected data on NAs and ADAs among different groups it is very difficult to draw any reliable conclusions.
Two things should be clear by now: Firstly, standardization of the assays must be achieved and only then it will be possible to draw conclusions on (1) incidences of NAs and then (2) possible influence on immunogenicity of rh therapeutics.

Conclusions

Autoreactive B-1 cells and the presence of autoreactive antibodies are pivotal for our immune system to be functional and to remain in a state of homeostasis. NAs participate in maintaining homeostasis by clearing waste products and provide a first line of defense against invading pathogens. Besides their crucial role in keeping the immune system in check, both B-1 cells and NAs are considered to be major players in autoimmune diseases. Considering the polyreactive properties of NAs and the fact that more and more NAs against self-proteins are discovered in healthy individuals, it is not surprising that they attract a lot of attention, especially in regards to their possible influence on disease outcome. They also may impact treatment with recombinant human proteins. The research in this area is hampered by the lack of standardized assays which makes it difficult to draw reliable conclusions and to compare study results. Furthermore, lack of standardization in measuring anti-drug antibodies makes it currently very

difficult link these two phenomena at all. Considering the exponential growth of recombinant human therapeutics, every aspect that might increase immunogenicity, regardless of how small that impact might be, needs further investigations, including NAs.

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Chapter 7

Natural antibodies against bone morphogenic proteins and interferons in healthy donors and patients with infections linked to type-1 cytokine responses

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Abstract

In patients receiving recombinant therapeutic proteins, the production of antibodies against the therapeutics is a rising problem. The antibodies can neutralize and interfere with efficacy and safety of the drugs, and even cause severe side-effects if they cross-react against the naturally, endogenous protein. Various factors have been identified to influence the immunogenic potential of recombinant human therapeutics, including several patients' characteristics. In recent years, so called naturally occurring antibodies against cytokines and growth factors have been detected in naïve patients before start of treatment with recombinant human therapeutics. The role of naturally occurring auto-antibodies is not well understood and their influence on production of anti-drug antibodies is not known. One might speculate that the presence of naturally occurring antibodies increases the likelihood of eliciting anti-drug antibodies once treatment with the corresponding recombinant therapeutic protein is started.
We screened serum samples from 410 healthy controls and patients for auto-antibodies against bone morphogenetic proteins 2 and 7, interferon-α, -β and -γ in a new three-step approach: rough initial screening, followed by competition and protein A/G depletion.
Naturally occurring antibodies against these proteins were detected in 3-4% of the tested sera. Individuals of 65 years or older had a slightly higher occurrence of naturally occurring antibodies. Auto-antibodies against BMP-7 and IFN-α were mainly comprised of IgM isotypes, and natural antibodies against BMP-2, IFN-β and –γ were mainly IgG. To ensure assay specificity the assays were also used to detect antibodies against BMP-7 in patients being treated with rh BMP-7 before and after surgical procedure. 50% of the treated patients had persistent anti-BMP-7 antibodies over time. The three-step approach provides an attractive tool to identify naturally occurring antibodies in naïve patients.

Keywords: Naturally occurring antibodies, cytokines, interferons, autoimmunity, immunogenicity, recombinant human therapeutics

Introduction

Recombinant human (rh) therapeutic proteins are increasingly prescribed to treat a variety of diseases. Many of these rh proteins induce antibodies in patients. In most cases these antibodies have no biological or clinical effect. Sometimes however they interfere with the efficacy of the therapeutic protein. Antibodies against rh therapeutics may lead to serious complications if the antibodies cross-neutralize an endogenous factor with important biological functions as has been described for antibodies induced by erythropoietin used to treat anemia in chronic renal failure [1].
Immunogenicity testing is now a regulatory requirement during the development of every new biologic and this increased screening for antibodies has led to an increase detection of natural antibodies (NAs) against cytokines and growth factors in healthy untreated individuals [2]. The occurrence of these NAs is already known for two decades by the pioneering work of Bendtzen and his colleagues [3-4]. They include auto-antibodies to the interferons, several interleukins, GM-CSF and TNF [5-6].
The biological significance of the NAs is still under debate [7]. Some are associated with diseases such as cyclic thrombocytopenia and myasthenia gravis [8-9]. It is argued that the auto-antibodies are part of a natural mechanism to prevent overstimulation by cytokines and growth factors [10]. Also a role in maintaining tolerance to these different factors has been suggested [11]. There remains controversy about the existence and function of NAs, one of the main reasons for this is the lack of standardization of the different assays. However, over the past few years a number of consensus papers and regulatory guidelines have appeared concerning the assay strategy, validation and specifications, also with regard to detection of anti-drug antibodies [12-13].
One may suspect a predisposition to developing anti-drug antibodies against a therapeutic to which the patient already has pre-existing natural antibodies. In order to determine whether there is an association between the presence of NAs and a predisposition to develop neutralizing anti-drug antibodies, an assay for detection of NAs is needed. We have developed an assay to detect NAs and anti-drug antibodies with the recommended assay strategy, which includes a confirmatory step to verify specificity, the identification of the binding factors as antibodies and subtyping of the immune globulins [14]. We subsequently studied the presence of NAs to bone morphogenic proteins (BMP) 2 and 7, interferon-α, -β and -γ, both in normal donors and patients

with different disease conditions. The three-step assay approach was also used to detect antibodies against BMP-7 in patients who had received rh BMP-7 in the course of a lumbar spinal fusion.

Materials and Methods

Antigens & Sera

BMP-2 and BMP-7 (Stryker Biotech, Hopkinton, MA, USA) are produced in CHO cells and are highly purified. Highly purified IFN-α2a, manufactured by recombinant DNA technology, was a kind gift of Hoffman-La Roche (Basel, Switzerland). IFN-β1a, produced in CHO cells and highly purified, was a kind gift of Biogen (Cambridge, MA, USA). Highly purified IFN-γ (Invitrogen, Breda, The Netherlands) is manufactured by recombinant DNA technology.
In total 410 sera from different sources were tested. Sera from 231 normal healthy individuals as well as samples from 100 patients with persistent or recurrent, atypical infections with otherwise poorly pathogenic mycobacteria and salmonellae were collected by the Department of Infectious Diseases of the Leiden University Medical Center (LUMC). Normal serum samples (n=79) were purchased from J.C. Biologics, Inc. (MA USA).
In addition, 89 longitudinal plasma samples from patients admitted the University Medical Center Utrecht for physical instability and who underwent a lumbar spinal fusion [15] were tested for BMP-7 antibodies. Samples were taken before operation, six weeks, three months, six months and 12 months later.
There were two treatment arms: osteogenic protein-1 (OP-1, another name of BMP-7) combined with locally available bone from laminectomy (osigraft group) or iliac crest autograft (autograft group). One unit Osigraft (Stryker Biotech, Hopkinton, MA) containing 3.5 mg lyophilized rh BMP-7 in 1 g of collagen type I carrier was used per side of spine, for a total of 2 units.

Assay design

A direct (coating with drug and detecting with labeled Ig) enzyme-linked immunosorbent assay (ELISA) was chosen to measure naturally occurring antibodies. Appropriate reagents were selected. Antibodies against interferon α and β to be used as positive controls were raised in house by immunization of mice with recombinant human interferon α or β. Plasma was collected and stored at -20°C. Rabbit polyclonal antibody against interferon γ was obtained from Abcam (Cambridge, UK) as a positive control for the interferon γ assay.

A polyclonal antibody raised in rabbits against BMP-2 as the positive control was purchased from AdD Serotec (Oxford, UK). In the case of BMP-7 mouse monoclonal 1B12 and rabbit polyclonal 1263 antibodies were bought from Creative Biomolecules (now Curis, Cambridge, MA) and mixed to create a positive control. In all assays pooled human serum (Golden West Biologicals, Temecula, CA) was used as both the negative control and the assay diluent to assess non-specific backgrounds. All plates included both positive and negative samples. To validate assay parameters such as minimum dilution, incubation time, and antibody concentrations were determined. Assay described in the following sections describe the optimized conditions. The minimal dilution at which the signal was close to the non-specific signal was determined to be 1:80. Each assay was performed twice on different days and the mean for each sample was calculated and presented in this paper. Samples being considered true positive after following the three-step approach were removed from the pool of samples and a frequency distribution was applied to assess the distribution pattern of the plasma samples, resulting in a non-normal distribution pattern. Samples not containing NAs were considered non-specific binders (NSB) and the 95th percentile of the averaged OD value of the NSB was determined. From that we subtracted the averaged mean of the negative controls to obtain a cut-point for a sample to be called positive, as suggested by Mire-Sluis et.al [14]. Cut-point was calculated for each plate.

Antibody binding assays for BMP-2, BMP-7 and IFN-γ

Microlon 96-well plates (Greiner, The Netherlands) were incubated overnight with 100 µl BMP-2 or BMP-7 (5 µg/ml) in borate buffered saline (BBS) per well at room temperature (RT). The wells were drained and washed 6 times with 300 µl wash buffer (0.1 % Tween 20 in BBS) and then carefully tapped dry on a tissue. Wells were blocked by incubating with 300 µl 4 % milk in wash buffer for 2 hours at 37°C, while the plates were shaking. The plates were drained and washed 6 times with 300 µl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Serum dilutions were added to the wells and incubated for 1 hour at 37°C while gently shaking. The plates were washed 6 times with 300 µl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. 100 µl peroxidase labeled anti-human Ig and IgE (Southern Biotech, USA) was added to the wells and incubated for 1 hour

at 37°C while gently shaking the plates. Plates were drained and washed 6 times with 300 µl wash buffer and twice with 300 µl purified water. After the last wash, wells were carefully tapped dry on a tissue. 100 µl of a ready-to-use 3,3',5,5'-Tetramethylbenzidine solution (TMB) (Roche, The Netherlands) was added and plates were incubated for 15 minutes. The reaction was stopped by adding 100 µl of 0.18 M H_2SO_4 to each well and absorbance was recorded on a Novapath microplate reader (Biorad) at wavelength 450 nm. During all incubation steps the plates were covered to prevent evaporation. The titer of anti-IFN-γ, anti-BMP-2 and anti-BMP-7 Ig in positive plasma was determined by plotting the absorbance values of a 2-fold serial dilution against log dilution. The plots were fitted to a sigmoidal dose-response curve using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego CA, USA). The reciprocal of the dilution of the EC50 value was defined as the titer.

Antibody binding assays for IFN-α and IFN-β

Microlon 96-well plates (Greiner, The Netherlands) were incubated overnight with 100 µl IFN-α or IFN-β (2 µg/ml) in phosphate buffered saline (PBS) per well at 4°C. The wells were drained and washed 6 times with 300 µl wash buffer (0.05 % Tween 20 in PBS) and then carefully tapped dry on a tissue. Wells were blocked by incubating with 300 µl 1 % bovine serum albumin in wash buffer for one hour at RT, while the plates were shaking. The plates were drained and washed 6 times with 300 µl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Serum dilutions were added to the wells and incubated for 1 hour at RT while the plates were gently shaking. The plates were washed 6 times with 300 µl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. 100 µl peroxidase labeled anti-human Ig and IgE (Southern Biotech, USA) was added to the wells and incubated for 1 hour at RT while gently shaking the plates. Plates were drained and washed 6 times with 300 µl wash buffer and twice with 300 µl PBS and then carefully tapped dry on a tissue. 100 µl of a ready-to-use TMB solution (Roche, The Netherlands) was added and plates were incubated for 15 minutes. Color reaction was stopped by adding 100 µl of 0.18 M H_2SO_4 to each well and absorbance was recorded on a Novapath microplate reader (Biorad) at wavelength 450 nm. During all incubation steps the plates were covered to prevent evaporation. The titer of anti-IFN-α

and anti-IFN-β Ig in positive plasma was determined by plotting the absorbance values of a 2-fold serial dilution against log dilution. The plots were fitted to a sigmoidal dose-response curve using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego CA, USA). The reciprocal of the dilution of the EC50 value was defined as the titer.

Confirmatory steps and subtyping

The sera which were positive in the initial screening were confirmed by adding a 200 fold excess of BMP-2 and -7 to the sera and a 2 fold excess in the case of interferons. Two different amounts of excess drug were used to show that the amount was not the limiting factor. After incubation for 30 minutes at RT the sera were tested with the antibody assays as described in previous sections above. A signal reduction of at least 30 % was considered proof that the antibodies were specific. To confirm that the reactivity was caused by antibodies the sera were incubated with protein A/G (Thermo Scientific) according to the manufacturer's instructions. In brief, sera were pre-incubated with Protein A/G for 2 hours at RT on a roller bench. After incubation, samples were centrifuged for 5 minutes at 3000 x g. Supernatant was collected and analyzed by ELISA as described in section *2.2* and *2.3* for the presence of auto-antibodies. If the reduction of binding was less or equal than the mean + 1.96*SD (complete absence of signal) the binding was considered to be due to immunoglobulins. The antibodies binding the different products were typed for total IgE (only for BMP-2 & BMP-7), IgG and IgM by ELISA essentially as described in the sections above except that now peroxidase labeled anti-human IgG, IgM and IgE (Southern Biotech, USA) were used as secondary antibodies.

Statistical analysis

A Shapiro-Wilk test was performed to assess distribution patterns. The student's T –test was performed to compare independent means of the antibody occurrences in cohort patient versus controls, individuals older and equal 65 years versus individuals younger than 65 years and independent means of signal reduction in the competition ELISA with GraphPad Prism,

Version 4.02 (GraphPad Software, San Diego, CA, USA). For all tests *P* values < 0.05 were considered statistically significant.

Results

Detection and confirmation of presence of natural antibodies

After setting up the assays, initial screens were conducted to identify samples containing naturally occurring antibodies against IFN-α, IFN-β, IFN-γ, BMP-2 and BMP-7 (Figure 1). In these initial screens 9 to 42% of samples were found to be positive (Table 1). To confirm the presence of NAs, first a competition experiment was performed: the initially positive samples were pre-incubated with an excess of protein followed by ELISA. A reduction of the signal of at least 30% was considered positive (Figure 2). A second confirmation experiment involved depletion of antibodies by exposure of the samples to protein A/G, followed by ELISA. The presence of antibodies in the samples was considered confirmed if complete loss of ELISA signal was observed.

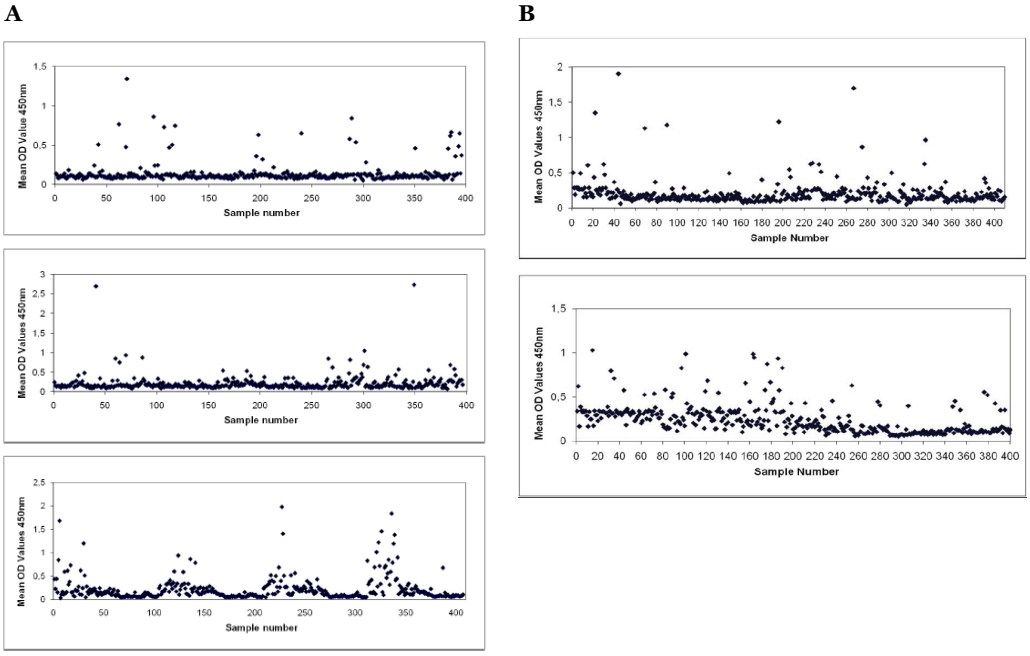

Figure 1 Scatter Plot of initial screen (n=410) identifies possible samples containing naturally occurring antibodies. Samples were screened for (A) anti-IFNalpha, anti-IFNbeta, anti-IFNgamma, and (B) anti-BMP-2 and anti-BMP-7 antibodies. Each symbol represents an individual plasma sample. Data show mean of two independent assays performed on two different days.

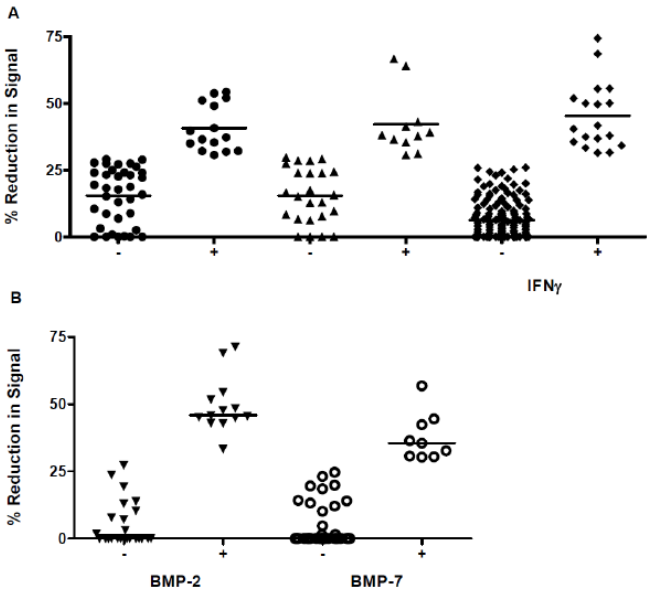

Figure 2 Competition Elisa eliminates false positive samples. All initial called positive samples were subjected to a competition Elisa after incubation with an excess of drug. (A) IFNalpha, IFNbeta, IFNgamma or (B) BMP-2 and BMP-7. A reduction of signal of at least 30% was considered evidence for presence of antibodies. A student's t test was performed to compare mean of reduction signal of samples being rejected (-) and samples being called true positive (+). For all groups a significant difference between rejected and true positive samples was obtained with a P value < 0,001. Data show mean of two independent assays performed on two different days.

As shown in Table 1, 36 of the 410 samples screened for BMP-2 were initially positive. After the confirmation steps 13 (3.2%) samples remained positive for NAs. Similar results were obtained for BMP-7; of the 400 samples screened for the presence of NAs against BMP-7 44 were initially found positive of which eight (2%) samples were confirmed. 398 samples were screened for the presence of NAs against the interferons. In the case of IFN-α, 11 (2.8%) of the 52 initially positive samples were confirmed. 35 samples were initially found positive for IFN-β, of which 11 (2.8%) could be confirmed. Nearly half of the samples were initially screened positive for NAs against IFN-γ. After the confirmation steps 17 (4.3%) were confirmed. Each individual being tested positive for naturally occurring antibodies was only positive for one protein. There was no overlap in presence of naturally occurring antibodies against multiple proteins in one individual.

TABLE 1. Number of samples containing natural antibodies

	Natural antibodies against				
	BMP-2	BMP-7	IFN-α	IFN-β	IFN-γ
Sera tested	410	400	398	398	398
Initially positive	36	44	52	35	167
Confirmed by competition	13	8	15	11	17
Confirmed by protein A/G depletion	13	8	15	11	17
Confirmed positive	3.2 %	2.0 %	3.8 %	2.8 %	4.3 %

Equal distribution of natural IFNγ natural antibodies between patients and controls

100 samples from the total 410 tested samples were collected from patients that have infections with otherwise poorly pathogenic mycobacteria and salmonellae. It has been reported that this unusual susceptibility to infection with these types of bacteria is sometimes due to the presence of auto-antibodies against IFN-γ [16].
We wanted to investigate whether a higher number of positive samples with NAs directed against IFNγ were present in the patient cohort. IFN-γ antibodies were dominantly present in the healthy individuals; 16 (5.2%) samples were found positive, in contrast to only one (1%) in the patients (Table 2).

TABLE 2. Natural antibodies present in healthy individuals and in patients

	Healthy individuals	Patients
Sera tested	310	100
Antibodies to BMP-2	8 (2.6 %)	5 (5 %)
Antibodies to BMP-7	7 (2.3 %)	1 (1 %)
Antibodies to IFN-α	10 (3.1 %)	5 (5 %)
Antibodies to IFN-β	10 (3.1 %)	1 (1 %)
Antibodies to IFN-γ	16 (5.2 %)	1 (1 %)

Tendency for higher incidences of auto-antibodies in individuals older than 65 years

It is generally thought that NAs occur more frequently in older people due to rearrangement of the immune system [17]. We tested this hypothesis by comparing the occurrence of antibodies in individuals younger than 65 to those in individuals 65 years or older. A tendency to a higher occurrence of auto-antibodies in people older than 65 years appeared to exist, although no statistically significant difference was detected (Table 3). Six (2.3%) individuals younger than 65 were found positive for BMP-2 auto-antibodies, in contrast to seven (5.3%) individuals older than 65 years. BMP-7 NAs were detected in four (1.6%) individuals younger than 65 years, whereas four (3%) individuals older than 65 tested positive for BMP-7 NAs. Within the population of people younger than 65, eight (3.1%) individuals showed auto-antibodies against IFN-α, IFN-β or IFN-γ. From people older than 65 six (4.5%) samples were positive for antibodies against IFN-α, three (2.8%) were positive for IFN-β antibodies and nine (6.8%) were confirmed positive for IFN-γ auto-antibodies (Table 3). None of these differences were statistically significant.

TABLE 3. Natural antibodies in younger and older individuals

	< 65 years	≥ 65 years	Unknown age
Samples tested (n)	257	132	21
Antibodies to BMP-2	6 (2.3 %)	7 (5.3)	-
Antibodies to BMP-7	4 (1.6%)	4 (3%)	-
Antibodies to IFN-α	8 (3.1%)	6 (4.5%)	1 (4.8%)
Antibodies to IFN-β	8 (3.1%)	3 (2.8%)	-
Antibodies to IFN-γ	8 (3.1%)	9 (6.8%)	-

Isotyping and titers of natural antibodies

We isotyped the naturally occurring antibodies to determine the dominant isotypes (Figure 3). In Table 4 the occurrence of the isotypes IgG, IgM and IgE in the confirmed positive samples is summarized. IgE was only analyzed in the samples positive for the bone morphogenetic proteins 2 and 7.

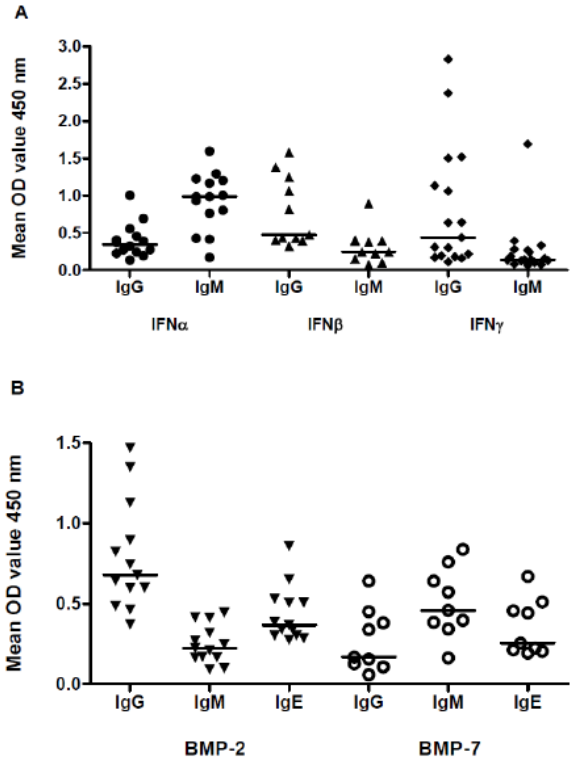

Figure 3 Subtyping of true positive samples reveals no common subtype among the different naturally occurring antibodies. In the case of the interferons (A) only IgG and IgM was measured with enzyme-linked immunosorbent assay (ELISA), whereas in case of the bone morphogenetic proteins (B) also IgE was measured. Data shows mean of two independent assays performed on two different days.

TABLE 4. Isotype occurrence in confirmed positive samples

	Percentage of samples containing		
	IgG antibodies	IgM antibodies	IgE antibodies
BMP-2	100%	38.5%	93%
BMP-7	50%	100%	50%
IFN-α	80%	73%	n.a.
IFN-β	100%	82%	n.a.
IFN-γ	94%	82.5%	n.a.

(n.a. = not analyzed)

All of the samples positive for BMP-2 NAs contained IgG BMP-2 antibodies, whereas only 38.5% samples contained IgM antibodies and 93% of the samples included antibodies of the IgE subtype. IgM antibodies were detectable in all samples that contained BMP-7 antibodies, whereas 50% of the samples contained either IgG or IgE antibodies. In case of IFN-α, 80% of

the positive samples contained IgG isotypes, whereas 73% contained IgM antibodies. All samples positive for IFN-β antibodies contained the IgG isotype, while 82% of the samples also contained IgM antibodies against IFN-β. NAs against IFN-γ showed in 94% of positive samples an IgG isotype and in 82.5% an IgM isotype (Table 4). NAs are of low affinity, thus making titration difficult. Nevertheless, where possible true positive samples were titered and all titrations started at a 1:20 dilution of the samples. Titers were on average low with no significant differences between the products (Figure 4).

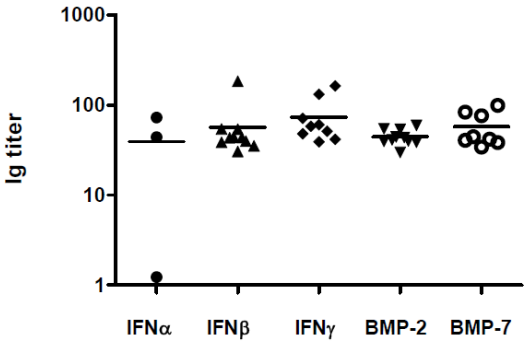

Figure 4 Ig titers of true positive samples are on average low. Where possible, the titers of true positive samples were determined by plotting the absorbance values of a 2-fold serial dilution against log dilution, starting at a 1:20 dilution. The plots were fitted to a sigmoidal dose-response curve and the reciprocal of the dilution of the EC50 value was defined as the titer.

50% of patients retrieving rh BMP-7(OP-1) showed antibodies persisting over time, confirming the specificity of the three-step approach

Immunogenicity of rh BMP-7 is a known phenomenon, though the clinical impact of the anti-BMP-7 antibodies has not been determined [18]. We used this knowledge to ascertain the specificity of the three step approach by applying the three-step assay to samples most likely containing high-affinity anti-BMP-7 antibodies. The initial screen showed that 70% of the patients treated with rh BMP-7 during lumbar spinal fusion (the osigraft group) showed antibodies against rh BMP-7 six weeks after operation, but no antibodies were detected in patients being autografted and not receiving rh BMP-7 (the autograft group) (Figure 5A). After competition ELISA 50% of the samples remained antibody positive (Figure 5B). Subtyping of the samples revealed that IgG was the main isotype (Figure 5C). Antibodies persisted for up to twelve months post-operation but declined over time.

Titers followed the same trend as the initial screen, presenting a decrease of titer values over time with the highest values six weeks after operation (time point 2, Figure 5D). In general, the antibody profile obtained from the patient samples treated with rh BMP-7 shows differences compared to the individual samples screened for autoantibodies against BMP-7. The number of false positives was much lower in the patients being screened for treatment associated BMP-7 antibodies, and most likely high-affinity antibodies, than in the individuals tested for BMP-7 natural antibodies.

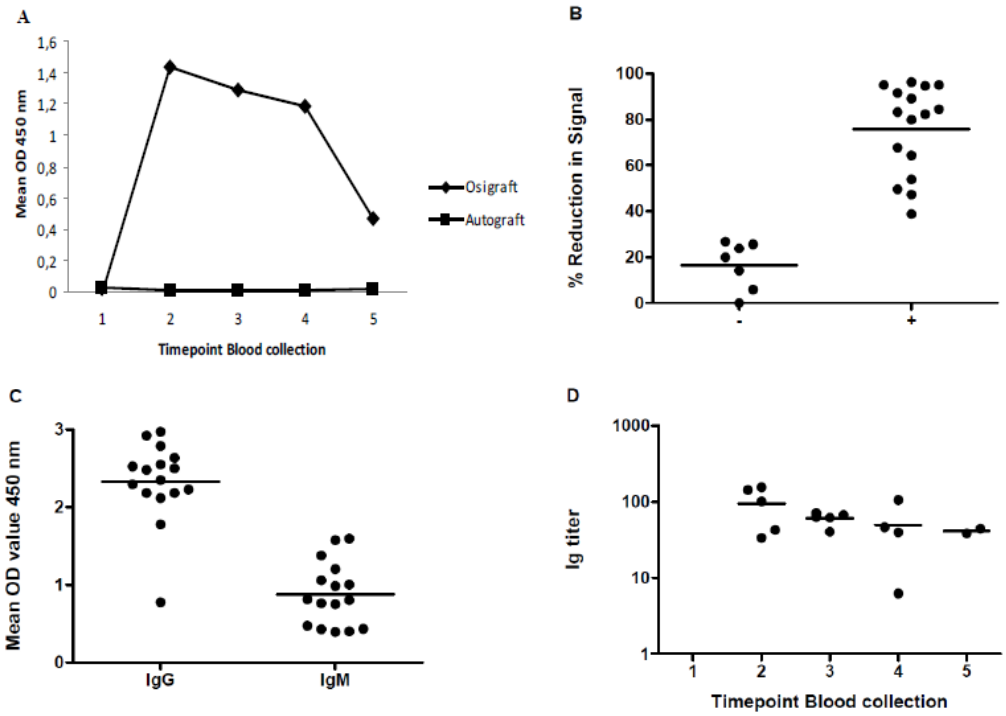

Figure 5 The three step approach also identifies antibodies against recombinant human BMP-7, administered to patients after surgical lumbar spinal fusion with locally available bone (osigraft, n=10). After an initial screen (A), samples were subjected to a competition Elisa and rejected (-) when the reduction of signal compared to the screen results was not greater than 30%, above 30% signal reduction samples were accepted (+) (B), subtyped for IgG, IgM and IgE (C) and titers were determined (D). Patients were monitored over time and five different time points per patient were available, (1) pre-operation (n=10), (2) six weeks post-operation (n=9), (3) three months post-operation (n=10), (4) six months post-operation (n=9) and (5) 12 months post-operation (n=8).

Discussion

With a new, three-step approach, we detected naturally occurring antibodies against BMP-2, BMP-7, IFN-α, -β and –γ in both healthy controls and a population of patients with persistent or recurrent infections with intracellular bacterial pathogens. NAs were present in 3 to 4 % of 410 tested samples. NAs tended to occur more frequently among individuals 65 years or older, although the difference with those younger than 65 years did not reach a level of statistical significance. Finally, isotyping revealed that antibodies to BMP-7 and IFN-α were mainly of the IgM type, whereas IgG was more predominant among the NAs to BMP-2, IFN-β and IFN-γ, titers were on average low.

Detection of NAs can be a challenge due to their low affinity. ELISA-based methods may lack the required level of sensitivity to detect auto-antibodies, other ELISAs may be so sensitive that they overrate the occurrence of auto-antibodies due to false-positive samples [19]. Other biological methods include usage of freshly isolated mononuclear cells [20] which makes it difficult to standardize the testing due to batch differences of the mononuclear cells. Another disadvantage is the low number of samples that can be tested. Our three-step approach allows high throughput of samples, elimination of false-positive samples and standardization, the main concern in biological assays. The cut-off point being determined for each assay ensures that the true positive samples reflect the actual occurrence in the cohort tested. Once auto-antibodies have been identified, further investigation of NAs characteristics such as binding kinetics performed with a Biacore can be applied. Also neutralization assays can be applied to investigate biological functions of these NAs. Both the use of the Biacore and mononuclear cells for assessing neutralizing capacities of antibodies are time consuming, therefore making the relatively fast three-step assay an appealing method to screen large cohorts for the presence of natural antibodies before continuing further analysis.

One of the main reasons for the current lack of data about the biological function of auto-antibodies is the lack of standardized assays for these antibodies. Because of the increasing regulatory and scientific interest in the immunogenicity of biologics there is an international consensus concerning the assay strategy of antibodies to products like the interferons and BMPs. This strategy includes screening with highly sensitive binding assays with proper confirmation of the specificity of the results with a displacement assay

and further characterization of the binding activity as antibodies. We have used the same principles in identifying auto-antibodies to BMPs and the interferons in contrast to the methodology of many other groups. These assay differences and thus the lack of standardization may explain the profound discrepancy of the reported incidences of auto-antibodies especially in the case of the interferons which range from 3-4 % in our experiments up to a 100 % in other publications [19, 21-23]. We have in addition found auto-antibodies to BMP-2 in untreated individuals which were apparently not identified by others.

The biological significance of NAs is not known. The commonly held opinion concerns their putative role in preventing overexposure to the activities of the cytokines and growth factors. However, the members of the ever-expanding family of cytokines and growth factors have many biological functions, including agonistic and antagonistic activities towards their family members. It is a delicately tuned system and it is presently unknown what the biological relevance is of the low level and low affinity auto-antibodies.

If the auto-antibodies are part of the regulatory network of the cytokine and growth factor production these antibodies are expected to increase when these individuals are treated with exogenous factors [21]. However, the presence of auto-antibodies is not related to the immune response to these products [22-23]. A more likely explanation for auto-antibodies is an autoimmunity phenomenon [24]. There are always auto-reactive B cells circulating against many self-antigens [25]. Low levels of circulating cytokines and growth factors are needed to maintain these peripheral tolerance mechanisms. In case of reduced production of these factors or activation of bystander auto-reactive B cells during normal B cell response to foreign antigens may temporarily lead to breakdown of this tolerance.

In conclusion, these data show the occurrence of NAs against the interferons and BMPs by a three-step approach. In addition, the three-step assay reveals that 50% of patients treated with OP-1 produced antibodies against OP-1 over time, thus showing specificity of the assay. But once more it is clear that it is difficult to compare results of antibody assays detecting NAs against growth factors and cytokines obtained by different laboratories and thus drawing conclusions about possible biological functions of NAs. It makes standardization essential, internationally and between different research groups in both academia and industry as already initiated in case of Fabry disease [26]. Our approach presents a rational and convenient technique to

measure NAs in a large cohort of individuals. The data presented here can stimulate more studies on the controversial topic of natural antibodies.

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Chapter 8

Summary, general discussion

& future perspectives

Summary

This thesis consists of two parts, both dealing with immunogenicity issues of rh therapeutic proteins, but with two different approaches. Part I reports data obtained from work with animal models, whereas part II summarizes the current literature on NAs and describes the analysis of human plasma samples.

Part I

Immunogenicity of recombinant human (rh) therapeutics is a rising problem, and due to the high numbers of newly developed recombinant human therapeutics in the pipeline it becomes pivotal to understand the mechanisms behind immunogenicity, on both the protein and the immunology side. Intensive protein characterizations and immunogenicity testing in tg animals have indicated that aggregation is a major cause of immunogenicity, but so far the immunological mechanisms behind it have been neglected.

Chapter 1 lies out the theories concerning the mechanisms leading to the formation of anti-drug antibodies (ADAs). It is believed that aggregates display repetitive epitopes and thereby mimic T-cell independent type 2 (TI-2) antigens. TI-2 antigens are known to be able to initiate an immune response in the absence of T cells by direct cross-linking of the B cell receptor (BCR). They also trigger other immune cells to secrete cytokines delivering positive signals to activated B cells in order to become IgG secreting plasma cells, the mechanisms behind it remain unclear. It has been reported by others that the source of the secreted cytokines may be natural killer cells. Marginal zone B cells, a subset of B lymphocytes, may also contribute to the formation of ADAs, due to their strategic location around the sinuses of the spleen and their function to rapidly respond towards TI-2 antigens. Most TI-2 antigens don't induce formation of an immunological memory, but may induce long-living plasma cells. Many basic questions with regard to the immunological processes leading to formation of ADAs are unanswered and build the basics for the research conducted in this thesis.
Due to the lack of immunological data on the adverse immune response against rh therapeutic proteins and based on the idea that aggregated rh therapeutic proteins mimic TI-2 antigens, the aim of the experimental work

done and presented in this thesis was to obtain the first *in vivo* data regarding the immunological aspects of the formation of ADAs. In order to achieve that aim the three basic immunological questions were addressed:

(1) Is there formation of memory after rechallenge with rh therapeutics?

(2) Are T cells necessary to produce ADAs?

(3) Can MZ B cells be the main source of ADAs?

All *in vivo* studies were carried out in immune tolerant mouse models being tolerant for either human interferon beta (IFNβ) or human interferon alpha (IFNα).

Chapter 2, a work performed with my colleague M.M.C. van Beers, describes the first attempts to characterize the immune response underlying the production of ADAs against aggregated human interferon beta in an immune tolerant mouse model. RhIFNβ-1b (Betaferon®) and three rhIFNβ-1a preparations were intensively characterized for content and structure of aggregates. RhIFNβ-1b is a product known for its strong tendency to form aggregates, its high immunogenicity in patients and also for its capacity to breach the tolerance in the immune tolerant mouse model and was used as a positive control. Reformulated rhIFNβ-1a was essentially aggregate free, bulk rhIFNβ-1a contained large but non-covalently bound aggregates, whereas stressed rhIFNβ-1a showed aggregates homogenous in size (100 nm) and mostly covalently bound. Besides the different levels of aggregation, another major difference between the products was that the native epitopes in the stressed rhIFNβ-1a product were destroyed. Due to these differences in aggregation level and presence of native epitopes, the products showed different levels of ADA production. As expected, RhIFNβ-1b induced the highest levels of binding ADAs in the transgenic (tg) animals, reformulated rhIFNβ-1a the lowest. None of the products showed formation of neutralizing antibodies or responded to a rechallenge with reformulated rhIFNβ-1a, a strong indication of a lack of immunological memory.

Chapter 3 presents the follow-up studies of chapter 2, but now animals were rechallenged with Betaferon®. As seen in the previous chapter, rhIFNβ-1b breached the tolerance in the tg animals, but neither neutralizing antibodies

nor memory could be detected upon rechallenge. Bulk rhIFNβ-1a showed no immune response in the tg animals. The binding antibodies found in the tg animals contained antibodies of the IgG1 and IgG2a subtype. As described in chapter 1, in general TI-2 antigens do not induce formation of memory due to the absence of CD4+ T cell help, but the presence of subtypes in our model indicated the need for the presence of CD4+ T cells. Therefore the next question was: If rh aggregated proteins do not induce memory but isotype switching, can they also activate a B cell in the absence of T cell help? T cell help consists of two components: (1) secretion of cytokines and (2) cell-contact mediated signaling. CD4+ T helper cells are the major group of T cells providing those two components, therefore we depleted all CD4+ T cells in our model, which resulted in a complete abolishment of ADA titers. These findings strongly indicate that the adverse reaction against rh aggregated therapeutics is dependent upon the presence of CD4+ T cells. A preliminary study to scan for differences in cytokine production also indicated a difference in cytokine release between tg animals and wildtype (wt) animals treated with Betaferon®.

Chapter 4 describes results obtained from a mouse model immune tolerant for human IFNα and immunized with aggregated rhIFNα, which was obtained by metal catalyzed oxidation of native rhIFNα. Three experimental set-ups were chosen of which two were the same than in the IFNβ tg mouse model: (1) Rechallenge with both aggregated and aggregate-free rhIFNα, (2) Depletion of CD4+ T cells and in addition, (3) marginal zone (MZ) B cells were depleted to explore their role in ADA production. Aggregated rhIFNα breached the tolerance of the tg animals resulting in production of binding ADAs but as seen in the IFNβ model, neither neutralizing antibodies nor memory could be detected. Upon CD4+ T cell depletion no ADAs were formed, indicating a T-cell dependency. Transient depletion of MZ B cells unexpectedly led to a burst of ADA production.
Another interesting observation to report was the significantly lower production of ADAs in tg animals compared to anti-IFNα antibodies in wt animals, both groups being treated with the same aggregated IFNα product.

In **chapter 5**, the MZ B cells of the mice transgenic for human IFNβ were depleted to investigate their role in the breach of tolerance after administration of aggregated IFNβ-1b (Betaferon®) but also to evaluate if the depletion showed the same effect than in the human IFNα mouse model

(chapter 4). Surprisingly, the absence of MZ B cells during the early days of immunization (Day 8, 10 and 14) resulted in significantly lower ADA titers compared to undepleted tg animals. Once the spleen was repopulated, a second round of depletion was ineffective and the ADA titers rose to comparable levels to those of the undepleted tg animals during the last phase (Day 18) of immunization. In the human IFNβ model MZ B cells play a role during the early onset of the adverse immune response against rh aggregated therapeutics, in contrast to the human IFNα model. This indicates that the mouse strain and/or aggregate type influence the immune response against aggregated rh therapeutic proteins. But one should also consider biological activities of the rh proteins in the tg animal models, leading to biased results. Rh IFNα has a biological activity in the mouse model, whereas rh IFNβ has not. How precisely rh IFNα may interfere with MZ B cell depletion and lead to boost of ADAs needs to be further investigated.

The research presented in the first part of this thesis describes the immunological data obtained from two different mouse models immune tolerant for either human IFNα or IFNβ being treated with the aggregated version of the rh therapeutic proteins. The formation of immunological memory, the dependency on CD4+ T cell help and the involvement of MZ B cells were investigated. The data emphasize that appropriate mouse models are useful tools to further study immunogenicity of rh therapeutic drugs and aggregated products in specific.

Part II

Chapter 6 summarizes the current literature on the source of naturally occurring antibodies (NAs) and their function, and discusses the possible role in immunogenicity of rh therapeutics. A major problem with rh therapeutic drugs is their immunogenic potential. As laid out in the first part of this thesis, aggregation is a major concern and aggregates can induce formation of ADAs, but there are also other factors influencing the immunogenicity of rh therapeutics, of which one may be NAs. Since NAs are more and more identified in naïve, untreated individuals it is suspected that patients having circulating NAs against the endogenous counterpart of the drug may be more likely to have an immune response against rh therapeutic drugs than patients without NAs, a theory which has not been properly investigated yet and so far

no evidence for such a correlation has been reported. NAs are in general low affinity antibodies. In rare cases, such as seen in patients suffering from severe mycobacterial infections, it is believed that NAs against IFNγ mature into high affinity antibodies being able to neutralize endogenous IFNγ, thus hampering the patients' defense against infection. NAs are produced by a subset of B cells, the B-1 cells. B-1 cells are autoreactive B cells residing in the peritoneal and pleural cavities and they are thought to function as a first line defense against invading pathogens. It is believed that the NAs bind to damaged self-proteins and thus mark them to be taken up and degraded by macrophages. The theory behind the possible influence of NAs on immunogenicity of rh therapeutics remains very speculative. It is hypothesized that high levels of circulating rh therapeutic can trigger autoreactive B-1 cell and thereby initiate isotype switching and hypermutation into antibody producing cells, thus causing immunogenicity. The clinical evidence for such an influence remains weak or even absent. But one of the major hurdles in identification of NAs and collecting clinical evidence is the lack of standardized assays. Only with standardized assays will it be possible to correlate the occurrence of NAs with possible influence of treatment with rh therapeutic drugs.

To initiate such standardization, **chapter 7** describes a three-step approach to screen 410 samples, of which 231 were obtained from healthy individuals, 100 samples were taken from patients with persistent infections and 79 normal serum samples were purchased commercially, for NAs against bone morphogenetic protein (BMP)-2 and -7, IFNα, IFNβ and IFNγ. The three-step approach consisted of an initial screening Elisa, spiking the samples with excess amount of drug and incubation with protein A/G, a fusion protein binding the Fc parts of human antibodies. Only if a sample passed all three steps it was called true positive and isotyped for total IgG, IgM and, in the case of BMP-2 and -7, also for IgE. On average, 3-4% of the 410 tested samples showed NAs against one of the proteins screened for. BMP-2 and IFNγ NAs were mainly of the IgM type whereas BMP-7, IFNα and IFNβ NAs were predominantly IgG antibodies. Some of the samples were taken from patients being admitted to the hospital for severe progression of normally harmless bacterial infections, with the cause of the severe progression being unknown. Since NAs against IFNγ were suspected to hamper a patient's capacity for fighting off harmless bacteria, it was expected that the samples being identified as true positive for NAs against IFNγ were correlated to

those patients, but no correlation could be found. To further assess the sensitivity and reliability of the three-step approach, samples knowing to contain antibodies against one of the proteins screened for, BMP-7, were subjected to the three-step approach. 89 samples of patients who had undergone surgical spinal fusion and had been treated with rhBMP-7 were tested for rhBMP-7 antibodies. Almost all patients receiving rhBMP-7 treatment showed ADAs after the surgical procedure and the antibodies persisted in most patients for a period up to at least 12 months, marking the last sample taken from those patients. The ADAs were mostly IgG and IgM, and no IgE could be detected. Most of the samples being positive in the first screen also remained true positive during the following two steps of the assay, confirming the sensitivity and reliability of the assay to pick up protein-specific antibodies. Due to its sensitivity and specificity the three-step approach can be used as a tool for screening naive individuals for NAs.

The data obtained from testing naïve blood samples indicate the presence of NAs against BMP-2, BMP-7, and the interferons. We developed and presented a three-step-approach to simplify the screen of big cohorts and reduce the number of false-positive samples. In the future, the true positive samples obtained by this approach could be subjected to more detailed characterization studies to investigate the possibility of NAs as an intrinsic factor contributing to the immunogenicity of rh therapeutics.

General Discussion

The effects of a breach of immune tolerance by aggregated rh protein drugs on the immune system were investigated in immune tolerant mouse models. In particular, the formation of memory, involvement of T cells and MZ B cells were studied.
In addition, the presence of naturally occurring antibodies in humans was discussed and investigated.

Lack of immunological memory and neutralizing antibodies

Memory response is characterized by an rapid increase of anti-antigen specific antibody levels of the IgG class upon a second (or repeated) encounter with the antigen [1]. In both animal models used for the *in vivo* study of the first part, no significantly higher amounts of anti-interferon antibodies could be detected after rechallenge with either aggregated or non-aggregated material. The absence of memory is an indicator for a T-cell independent mechanism. TI-2 antigens present repetitive epitopes which enables them to directly cross-link the BCR, thus activating the B cell without T cell help. There are two possible explanations for the incapability of the TI-2 antigens to elicit a memory formation: (1) Lack of co-stimulatory signals, normally provided by T helper cells, and (2) cross-linking of the BCR is too weak to sustain an activated status of the B cell [2]. In addition, immune responses differ between TI-2 antigens which have been correlated with dose of antigen and repetitive epitope structure, like spacing [3].
Aggregated rh therapeutic proteins also display repetitive epitopes and it is very likely that they are also capable of direct cross-linking of the BCR, thus leading to activation of the B cell. It is possible that the absence of memory and neutralizing antibodies in our models were due to the aggregated structures, which may have induced B cell activation like the TI-2 antigens, but it lacked any other kind of signals to provide help for the B cell to further mature and become a memory B cell. In the end, the aggregated rh therapeutic protein is still a self-protein.

Need for T cell help

A rather conflicting finding to the above described way how aggregated rh therapeutics may induce B ell activation is the need for CD4+ T cells to form ADAs. As seen in chapter 3 and 4, depletion of CD4+ T cells led to a complete absence of ADA production. So, how is it possible that although T cells are involved and thus could provide co-stimulatory signals, but still aggregated rh therapeutics do not induce memory and formation of neutralizing antibodies? And how are the naïve T cells activated if we previously hypothesized that the aggregated drugs may not display foreign epitopes or structures?
It is known that B cells can act themselves as antigen presenting cells, thereby priming naïve T cells to mature into T helper cells [4]. Small differences between endogenous protein and rh therapeutic protein may lead to minimal amount of foreign epitopes which in turn can be presented by B cells and activate naïve T cells to differentiate into T helper cells. But yet again, the signal provided may be too weak to initiate a full T cell activation. The preliminary cytokine profiling indicates that cytokines involved in T cell maturation are in general less expressed in the tg compared to wt animals treated with rhIFNβ. This lack of supporting cytokines and subsequently lack of proper activation of both T and B cells could also be reflected by the significantly lower ADA titers of tg *versus* wt animals observed in both models in all experiments (chapter 4, Fig. 7; Fig. 1).

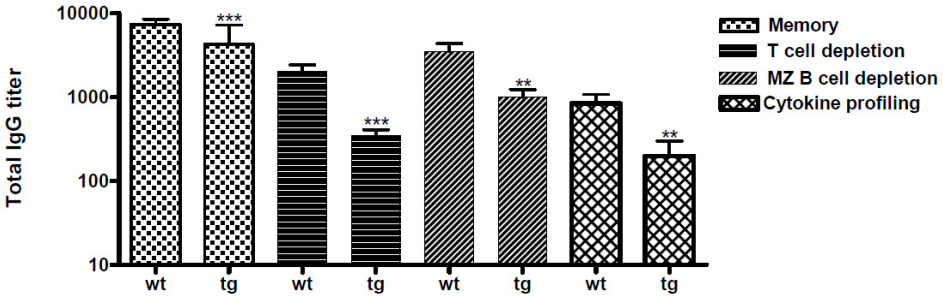

Figure 1. Total anti-IFNβ IgG titers of tg and wt animals treated with Betaferon® of all *in vivo* studies conducted within the human IFNβ mouse model on day 7, 11, 14, 18 and 21. Legend describes the *in vivo* study during which the titers were measured. Bars represent averaged titers of at least 10 animals plus SEM. P values indicate significantly lower titers in the tg animals compared to the wt animals. *p<0.05, **p<0.01, ***p<0.001.

MZ B cells and their role in formation of ADAs

Due to the aggregated structure of the rh therapeutics a group of B cells, so called marginal zone (MZ) B cells remained an interesting group of cells to be exploited, due to their strategic location in the spleen. MZ B cells are probably the first cells to encounter the aggregated therapeutic and they are known to act fast upon administration of TI-2 antigens mostly due to their repetitive epitopes [5]. MZ B cells are also thought to bridge the innate with the adaptive arm of the immune system. After activation MZ B cells capture aggregated structures, migrate to the site of conventional B cells (B-2) and present the aggregated structure, thus cross-linking their BCR and leading to activation of the B-2 B cells [6]. The B-2 B cells will also be directly activated by the aggregated rh therapeutic in the presence of MZ B cells.
Though the human IFNα and human IFNβ models showed lack of memory and neutralizing antibodies, their immune response towards aggregated therapeutic after MZ depletion differed. On the one hand, depletion of MZ B cells in the alpha model boosted the production of ADAs, on the other hand it significantly reduced ADA levels in the beta model. There are several explanations at hand, e.g. the alpha mice are bred on a pure FVB/N background, whereas the beta mice are hybrid mice, bred on a mixed C57BL/6-FVB/N background. It is known that different strains have different amounts of immune cells. Comparing the depletion efficacy of the MZ B cells between alpha (Fig. 2 A) and beta mice (Fig. 2 B) showed differences, though both models received the same treatment (chapter 4 and 5).
One could hypothesize that the complete depletion of MZ B cells in the beta model slowed down the immune response at the beginning and thus leading to reduced ADA titers. It is noticeable that within a few days after repopulation of the spleen and even after a second round of MZ B cell depletion on day 11 the ADA levels were fully restored compared to the undepleted tg animals on day 18, thus indicating a compensation mechanism, maybe fulfilled by conventional B-2 B cells.
The boost in ADAs in the alpha model could be due to an earlier onset of that compensation mechanism by B-2 B cells to substitute for the lost MZ B cells. The depletion of MZ B cells were less successful in the alpha model, thus replacement of the absent MZ B cells was achieved faster eventually leading to an overcompensation and a boost in total IgG antibodies. It can be speculated that this may have also been encountered in the beta model, but after a longer period of time.

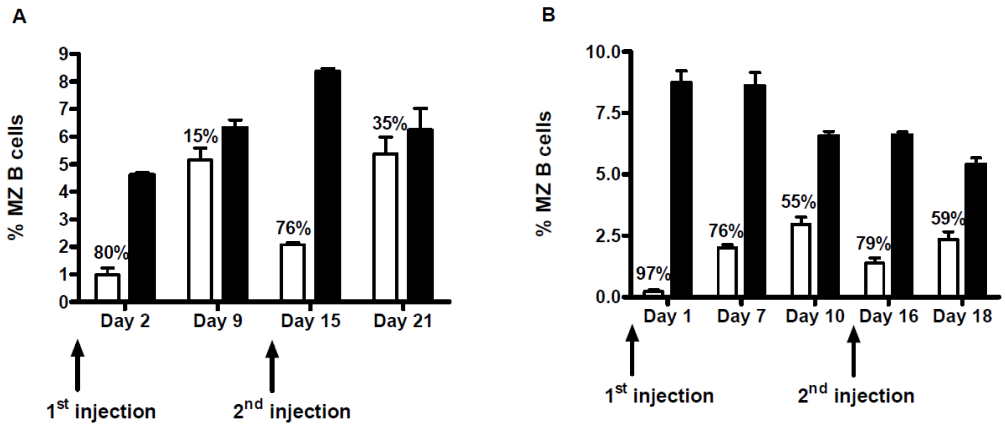

Figure 2. Efficiency of the MZ B cell depletion in two models. (A) Efficiency in the IFNα model (B) Efficiency in the IFNβ model. White bars represent percentage of MZ B cells in depleted animals, whereas black bars show occurrence of MZ B cells in undepleted animals. Percentage of MZ B cell pool is calculated from the number of all measured B cells (IgM^+ and $B220^+$). MZ B cells were identified by being $CD21^{high}$ and $CD23^{low}$. Error bars represent the SEM of at least four animals per time point. Numbers on bars indicate the reduction of MZ B cells in the depleted animals compared to the undepleted animals.

Another explanation may be the difference in aggregated products. RhIFNβ-1b is a commercially available product which already contains aggregates when obtained, whereas the aggregated IFNα was induced by metal oxidation. The aggregation profile differed between the therapeutics. One should also keep in mind that two different proteins were used, one (rhIFNα) being biologically active in mice and one (rhIFNβ) not. How exactly that may have influenced the outcome of the MZ B cell depletion experiment remains a matter of further investigation. The influence of interferons on MZ B cells is currently unknown.

In general it is difficult to compare the two strains due to strain differences and structural differences of the aggregated therapeutics. One thing is clear though, MZ B cells are not the only source of ADAs, which is was not unexpected since both MZ B and B-2 B cells respond to threats, just at different time points [7]. Nevertheless, at an early time of immunization MZ B cells play a role, but which one needs further exploration.

B-1 cells and NAs – self/non-self versus danger theory

The second part of the thesis introduces the concept of intrinsic element, such as the presence of NAs, as possible factors being able to influence the immunogenicity of recombinant human therapeutics. But it also laid out that the immunological dogma of self/non-self is changing and that naturally occurring antibodies and autoreactive B-1 cells are a natural part of the immune system. The danger theory is becoming more and more popular and it may help to understand why rh self-proteins are capable of inducing ADAs. It is still questionable if the presence of NAs in a patient predetermines the risk of developing ADAs and if any clinical consequences may evolve from them.

Conclusions

It can be said that aggregation plays an important role in the adverse immune reaction against rh therapeutic proteins. Though aggregated rh therapeutics induce formation of ADAs, probably due to direct cross-linking of the BCR, they fail to give rise to an immunological memory. Aggregated rh therapeutic proteins may lack certain signals to initiate and sustain a proper innate and/or adaptive immune response. Figure 3 illustrates a possible mechanism of how ADAs are induced, based on the data obtained in this thesis. Though some interesting data has been collected in this thesis, more research needs to be done to support and confirm the observed phenomena. There is one thing that can be said so far: rh aggregated therapeutics differ from both T-cell dependent and classical T-cell/B-cell interactions and thus represent a challenging field of research.

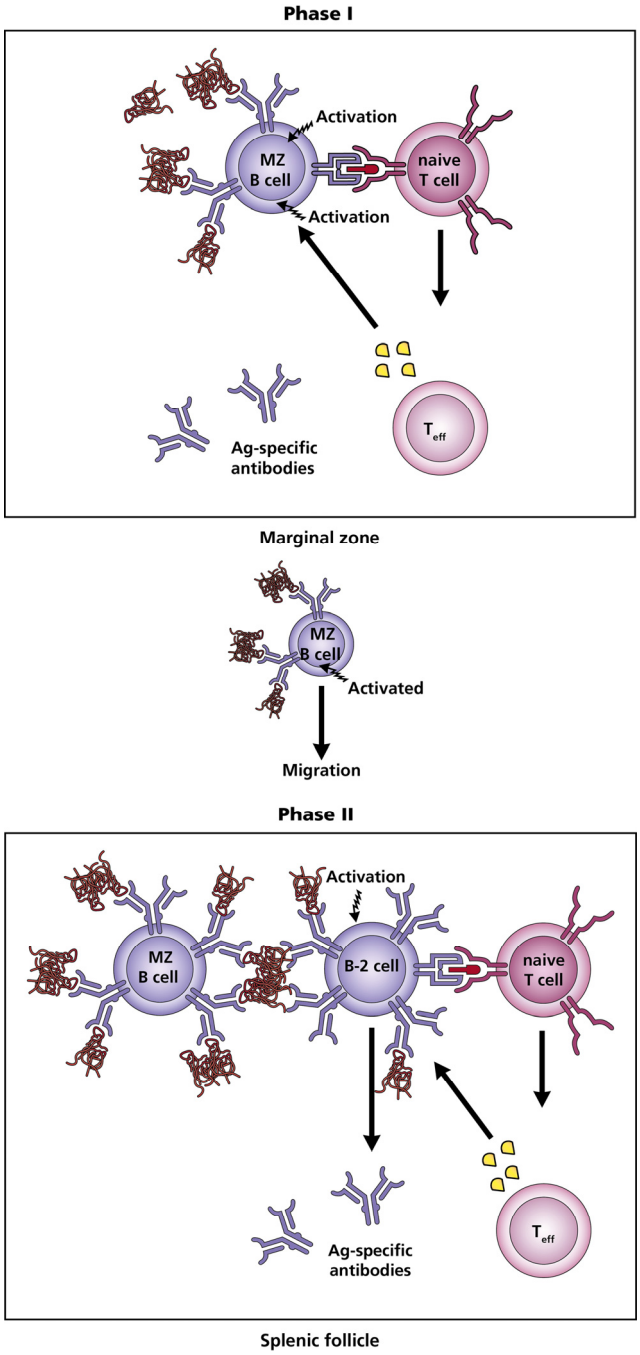

Figure 3. Schematic illustration of the possible mechanisms taking place after administration of aggregated rh therapeutic protein. After entry into the body the aggregates encounter MZ B cells near the sinuses during the early phase, phase I. Direct-cross linking leads to activation of the MZ B cells. In parallel, the MZ B cells also internalize the aggregate, process it and present it via their MCHII to naïve T cells. Recognition of the presented epitopes induces differentiation of the naïve T cells into T helper cells. The activated T helper cells produce cytokines to support isotype switching of the MZ B cell and becoming an IgG secreting plasma cell. In phase II, the activated MZ B cells migrate to the site of B-2 B cells and present the aggregate. Aggregated protein may also directly cross-slink the BCR of the B-2 B cell without presentation by the MZ B cell. In either case, B-2 B cells get activated and in the same manner than MZ B cells recruit their own T cell help to support secretion of antigen-specific ADAs.

Future Perspectives

The immunogenicity of rh therapeutic proteins is a broad field and the data presented in this thesis was collected during investigating the immunological aspects underlying the formation of ADAs. Though the data presented in this thesis marks a first step in characterizing the processes leading to ADAs, further research is necessary to expand our understanding of immunogenicity of rh protein therapeutics. In general, a direct comparison of aggregated versus de-aggregated protein could give new insights.

Cytokines – important mediators of the immune system

The preliminary cytokine screen described in chapter 3 showed some intriguing results, but a more in-depth study should be conducted with appropriate controls, such as non-aggregated rhIFNβ in order to achieve direct comparison of rh aggregated *versus* non-aggregated therapeutic protein. Cytokines are powerful mediators of the immune system and their expression profile can indirectly indicate the immune cells being involved, since most cell types have a unique or distinguishable cytokine profile. E.g. it could be helpful to evaluate if the immune response against rh aggregated therapeutics is mainly accompanied by T helper cells type 2 or 1, and if that differs between tg and wt animals.
Once one has identified a few cytokines of highest interest, a real-time PCR method could be established to measure mRNA levels of expressed cytokines to properly quantify the cytokine amount being expressed during an immune response.

More defined depletion studies of T cell subsets

In chapter 3 and 4 all CD4+ T cells were depleted in the mouse models. This is a rather rough approach since there are specific subsets of CD4+ T cells, such as induced regulatory T cells , T helper cell 1, T helper cell 2, and Th17, all fulfilling different functions [8]. All subsets can be distinguished by other cell surface marker than CD4+, which they all have in common, so more specific depletion is possible.

The CD4+ T cells were also depleted throughout the whole experiment and a more structured depletion as a function of time might give ideas when the CD4+ T cells are recruited to enter the immune response against rh aggregated therapeutic proteins.

Further characterization of MZ and B-2 B cells involvement

Based on the data obtained in this thesis we hypothesized that a lack of stimulatory signals could be a reason for the absence of memory, neutralizing antibodies and presence of low IgG titers, possibly by causing antibody-secreting B cells to undergo early apoptosis compared to the long-lived and memory B cells of the wt animals. It was shown by another group that TI-2 antigen triggered B cells died earlier than their counterparts being triggered by TD antigens [2]. This could be studied by creating a proliferation profile and studying cell death over time, but also indirectly by measuring cytokine production.
As indicated in chapter 5, it seems that the MZ B cells are only important during the early immune response. One could use bigger groups of animals in order to draw blood every day during early immunization to characterize the influence of depletion of MZ B cells in more details.

'Fine-tuning' of the mouse models

The *in vivo* studies conducted through this thesis were done in immune tolerant mouse models. For the last 10 years the study of immunogenicity of rh therapeutic drugs in immune tolerant mouse models has gained substantial insight into the key factors causing immunogenicity and the models are also increasingly used to describe the immunological mechanism underlying the formation of ADAs, as presented in this thesis. In this thesis, two models were used: (1) human IFNα tolerant mice on an FVB/N background, and (2) human IFNβ tolerant mice on the hybrid background of C57Bl/6 × FVB/N. Although the two mouse models used were bred on a different background, they showed similar results in regards to lack of immunological memory and neutralizing antibodies, dependency on CD4+ T cells and the significantly lower IgG titers, thus showing their scientific value. The difference seen during depletion of the MZ B cells could be due to

different numbers of immune cells between the strains, but also due to biological activity of the injected therapeutic.
'Fine-tuning' of the models could even enhance their use for immunogenicity testing, such as by introducing the same strain background. In addition, the identification of tg animals could be improved by measuring the human protein expression with a quantitative PCR based on mRNA copies to avoid possible 'outliers', e.g. animals in which the transgene is present but not active and therefore no tolerance was formed.

Final Remarks

The formation of ADAs due to immunogenicity of rh therapeutic drugs has always been termed 'breaking of tolerance', which is a misinterpretation of the term tolerance. The data obtained here showed that we temporarily breached or circumvented the tolerance against the rh therapeutic, but it was not broken. As soon as the treatments had stopped, ADA titers started to decline and no formation of either neutralizing antibodies or immunological memory could be found.
If one properly characterizes and identifies the key factors and the mechanism behind immunogenicity, these factors can be avoided or removed to make the rh therapeutic drug safer and more reliable. Though it is possible that each aggregated therapeutic protein induces different immune responses due to structural differences, key features, such as the ones leading to lack of memory, and the involvement of specific immune cells, such as MZ B cells, may be identified and interfered with. Animal models immune tolerant for the rh therapeutic protein of interest are powerful tools for continuing this line of research.
Eventually the deep waters of immunogenicity may be breached and enough insight will be gained to be able to avoid or significantly lower immunogenic potentials and thereby leading to safer and more reliable rh protein drugs.

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The future of the animal model?!

Appendices

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Abbreviations

ADAs	anti-drug antibodies
Ag	antigen
BABs	binding antibodies
BAFF	b cell activating factor
BBS	borate buffer saline
BCR	B cell receptor
BM	bone marrow
BMP	bone morphogenetic protein
C	constant
CRS	constant region switch recombination
CWPS	pneumococcal cell wall polysaccharide
DC	dendritic cells
DLS	dynamic light scattering
ECD	equivalent circular diameter
ELISA	enzyme-linked immunoabsorbent assay
EPO	erythropoietin
FO	follicular
GC	germinal centers
GPC	gel permeation chromatography
hIFNα	human interferon alpha
hIFNβ	human interferon beta

HLA	human leukocyte antigen
HP-SEC	high performance size exclusion chromatography
HRP	horse radish peroxidase
HSA	human serum albumin
IFN	interferons
IgG	immunoglobulin G
i.p.	intraperitoneally
LDA	limiting dilution assay
mAb	monoclonal antibody
MBC	memory B cell
MHC	major histocompatibility complex
MO	oxidized
MxA	myxovirus resistant protein A
MZ B cell	Marginal zone B cells
N	native
NA	naturally occurring antibody
NABs/nAbs	neutralizing antibodies
NBS	non-specific binders
NK	natural killer
OD	optical density
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline

PDI	polydispersity index
PEG	poly(ethylene glycol)
PRCA	pure red cell aplasia
rh	recombinant human
rhIFNα	recombinant human interferon alpha
rhIFNβ	recombinant human interferon beta
RR-MS	relapsing-remitting multiple sclerosis
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulfate
SLC	surrogate light chain
TCR	T cell receptor
TD	T cell dependent
tg	transgenic
TI	T cell independent
TI-2	T cell independent type 2
TLR	toll like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TRU/ml	ten-fold reduction units per ml
V	variable
wt	wildtype

In alphabetical order:

Aggregates –

Accumulation of rh therapeutic protein to species with higher molecular weight than the monomer.

Anti-drug antibodies (ADAs) –

Antibodies directed against a rh therapeutic drug, produced during the course of an adverse immune response.

B cell receptor (BCR) –

The B cell receptor (BCR) is an integral membrane protein complex on the surface of the B cells. The BCR complex is composed of two immunoglobulin (Ig) heavy chains, two Ig light chains and two heterodimers of Ig-alpha and Ig-beta. Main function of the BCR is to bind a specific antigen and initiate signaling pathways leading to proliferation and maturation of the B cell.

Break of tolerance –

Temporally failure of the tolerance leading to formation of autoantibodies.

Classical immune response –

Immune response initiated against a foreign antigen in a classical T cell/B cell dependent way.

Conventional (B-2/FO) B cells –

Majority of B cells during an adaptive immune response against TD antigens. They also can react towards TI-2 antigens.

Cytokines –

Immunomodulatory molecules.

Hypermutation -

Mechanism by which activated B cells in the course of an immune response produce a diversity of immunoglobulins to give raise to high-affinity antibodies.

Immunogenicity -

Capability of a rhu therapeutic to provoke an immune response, leading to the production of ADAs.

Isotype switching -

Process of recombination of immunoglobulin constant regions in the heavy chain to switch from production of IgM antibodies to the isotypes IgG, IgA or IgE. Each of which have different effector functions and initiate diverse downstream mechanisms.

Marginal zone (MZ) B cells –

Distinct B lymphocyte lineage of cells residing close to the sinuses of the spleen. They are thought to be the first line of defense against blood-borne pathogens and bridge the innate with the adaptive immune system.

Naturally occurring antibodies (NAs) –

Low autoreactive antibodies secreted by B-1 cells, most likely part of regulatory immune mechanisms.

Recombinant human (rh) therapeutics –

Genetically engineered class of therapeutics produced in either bacterial strains or human cell lines to obtain drugs identical to the endogenous human protein.

T cell dependent type 2 (TI-2) antigens –

Group of antigens being able to activate B cells in the absence of T helper cells by direct cross-linking of the B cell receptor.

T cell dependent (TD) antigens –

Group of antigens which are dependent on the presence of T helper cells to initiate an adaptive immune response.

T helper (Th) cell –

Subgroup of T cells helping other immune cells to become activated and develop into effector cells by either direct cell contact or indirectly by secreting cytokines.

Tolerance –

Immunological status initiated during development to prevent autoimmune disease. The immune cells are taught to distinguish between self and non-self and thus later on the immune cells are tolerant for self proteins.

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Curriculum Vitae

Melody Sauerborn was born on the 28th of November in Krefeld, Germany. After finishing pre-university education (Gymnasium) at St. Wolfhelm in 2000, she started studying human biology at the Philipps University of Marburg, Germany. In 2006 she received her Master's degree, a combined degree from the University of Marburg and the University of Manitoba, Canada where she had done most of the work presented in her Master's thesis (Health Canada, National Microbiology Laboratories under the supervision of Dr. Feldmann). She graduated with a major in infectious diseases and a minor in neuroanatomy. Her studies were complemented with international internships such as at the National Australian University (Canberra), the Centers for Disease Control and Prevention (CDC) in Atlanta, USA and at the Robert Koch Institute (RKI) in Berlin, Germany. During the year 2006 she worked for a public relations agency in Frankfurt, Germany and was responsible for health communication. In March 2007 she started her PhD at the University of Utrecht under the supervision of prof.dr. Huub Schellekens and prof. dr. Wim Jiskoot. The results of her work are presented in this thesis.

Nederlands samenvatting

Nederlandse samenvatting

Dit proefschrift bestaat uit twee delen, die beide gaan over de immunogeniciteitsproblemen van recombinant humane (rh) therapeutische eiwitten, maar met twee verschillende benaderingen. Deel I gaat over data verkregen uit werk met diermodellen, terwijl deel II de huidige literatuur over natuurlijk voorkomende antilichamen (NA's) samenvat en de analyse van menselijke plasmamonsters beschrijft.

Deel I

Ongewenste immunogeniciteit van rh therapeutische eiwitten is een toenemend probleem. Vanwege het hoge aantal nieuwe rh therapeutische eiwitten in ontwikkeling, is het van cruciaal belang om de mechanismen achter hun immunogeniciteit te begrijpen, aan zowel de eiwit- als de immunologie kant. Intensieve eiwitkarakterisaties en immunogeniciteitstesten in transgene (tg) dieren hebben laten zien dat aggregatie een belangrijke oorzaak is van immunogeniciteit, maar tot nu toe zijn de immunologische mechanismen erachter onbekend.

Hoofdstuk 1 beschrijft de theorieën over de mechanismen die leiden tot de vorming van anti-drug antilichamen (ADA's). Men neemt aan dat aggregaten repetitieve epitopen vertonen en zo T cel onafhankelijke type 2 (TI-2) antigenen nabootsen. Het is bekend dat TI-2 antigenen een immuunreactie kunnen initiëren in afwezigheid van T cellen door directe crossslinking van de B cel receptor (BCR). Zij stimuleren ook andere immuuncellen tot het afgeven van cytokines, die positieve signalen afgeven aan geactiveerde B cellen om uit te groeien tot IgG-secreterende plasmacellen. De achterliggende mechanismen zijn nog steeds onduidelijk. Door anderen is beschreven, dat de bron van de uitgescheiden cytokines natuurlijke killercellen zouden kunnen zijn. Marginale zone (MZ) B cellen, een subset van B lymfocyten, zouden ook kunnen bijdragen aan de vorming van ADA's, vanwege hun strategische ligging rond de sinussen van de milt en hun functie om snel te reageren op TI-2 antigenen. De meeste TI-2 antigenen leiden niet tot immunologisch geheugen, maar zouden langlevende plasmacellen kunnen induceren. Veel fundamentele vragen over de immunologische processen die leiden tot de

vorming van ADA's zijn onbeantwoord en vormen de basis voor het in dit proefschrift beschreven onderzoek.
Het gebrek aan kennis van immunologische mechanismen achter de ongewenste immuunresponsen tegen rh therapeutische eiwitten en het idee dat geaggregeerde rh therapeutische eiwitten TI-2 antigenen nabootsen, hebben samen geleid tot het doel van het onderzoek in dit proefschrift: het verkrijgen van inzicht in de immunologische aspecten van de vorming van ADA's. Om dat doel te bereiken werden drie fundamentele immunologische vragen behandeld:

1. Is er geheugenvorming na herhaalde toediening van een rh therapeutisch eiwit?
2. Zijn T cellen nodig om ADA's te produceren?
3. Kunnen MZ B cellen de belangrijkste bron van ADA's zijn?

Alle *in vivo* studies werden uitgevoerd in transgene muizen die immuuntolerant zijn voor humaan interferon bèta (hIFNβ) of humaan interferon alfa (hIFNα).

Hoofdstuk 2, werk verricht met mijn collega M.M.C. van Beers, beschrijft de eerste pogingen tot het karakteriseren van de immuunrespons onderliggend aan de productie van ADA's tegen rhIFNβ. Hiertoe werd rhIFNβ toegediend aan transgene muizen immuuntolerant voor hIFNβ. RhIFNβ-1b (Betaferon ®) en drie rhIFNβ-1a preparaten werden intensief gekarakteriseerd op de aanwezigheid en structuur van aggregaten. RhIFNβ-1b is een product dat bekend staat om zijn sterke neiging tot aggregatie, zijn hoge immunogeniciteit bij patiënten en ook voor zijn vermogen om de tolerantie te doorbreken in het immuuntolerante muismodel. Daarom werd dit product gebruikt als positieve controle. Geherformuleerd rhIFNβ-1a was nagenoeg vrij van aggregaten, bulk rhIFNβ-1a bevatte grote maar niet covalent gebonden aggregaten, terwijl gestrest rhIFNβ-1a aggregaten van homogene grootte (100 nm) bevatte, veelal covalent gebonden. Naast de verschillen in gehalte aan aggregaten, was een ander belangrijk verschil tussen de producten, dat de natieve epitopen in het gestreste rhIFNβ-1a product vernietigd waren. Vanwege deze verschillen in aggregatieniveau en de aanwezigheid van natieve epitopen, vertoonden de producten verschillende niveaus van ADA productie. Zoals verwacht induceerde RhIFNβ-1b het hoogste niveau van bindende ADA's in de transgene (tg)

dieren, en geherformuleerd rhIFNβ-1a het laagste. Geen van de producten vormde neutraliserende antilichamen of reageerde op een herhaalde toediening met geherformuleerd rhIFNβ-1a, hetgeen wijst op het ontbreken van immunologisch geheugen.

Hoofdstuk 3 presenteert de vervolgstudie van hoofdstuk 2, maar nu kregen de dieren Betaferon ® herhaaldelijk toegediend. Zoals in het vorige hoofdstuk, doorbrak rhIFNβ-1b de tolerantie in de tg dieren, maar neutraliserende antilichamen, noch geheugen kon worden gedetecteerd bij herhaalde toediening. Bulk rhIFNβ-1a toonde geen immuunrespons in de tg dieren. De bindende antilichamen gevonden in de tg dieren bevatten antilichamen van het IgG1 en IgG2a isotype. Zoals beschreven in hoofdstuk 1, induceren TI-2 antigenen in het algemeen geen immunologisch geheugen door het ontbreken van CD4 + T cel hulp. De aanwezigheid van IgG isotypen in ons model duidde echter op de aanwezigheid van CD4 + T cellen. Daarom was de volgende vraag: Als geaggregeerde rh eiwitten geen geheugen, maar wel isotype switching kunnen induceren, kunnen ze dan ook een B cel activeren in de afwezigheid van T cel hulp? T cel hulp bestaat uit twee onderdelen: (1) secretie van cytokines en (2) celcontact-gemedieerde signaalvorming. CD4 + T helper cellen zijn de belangrijkste T cellen met beide functies. Daarom hebben we alle CD4 + T cellen verwijderd in ons model, wat resulteerde in een volledige opheffing van ADA titers. Deze bevindingen wijzen er sterk op dat de immuunresponse tegen geaggregeerde rh eiwitten afhankelijk is van de aanwezigheid van CD4 + T cellen. Een voorlopige studie duidde op een verschil in cytokine productie tussen tg dieren en wildtype (wt) dieren behandeld met Betaferon ®, hetgeen ook duidt op een afwijkende immuunrespons tegen rh therapeutica.

Hoofdstuk 4 beschrijft de resultaten verkregen uit een muismodel dat immuuntolerant is voor humaan IFNα en geïmmuniseerd is met geaggregeerd rhIFNα, dat werd verkregen door metaal gekatalyseerde oxidatie van natief rhIFNα. Drie experimentele set-ups werden gekozen waarvan er twee hetzelfde waren als in het IFNβ tg muismodel: (1) Herhaalde toediening met zowel geaggregeerd als aggregaat-vrij rhIFNα, (2) verwijdering van CD4 + T cellen en additioneel, (3) verwijdering van marginale zone (MZ) B cellen om hun rol in ADA productie te bestuderen.

Geaggregeerd rhIFNα doorbrak de tolerantie van de tg dieren, hetgeen resulteerde in de productie van bindende ADA's, maar zoals gezien in het IFNβ model, konden neutraliserende antilichamen en geheugen niet gedetecteerd worden. Na de CD4 + T cel verwijdering werden geen ADA's gevormd, hetgeen duidt op T cel afhankelijkheid. Tijdelijke verwijdering van de MZ B cellen leidde onverwachts tot een verhoogte ADA productie.
Een andere interessante observatie was de significant lagere productie van ADA's in tg dieren in vergelijking met anti-IFNα antilichamen in wt dieren, terwijl beide groepen behandeld werden met hetzelfde geaggregeerde rhIFNα product.

In **hoofdstuk 5** werden de MZ B cellen verwijderd van muizen transgeen voor humaan IFNβ, om hun rol bij de doorbreking van de tolerantie te onderzoeken na toediening van geaggregeerd rhIFNβ-1b (Betaferon ®), maar ook om te evalueren of de verwijdering hetzelfde effect liet zien als in het humaan IFNα muismodel (hoofdstuk 4). Het was verrassend dat de afwezigheid van MZ B cellen in tg muizen tijdens de eerste dagen van de immunisatie (Day 8, 10 en 14) resulteerde in significant lagere ADA titers in vergelijking met normale tg muizen. Wanneer de celpopulatie in de milt hersteld was, was een tweede ronde van MZ B cel verwijdering ineffectief, en stegen de ADA titers tot op een niveau vergelijkbaar met die van de tg dieren die geen tweede ronde van MZ B cel verwijdering hadden ondergaan. In het humaan IFNβ model spelen MZ B cellen een rol tijdens het vroege begin van de ongewenste immuunrespons tegen geaggregeerde rh therapeutische eiwitten, in tegenstelling tot het humaan IFNα model. Dit geeft aan dat de muisstam en/of het aggregaattype invloed hebben op de immuunrespons tegen geaggregeerde rh therapeutische eiwitten. Maar men moet ook rekening houden met de verschillen in biologische activiteit van de rh eiwitten in de tg diermodellen, hetgeen de resultaten kan beïnvloeden. In tegenstelling tot rhIFNβ is rhIFNα biologisch actief in het muismodel. Wat hiervan de consequenties zijn moet verder worden onderzocht.
Het onderzoek gepresenteerd in het eerste deel van dit proefschrift beschrijft de immunologische data die zijn verkregen uit twee verschillende muismodellen. Immuuntolerante muizen voor humaan IFNα of IFNβ werden behandeld met geaggregeerde versies van de rh therapeutische eiwitten. De vorming van immunologisch geheugen, de afhankelijkheid van CD4 + T cel

hulp en de betrokkenheid van MZ B cellen werden onderzocht. De data benadrukken dat de juiste muismodellen nuttig zijn voor het bestuderen van immunogeniciteit van rh therapeutische eiwitgeneesmiddelen en in het bijzonder geaggregeerde producten.

Deel II

Hoofdstuk 6 geeft een samenvatting van de huidige literatuur over de bron van natuurlijk voorkomende antilichamen (NA's) en hun functie, en bespreekt de mogelijke rol bij immunogeniciteit van rh therapeutische eiwitten. Een groot probleem met rh eiwitgeneesmiddelen is hun immunogene potentie. Zoals uitgelegd in het eerste deel van dit proefschrift, is aggregatie een bron van zorg omdat aggregaten de vorming van ADA's kunnen induceren. Er zijn echter ook andere factoren die van invloed zijn op de immunogeniciteit van rh therapeutische eiwitten, waarvan NA's er een kan zijn. Aangezien NA's steeds vaker geïdentificeerd worden in naïeve, onbehandelde individuen bestaat het vermoeden dat patiënten met circulerende NA's tegen de endogene tegenhanger van het geneesmiddel meer kans lopen een immuunrespons tegen rh therapeutische eiwitten te ontwikkelen, dan patiënten zonder NA's. Dit is een theorie die nog niet grondig onderzocht is en tot nu toe is er nog geen bewijs gerapporteerd voor een dergelijke correlatie. NA's zijn in het algemeen antilichamen met lage affiniteit. In zeldzame gevallen, zoals bij patiënten die lijden aan ernstige mycobacteriële infecties, wordt gedacht dat NA's tegen IFNγ uitgroeien tot hoog-affiene antilichamen die endogeen IFNγ kunnen neutraliseren, zodat de afweer van de patiënten tegen infecties belemmerd wordt. NA's worden geproduceerd door een subset van B cellen, de B-1 cellen. B-1 cellen zijn autoreactieve B cellen die verblijven in de peritoneale en pleurale holten en verondersteld worden te functioneren als een eerste verdediging tegen aanvallende ziekteverwekkers. Men denkt dat de NA's aan beschadigde eigen eiwitten binden en deze zo markeert om opgenomen en afgebroken te worden door macrofagen. De theorie achter de mogelijke invloed van de NA's op de immunogeniciteit van rh therapeutische eiwitten blijft zeer speculatief. Er wordt verondersteld dat hoge niveaus van circulerende rh therapeutica autoreactieve B-1 cellen op gang kunnen brengen en dat daardoor isotype switching en hypermutatie naar antilichaam producerende cellen geïnitieerd

kan worden, hetgeen immunogeniciteit veroorzaakt. Het klinisch bewijs voor een dergelijke invloed blijft zwak of zelfs afwezig. Maar een van de grootste hindernissen bij de identificatie van NA's en het verzamelen van klinisch bewijs is het ontbreken van gestandaardiseerde testen. Alleen met gestandaardiseerde testen zal het mogelijk zijn om het verschijnen van NA's te correleren met de mogelijke invloed van behandeling met rh therapeutische eiwitten

Om een dergelijke standaardisering te initiëren, beschrijft **hoofdstuk 7** een drie-stappen-aanpak om 410 monsters te onderzoeken, waarvan er 231 werden verkregen van gezonde personen, 100 monsters werden genomen van patiënten met hardnekkige infecties en 79 normale serummonsters werden commercieel gekocht. De monsters werden onderzocht op NA's tegen bot morfogenetisch proteïne (BMP)-2 en -7, IFNα, IFNβ en IFNγ. De drie-stappen-aanpak bestond uit een initiële screening-Elisa, het pulsen van de monsters met een overmaat aan medicijn en incubatie met het eiwit A/G, een fusie-eiwit dat de Fc delen van humane antilichamen bindt. Alleen als een monster positief was in alle drie de stappen werd het als echt-positief genoemd en werd het geclassificeerd in totaal IgG, IgM en, in het geval van BMP-2 en -7, ook in IgE klassen. Gemiddeld had 3-4% van de 410 geteste monsters NA's tegen een van de geteste eiwitten. Anti-BMP-2 en anti-IFNγ NA's waren voornamelijk van het type IgM, terwijl anti-BMP-7, anti-IFNα en anti-IFNβ NA's overwegend IgG antilichamen waren. Sommige monsters waren afkomstig van patiënten die in het ziekenhuis opgenomen waren voor ernstige progressie van normaal gesproken onschadelijk bacteriële infecties, waarvan de oorzaak van de ernstige progressie onbekend was. Aangezien NA's tegen IFNγ ervan verdacht werden het vermogen van een patiënt om onschadelijke bacteriën aan te vallen te belemmeren, was de verwachting dat de monsters die waren geïdentificeerd als echt-positief voor NA's tegen IFNγ gecorreleerd konden worden aan die patiënten. Een correlatie kon echter niet worden gevonden. Om de gevoeligheid en betrouwbaarheid van de drie-stappen-aanpak verder te beoordelen, werden monsters waarvan bekend was dat ze antilichamen bevatten tegen een van de gescreende eiwitten, BMP-7, onderworpen aan de drie-stappen-aanpak. 89 monsters van patiënten die een chirurgische spinale fusie hadden ondergaan en behandeld waren met rhBMP-7 werden getest op rhBMP-7 antilichamen. Bijna alle van deze

patiënten hadden ADA's na de chirurgische ingreep en de antilichamen bleven bij de meeste patiënten aanwezig voor een periode tot ten minste 12 maanden, het tijdstip waarop het laatste monster werd genomen. De ADA'S waren meestal IgG en IgM, terwijl geen IgE kon worden gedetecteerd. De meeste monsters die positief waren in de eerste screening bleven ook echt-positief tijdens de volgende twee stappen van de test, hetgeen de gevoeligheid en betrouwbaarheid van de test bevestigde om eiwit-specifieke antilichamen te detecteren. Vanwege de gevoeligheid en specificiteit kan de drie-stappen-aanpak worden gebruikt als een methode voor het screenen van naïeve personen voor NA'S.

De data verkregen uit het testen van naïeve bloedmonsters wijzen op de aanwezigheid van NA's tegen BMP-2, BMP-7, en de interferonen. We ontwikkelden en presenteerden een drie-stappen-aanpak om het screenen van grote cohorten te vereenvoudig en het aantal vals-positieve monsters te verminderen. In de toekomst kunnen de echt-positieve monsters verkregen uit deze aanpak worden onderworpen aan meer gedetailleerde karakterisatiestudies voor het onderzoeken van de mogelijke bijdrage van NA's als intrinsieke factor aan de immunogeniciteit van rh therapeutische eiwitten.

Deutsche Zusammenfassung

The following chapter is only for Germans...and you know you're German if...

- You separate your trash into more than five different bins
- You eat a cold dinner at 6pm
- You call your cell phone "handy" and a projector "beamer"
- You have no problems with nude beaches and saunas
- You are shocked when you have to pay for dental care
- People start talking about Hitler and Hofbräuhaus when you tell them where you're from
- Tenth grade was all about dancing lessons
-Your childhood diet consisted of Alete and Zwieback. Your college diet consisted of Miracoli and Döner
- You were educated about sex by Dr. Sommer
- You grew up watching "Löwenzahn" and "Die Sendung mit der Maus"
- You think college tuition is an outrage
- You routinely go 100mph on the highway and tailgate heavily
- Your first audio tape was Benjamin Blümchen and Bibi Blocksberg
- You can tell at least one Manta joke
- Your first sexual experience was on Sat1, Saturday night at 11pm
- You spent hours in school learning to pronounce "th"
- You expect chocolate in your shoes on December 6th
- You complain that in other countries everything is dirty

If you discovered you are German, please proceed...

Deutsche Zusammenfassung

Diese Dissertation besteht aus zwei Teilen, die sich beide mit der Immunogenität von therapeutischen Proteinen beschäftigen, aber mit unterschiedlichen Ansätzen. Teil I beschreibt Daten, die von der Arbeit mit Mausmodellen stammen, während Teil II die aktuelle Literatur zum Thema natürlich vorkommender Antikörper zusammenfasst und die Analyse von humanen Blutproben beschreibt.

Teil I

Immunogenität von therapeutischen Proteinen ist ein immer größer werdendes Problem und vor dem Hintergrund der hohen Anzahl von neu entwickelten rekombinanten humanen (rh) therapeutischen Proteinen in der Pipeline ist es äußerst wichtig, die immunologischen Mechanismen hinter Immunogenität zu verstehen, sowohl auf Protein-Seite als auch auf immunologischer Seite. Intensive Charakterisierung der Proteine und Immunogenitäts-Tests in transgenen Tieren haben gezeigt, dass Aggregate einen wichtige Ursache von Immunogenität sind, die immunologischen Mechanismen sind bis jetzt jedoch vernachlässigt worden.

Das **erste Kapitel** fasst die aktuellen Theorien betreffend der Mechanismen, die zur Bildung von Anti-Medikament Antikörpern (AMA) führen, zusammen. Aufgrund der repetitiven Epitope auf Aggregatoberflächen wird vermutet, dass rh therapeutische Proteine sogenannte T-Zell Typ 2 (TI-2) Antigene imitieren. TI-2 Antigene sind dafür bekannt, dass sie in der Lage sind, in Abwesenheit von T Zellen Immunreaktionen zu initieren, indem sie den B Zell Rezeptor (BZR) verlinken. TI-2 Antigene sind auch fähig andere Immunzellen zur Cytokin-Produktion anzuregen, um so ein positives Signal zu liefern, welches B Zellen stimuliert antikörper-produzierende B Zellen zu bekommen. Der Mechanismus ist bis jetzt noch nicht vollständig geklärt. Andere Untersuchungen haben gezeigt, dass die Quelle von Cytokinen auch natürliche Killer-Zellen sein können. Marginale Zonen (MZ) B Zellen sind eine Unterart von B Zellen, welche aufgrund ihrer strategischen Position an den Sinuses der Milz auch zur Produktion von AMA beitragen können.. MZ B Zellen sind dafür bekannt, dass sie schnell auf TI-2 Antigene reagieren. Die meisten TI-2 Antigene führen nicht zur Entwicklung eines immunologischen Gedächtnisses, aber einige TI-2 Antigene induzieren lang-lebende

Plasmazellen. Viele grundlegende Fragen in Bezug auf die Mechanismen, die zur Produktion von AMA führen, bleiben offen und bilden die Grundlage für die Forschung, die in dieser Dissertation präsentiert wird.

Das Fehlen von immunologischen Daten in Bezug auf die ungünstigen Immunreaktionen gegen rh therapeutische Proteine und die Idee, dass aggregierte rh Proteine TI-2 Antigene imitieren, legten die Bausteine für diese Dissertation. Das Ziel der Forschung war, die ersten *in vivo* Daten über die immunologischen Aspekte der Formation von AMA zu sammeln. Um dieses Ziel zu erreichen wurden drei grundlegende Fragen gestellt und im Rahmen dieser Dissertation bearbeitet:

(1) Führt die erneute Immunisierung mit rh therapeutischen Proteinen zur Bildung eines immunologischen Gedächtnisses?
(2) Sind T Zellen essentiell wichtig für die Formation von AMA?
(3) Sind MZ B Zellen die Hauptquelle für AMA?

Alle *in vivo* Versuche wurden in zwei transgenen Mausmodellen durchgeführt, ein Mausmodell immuntolerant für humanes Interferon Beta (IFNβ) und ein Mausmodell immuntolerant für humanes Interferon Alpha (IFNα).

Das **zweite Kapitel** behandeltIm **zweiten Kapitel**, eine Arbeit, die zusammen mit meiner Kollegin M.M.C. van Beers durchgeführt worden ist, und beschreibt die ersten Versuche, die Immunantwort hinter der Produktion von AMA gegen aggregierte humane IFNβ in einem immuntoleranten Mausmodell zu charakterisieren. RhIFNβ-1b (Betaferon®) und drei weitere IFNβ-1a Produkte wurden intensiv auf Inhalt und Struktur der Aggregate untersucht. RhIFNβ-1b ist ein Produkt welches für seine hohe Affinität Aggregate zu formen bekannt ist, außerdem für seine hohe Immunogenität in Patienten und sein Potential, die Toleranz in den immuntoleranten Mausmodellen zu umgehen, und wurde darum als positive Kontrolle genutzt (An dem Satzbau solltest du nochmal arbeiten:-) Da ich inhaltlich aber nichts verstehe, traue ich mich nicht, den umzustellen :-)). Reformuliertes rhIFNβ-1a war vorwiegend frei von Aggregaten, rh-IFNβ-1a in Bulkware beinhaltete große, aber nicht covalent gebundene Aggregate, wohingegen gestresstes rhIFNβ-1a einheitliche Aggregate von einer durchschnittlichen Größe von 100nm und covalent gebunden Aggregate

beinhaltete. Neben den Unterschieden in Aggregaten bestand auch ein großer Unterschied in der Präsenz von nativen Epitopen. Durch die Stressmethoden bedingt besaß gestresstes rhIFNβ-1a keine nativen Epitope mehr. Aufgrund der oben genannten Unterschiede zeigten die rhIFNβ Produkte auch verschiedene Mengen an produzierten AMA. Wie erwartet regte rhIFNβ-1b die Produktion von AMA am meisten an, wohingegen reformuliertes rhIFNβ-1a die niedrigsten Level an AMA Formation provozierte. Keines der Produkte führte zur Formation von neutralisierenden Antikörpern und eine erneute Immunisierung mit reformuliertem rhIFNβ-1a zeigte keinen Einfluss auf die produzierte Menge an AMA. Zusammen betrachtet deutet dies stark auf die Abwesenheit eines immunologischen Gedächtnisses hin.

Im **dritten Kapitel** werden die Studien aus dem zweiten Kapitel weitergeführt, indem erneut Immunisierungsversuche aufgegriffen werden, diesmal mit aggregiertem rhIFNβ-1b (Betaferon®). Wie bereits im zweiten Kapitel dargestellt, konnte Betaferon® die Toleranz der transgenen Tiere umgehen, aber auch hier führte die erneute Immunisierung mit Betaferon® weder zur Formation von neutralisierenden Antikörpern noch zu einem immunologischen Gedächtnis. Bulkmaterial von rhIFNβ-1a führte nicht zur Produktion von AMA in den transgenen Tieren. Die bindenden Antikörper in den transgenen Tieren beinhalteten auch die Antikörper-Subtypen IgG1 and IgG2a. Wie im ersten Kapitel beschrieben, erzeugen TI-2 Antigene kein immunologisches Gedächtnis auf Grund der Abwesenheit von T Zellen, die Antikörper-Subtypen in unserem Tiermodell deuteten aber auf die Notwendigkeit von T Zellen hin. Daher war die nächste aufkommende Frage: Können aggregierte rh Proteine auch B Zellen in der Abwesenheit von T Zellen aktivieren, da sie kein immunologisches Gedächtnis hervorrufen aber Antikörper-Subtypen? Die T Zell Hilfe besteht aus zwei Komponenten: (1) Ausscheidung von Cytokinen, und (2) Zell-Kontakt bedingte Signalgebung. CD4+ T Zellen sind die Hauptgruppe von T Zellen, die diese beiden Komponenten liefern können. Aus diesem Grund wurden die CD4+ T Zellen in unserem Mausmodell zerstört, was zu einer völligen Abwesenheit von AMA führte. Diese Daten deuteten stark auf eine T-Zell-Abhängigkeit hin. Eine vorläufige Studie, in welcher Cytokineausschüttung gemessen wurde, deutete auf unterschiedliche Cytokin-Profile bei trangsgenen und nicht-transgenem Tieren hin.

Das **vierte Kapitel** beschäftigt sich mit den Resultaten, die von Versuchen mit einem transgenen Mausmodell immuntolerant für humanes IFNα stammen. Diese Tiere wurden mit aggregiertem rhIFNα Produkt immunisiert, welches durch Metall-katalysierte Oxidation gestresst und somit aggregiert wurde. Drei verschiedene Experimente wurden durchgeführt, wobei zwei davon identisch mit den Versuchen mit den humanen IFNβ transgenen Mäusen waren: (1) Erneute Immunisierung mit sowohl ungestresstem als auch gestresstem/aggregiertem rhIFNα, um das immunologische Gedächtnis zu untersuchen, (2) Depletierung der CD4+ T Zellen, und (3) Depletierung der Marginal Zonen (MZ) B Zellen, um ihren Einfluss auf die Formation von AMA zu erforschen. Aggregiertes rhIFNα resultierte in Produktion von AMA bei Überwindung der Toleranz in den transgenen und immuntoleranten Tieren, aber wie bereits im humanen IFNβ Model gesehen, konnten weder neutralisierende Antikörper noch ein immunologisches Gedächtnis detektiert werden. Die Depletierung der CD4+ T Zellen führte zu einer völligen Abwesenheit von AMA, was auf eine T Zell-Abhängigkeit hindeutet. Überraschenderweise kam es zu einem Überschuss an AMA Formation, nachdem die MZ B Zellen depletiert wurden.
Eine weitere interessante Beobachtung war, dass die AMA Produktion in den transgenen Tieren immer signifikant niedriger war als in den nicht-transgenen Tieren, obwohl beide Gruppen mit dem selben aggregierten rhIFNα behandelt worden sind.

Das **fünfte Kapitel** untersucht den Einfluss der Depletierung von MZ B Zellen im humanen IFNβ Modell, zum einen auf die Produktion von AMA aber auch im Vergleich zu den Resultaten vom humanen IFNα Tiermodell (siehe Kapitel Vier). Überraschenderweise führte die Abwesenheit von MZ B Zellen in den ersten Tagen (Tag 8, 10 und 14) der Immunisierung zu signifikant niedrigeren AMA Levels als in den nicht-depletierten Tieren. MZ B Zellen werden stets im Rückenmark neu produziert und wandern dann in die Milz. Nachdem die Milz wieder ihre normale Anzahl von MZ B Zellen nach der Depletierung erreicht hatte, hatte eine zweite Runde von Depletierung der MZ B Zellen in der Milz keinen Einfluss mehr auf die Produktion von AMA (Tag 18). Die AMA Niveaus waren nun mit nicht-depletierten Tieren vergleichbar. Es scheint, dass im humanen IFNβ Modell die MZ B Zellen einen Einfluss auf die frühe Phase der Produktion von AMA haben, im Unterschied zum humanen IFNα Modell. Dies kann darauf hindeuten, dass der Mausstamm eine Rolle spielt und/oder die Struktur des

aggregierten Proteins. Es sollte aber auch in Betracht gezogen werden, dass die biologische Aktivität des humanen Proteins einen Einfluss haben kann. Humanes IFNβ ist im Gegensatz zu humanem IFNα nicht aktiv in Mäusen.. In welcher Weise die biologische Aktivität von humanem IFNα eine Rolle gespielt hat, ist noch unklar.

Der erste Teil dieser Doktorarbeit beschreibt die immunologischen Daten, die von zwei verschiedenen transgenen Mousmodellen gesammelt worden sind, ein Modell immuntolerant für humanes IFNβ, das andere immuntolerant für humanes IFNα. Beide Mausmodelle wurden mit aggregiertem rh Protein behandelt. Die Anwesenheit eines immunologischen Gedächtnisses sowie die Abhängigkeit von CD4+ T Zellen und MZ B Zellen wurde untersucht. Die erhaltenen Daten unterstreichen, dass geeignete Mausmodelle wertvolle Instrumente sind, um die Immunogenität von rh therapeutischen Proteinen, insbesondere aggregierten rh Proteine, zu untersuchen.

Teil II

Im sechsten Kapitel wird die aktuelle Literatur, die sich mit natürlichen Antikörpern (NA)und ihren Funktionen beschäftigt, zusammengefasst und die Rolle von NA in der Immunogenität von rh therapeutischen Proteinen besprochen. Ein großes Problem von rh Proteinen ist ihr Potential immunogen zu sein. Wie im ersten Teil der Doktorarbeit beschrieben, ist Aggregation eine bedeutende Ursache von Immunogenität, aber es gibt auch noch andere Faktoren, die die Immunogenität eines rh Proteins beeinflussen können. Einer dieser Faktoren könnten NA sein. Seit einiger Zeit werden mehr und mehr NA in naiven und unbehandelten Individuen identifiziert. Die Theorie besagt nun, dass Patienten mit NA gegen ein körpereigenes Protein eine höhere Chance haben AMA zu produzieren, wenn sie nun mit dem selben Protein, nun aber rh, behandelt werden. Diese Theorie ist bis jetzt wederbelegt noch existieren Daten, die diese Theorie unterstützen könnten. NA sind im allgemeinen Antikörper mit niedriger Affinität. In sehr seltenen Fällen, wie bei Patienten mit schweren mycobakteriellen Infektionen beobachtet, wird davon ausgegangen, dass NA gegen körpereigenes Interferon Gamma eine hohe Affinität entwickeln und somit sowohl das körpereigene, als auch das rh Interferon Gamma neutralisieren und es somit ineffektiv machen. Das Immunsystem des Patienten ist dann eingeschränkt in seiner Möglichkeit die Infektion zu bekämpfen. NA werden von einer

Unterart von B Zellen, den B-1 Zellen, produziert. B-1 Zellen bewohnen die peritonealen und pleuralen Körperöffnungen und ihre Hauptfunktion ist nach derzeitigem Kenntnisstand die schnelle Bekämpfung von eindringenden Pathogenen. Zusätzlich wird angenommen, dass NA beschädigte Proteine binden und sie somit als `Müll´ markieren, damit sie dann beseitigt werden. Die Theorie, wie NA die Immunogenität von rh therapeutischen Proteinen beinflussen ist hoch spekulativ. Es wird angenommen, dass die hohe Rate an rh Protein im Blut B-1 Zellen aktiviert und somit dazu anregt, sich zu Plasmazellen weiterzuentwickeln, die Antikörper mit hoher Affinität produzieren, die dann Immunogenität hervorrufen. Die klinischen Fakten, die diese Theorie unterstützen würden, sind schwach oder sogar nicht vorhanden. Eine große Hürde um klinische Fakten über NA und deren Einfluss zu sammeln ist das Fehlen von standardisierten Tests für die Detektierung von NA. Nur mit standardisierten Tests kann man das Vorkommen von NA mit klinischen Effekten und Immunogenität in Verbindung bringen.

Um solch eine Standardisierung zu initieren wurde im **siebten Kapitel** ein Drei-Stufen Test für die Identifizierung von NA in 410 humanen Blutproben entwickelt . Von diesen 410 Blutproben waren 231 von gesunden Individuen, 100 Proben von Patienten mit schweren Infektionen und 79 Proben wurden kommerziell gekauft (freiwillige Blutspenden). Die Proben wurden auf NA gegen Interferon Alpha, Beta und Gamma und zusätzlich auf die beiden Knochen-morphogenen Proteine (BMP) 2 und 7 untersucht. Der Drei-Stufen Test begann mit einer breiten Untersuchung der Proben für NA. Danach wurden die vorläufig positiven Proben mit einem Überschuss an Protein versehen, welches alle NA absorbieren sollte. Erneutes Untersuchen der Proben auf NA sollte nun negativ sein. Falls die Probe diesen Test auch bestanden hat, wurde im letzten Teil Protein A/G zur Probe gegeben. Protein A/G bindet an einen Teil der NA und macht sie somit schwerer. Nach einem Zentrifugationsschritt sollte dann der NA-Protein A/G Komplex zum Boden des Gefäßes gesunken sein und ein Test des Überstandes sollte kein Signal geben. Nur wenn eine Probe alle Test durchlaufen und bestanden hatte, wurde sie als echt-positiv für NA angesehen und die Antikörper Subklassen, IgG, IgM und im Fall von BMP 2 und 7 auch IgE, bestimmt. Im Durchschnitt zeigten 3-4% der Proben die Anwesenheit von NA gegen eines der untersuchten Proteine. BMP-2 und Interferon Gamma NA waren hauptsächlich von der IgM Subklasse, wohingegen BMP-7, Interferon Alpha

und Beta meist IgG NA besaßen. Einigeder Proben wurden von Patienten genommen, die im Krankenhaus aufgenommen wurden, weil sie eine schwere Infektion hatten, die durch eigentlich harmlose Pathogene verursacht wurde, (??? unklar) und die Ursache des schweren Verlaufes der Infektion unbekannt war. Da NA gegen Interferon Gamma unter Verdacht stehen, die Funktion des Immunsystems negativ zu beeinflussen, wurde erwartet, dass diese Patienten vornehmlich NA gegen Interferon Gamma in unserem Test zeigten. Diese Korrelation konnte nicht gefunden werden. Um die Sensibilität und Spezifizität unseres Drei-Stufen Tests einzuschätzen, wurden Proben untersucht, die von Patienten stammen, von denen bekannt war, dass sie AMA gegen rh BMP-7 hatten. 89 Proben von Patienten, deren Rückenmark chirurgisch fusioniert wurde und dann mit rh BMP-7 behandelt wurde, um den Heilungsprozess zu unterstützen, wurden auf Antikörper gegen BMP-7 untersucht. Beinahe alle Patienten, die mit rh BMP-7 behandelt wurden, zeigten AMA gegen BMP-7 in unserem Test. Die Antikörper konnten bis zu 12 Monate nach der Operation nachgewiesen werden, wobei nach diesem Zeitraum keine weiteren Proben genommen wurden. Die AMA gehörten meist zur IgG und IgM Subklasse, IgE konnte nicht gemessen werden. Die meisten der Proben, die beim ersten Schritt als positiv erachtet worden waren, bestanden auch die beiden anderen Stufen im Test. Zusammengefasst zeigte dies, dass der Drei-Stufen Test sensitiv war und Protein-spezifische Antikörper detektierte. Aufgrund der Sensitivität und Spezifität des Drei-Stufen Test kann er als nützliches Instrument angesehen werden um naive Individuen auf NA zu untersuchen.

Das Testen von naiven Blutproben zeigte die Anwesenheit von NA gegen BMP-2 und 7 und den Interferonen. Wir entwickelten und präsentierten einen unkomplizierten Drei-Stufen Test für das Messen von NA in großen Mengen von Blutproben und somit vereinfachten wir die Identifikation von falsch-positiven Proben. In der Zukunft könnten die wahren positiven Proben, welche durch diesen Drei-Stufen Test gefunden wurden, aufwendigeren und teureren Charakertisierungsstudien unterzogen werden, um die Möglichkeit zu untersuchen, ob NA ein intrinsischer Faktor ist, der die Immunogenität von rh therapeutischen Proteinen beinflussen kann.

Abschließend kann gesagt werden, dass Immunogenität von rh therapeutischen Proteinen ein Gebiet mit vielen offenen Fragen ist, wobei

einige in dieser Doktorarbeit angesprochen worden sind. Viele Fragen müssen noch beantwortet werden, aber jede Antwort bringt uns einen Schritt weiter, Immunogenität zu verstehen und präventiv zu arbeiten. Verringerung von Immunogenität wird in sicheren und besser verträglicheren Medikamenten resultieren.

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Dear Wim and Huub. You are for sure a great team together and sometimes I might have been a bit frustrated by the quite different styles of supervision but looking back at the PhD time, you were the best supervisors I could have ever found.
Huub, thanks for giving me the freedom and the trust to realize my own ideas. Wim, thanks for your instant feedbacks and critical views of my work and, in special, papers. Your input helped a lot to improve my work.

Vera, you entered our little group of 'immunogenicity' about 2 years ago as a postdoc. Thank you for being such a dedicated person and thank you for all the fruitful discussion and brainstorming we had. Hafid, you are a good technician and I think we could help each other over the years.

This work would have also not been able without the support of my colleagues in Leiden. First, Miranda, who left not too long ago to try her luck for a while in Singapore, and now Riccardo. You are great colleagues, very reliable, fast and precise. It was a pleasure to work with you. Riccardo, I hope our work will continue.

My dear friend, Maryam. I remember that your defense party was my first one right after I started my PhD, and what a start into my PhD! I think a lot of people remember that time. You once said that when someone told you that we had now a German immunologist in the team you thought I was a nerd ☺ I guess I convinced you that this is not the case through many occasion and our little trips to Paris, Venice, Switzerland, Skiing in France and so on. You supported me as a colleague when I needed inspiration or when I was frustrated, but also as a very good friend in personal matters. I owe you a lot for my personal development, you are a true friend.

Steffi, my other very dear friend. Our first encounter was more than unusual, I would say. It was the year 2000 during the summer before I left off to university. To earn some money I worked for a promotion company and on one of the trips I met you. We were driving on the highway when we were suddenly stopped by the highway patrol and they checked our passports. I remember the cop said: So Miss Sauerborn, your first name is Melody and you were born November 28th 1980? And all of a sudden you said 'What, no,

this is my birthday', and that was the beginning of a truly magnificent friendship. You accompanied me through all the years of university and PhD and gave me tremendous mental support along the way. Not many people can claim to have such a great friend.

Daniel & Alex, my two buddies ☺
Daniel, I met you somewhat 16 years ago when you were transferred into our class from another school. Thinking back, I do not even remember how we became friends but that is not even important anymore. More important is that though you are not a keen person on phone calls or emails we managed to stay good friends over the years, even best friends. I am very thankful for that and I wish that this will continue like that.
Alex, like with Daniel, our friendship is not based on regular contact via phone or email, but this did not stop us to keep our friendship alive and still be involved in each other's life! That is amazing and I am very grateful for that.
Now you both are Daddies and I am so happy for you!

How many movies did we watch together, Inge & Mike? I cannot remember nor can I count them on my two hands ☺ It has been many…some good ones, some bad ones…but we always had fun. I am very happy I met you two and that is also why I made Inge my paranimf. I am also quite honored that I can return the favor next year at her promotion!

Many thanks to my friends back in Germany, some of which I know already for over 20 years. Yeah man, time flies! There is Julia, Annika, Kira, Daniel (the other Daniel☺), Nadja, Michael, Thomas and Melanie.
I am happy you will join me at my defense and you will share this day with me.

Denise, we certainly went through some rough, interesting and intensive times in our friendship. Though contact is becoming less due to life getting busier and you living 600 km away in Berlin, you are still a special friend to me and I am sure we will manage to hang out together once a year somewhere on this planet ☺

Francisco, what a time in the lab it was with you, Cor and Manuela back in the days. People from other labs would come by to have fun at our lab ☺ Good old times…now everyone of you left me…but lucky me, you found a position as a PhD only three floors above me and I can still enjoy your company for lunch every now and then at the Gutenberg Café. You are my 'special' friend with 'special' needs!! Never lose them cause then you would lose yourself. Stay as you are, you are a great guy!! Happy to have you as my paranimf…and promise me something: Do NOT behave at my defense ☺

'I swear I was there…but the ball was not anymore!' I am sure I dropped this phrase many times during the squash sessions with Steffen. Steffen, we met during your internship at the Biopharmacy group and starting out with occasionally playing squash together. Now we became good friends, still playing squash and me still losing big time☺ But you are a patient teacher and a patient friend!

What would have been my lab and personal life without Emmy, Afrouz and Isil? Boring!!! Thanks for being such great people!! At this point I would also like to mention my dear colleagues of the Biopharmacy group, I always felt welcomed and comfortable in the group.

A big thanks to my talented students from the Erasmus program, especially Grzegorz, who is now a PhD in our group. Also worth mentioning are my Portuguese student José and my French student Marión. Thanks for your help, enthusiasm and team work. It was great to have you.

Very special thanks go to the people at the animal facility (GDL). I had great times with you and it did not matter with how many questions I came to bug you, you were always patient with me! Thank you: Marco, Sabine, Joyce, Anja, Rika, Willeke, Ajit, Helma, Tamara and everyone else.

I would also like to mention my collaborating partners such as Louis Boon and John den Engelsman. You provided both materials and scientific support.

Harald, I think we not only worked together very successfully but we also became friends. I appreciated your input over the years very much and I am happy that we will keep in touch after my PhD.

The team at the Gutenberg Café saved my life and probably PhD by regularly serving me a great Latte Macchiato on my countless ways back and forth to the GDL!! Great place, great people.

Last, but surely not least, my dear Andreas. Since end 2009 we share our lives, so also my thesis-ending time ☺ You supported me in every possible way and encouraged me to continue and always told me how proud you were of me. That kept me going even when I was fed up with everything. Thank you so much and I hope that we will continue sharing our lives.

Now I am really at the end and looking back at everything I am blessed with the people I have in my life!

This is dedicated to all of you!!