Development of ADA Against Recombinant Human Interferon Beta in Immune Tolerant Mice Requires Rapid Recruitment of CD4$^+$ T Cells, Induces Formation of Germinal Centers but Lacks Susceptibility for (Most) Adjuvants

GRZEGORZ KIJANKA,[1] MELODY SAUERBORN,[1] LOUIS BOON,[2] HUUB SCHELLEKENS,[1,3] VERA BRINKS[1]

[1]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands
[2]Bioceros, Utrecht, The Netherlands
[3]Department of Innovation Management, Utrecht University, Utrecht, The Netherlands

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ABSTRACT: Immunological processes leading to formation of antidrug antibodies (Abs) against recombinant human proteins remain poorly understood. Animal and clinical studies revealed that immunogenicity shares both T-cell-dependent (requirement of CD4$^+$ T cells, isotype switching) and T-cell-independent (involvement of Marginal Zone B cells, apparent lack of memory) characteristics. We used immune tolerant mice to study the mechanism underlying immunogenicity in more detail. We found that CD4$^+$ T cells were crucial at early stages of Ab responses against rhIFNβ. In addition, we found a similar number of germinal centers (GCs) in spleen after rhIFNβ treatment as after treatment with a foreign protein. However, neither Ab titers nor the number of GCs was increased by adsorption of rhIFNβ on aluminum hydroxide. Therefore, we tested the effect of several immune adjuvants in a follow-up study. We found that only conjugation of rhIFNβ to a carrier protein (cholera toxin subunit B) was effective in boosting Ab titers. However, these conjugates failed to trigger rhIFNβ specific memory formation. Our findings show that early events of the immunogenicity reaction to self-proteins are CD4$^+$ T-cell dependent. Nevertheless, despite those similarities, immunogenicity of human proteins is clearly not a classical CD4$^+$ T-cell-dependent response. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:396–406, 2015

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INTRODUCTION

The introduction of therapeutic proteins into clinical practice was a breakthrough in the therapies of many severe diseases such as diabetes, hemophilia, and multiple sclerosis (MS). Development of recombinant technology allowed large-scale production of highly purified proteins with sequences identical to endogenous counterparts. Unfortunately, the therapy outcome is often affected by the production of antidrug antibodies (ADA).[1–3] ADA can neutralize the biological activity of the drug, resulting in a decreased efficacy of the therapy. Moreover, in rare cases, ADA can also cross-react with endogenous proteins leading to severe, even life-threating, side effects.[4,5] Although many factors have been linked to an increased risk of immunogenicity, the immunological processes leading to ADA development are poorly understood. That lack of knowledge hampers efforts aiming at production of nonimmunogenic therapeutic proteins.

Two distinct mechanisms may lead to the production of ADA. The classical immune response against foreign proteins triggers B cells to produce antibodies (Abs) via a CD4$^+$ T-cell-dependent (TD) mechanism, which is characterized by (i) isotype class switching, (ii) affinity maturation of secreted Abs, as well as (iii) the formation of germinal centers (GCs) in lymph nodes and spleens. In these GCs, maturation and differentiation of B cells occur.[6–9] Moreover, immune responses against TD antigens can be easily enhanced by multiple adjuvants.

The second mechanism is a T-cell-independent (TI) immune response resulting in a direct activation of B lymphocytes by antigen without the involvement of CD4$^+$ T cells. A TI immune response is usually triggered by bacterial molecules such as CpG, LPS (TI antigens type I) or polysaccharides (TI antigens type II). In contrast to the TD response, a TI Ab response is mainly restricted to the IgM isotype, involves marginal zone (MZ) B-cells, typically leads to no or a reduced GC formation and usually lacks the formation of immunological memory.[10,11] TI responses usually, in contrast to TD reaction, cannot be easily enhanced by adjuvants.

Although the rhIFNβ has been used for the treatment of MS for over 20 years, the immune processes leading to ADA formation remain poorly understood. Both clinical studies as well as our studies in immune tolerant mice revealed an immune response with both TD and TI characteristics. Depending

Abbreviations used: Ab, antibody; ADA, antidrug antibodies; CFA, complete Freund's adjuvant; CTB, cholera toxin subunit B; GCs, germinal centers; HSA, human serum albumin; IFA, incomplete Freund's adjuvant; i.p., intraperitoneal; MS, multiple sclerosis; MZ, marginal zone; PBS, phosphate-buffered saline; PNA, peanut agglutinin; TD, T cell/thymus dependent; tg, transgenic; TI, T cell/thymus independent; TMB, 3,3′,5,5′-tetramethylbenzidine; rhIFNβ, recombinant human interferon beta.

Correspondence to: Huub Schellekens (Telephone: +31-65-468-6557; Fax: +31-71-527-4565; E-mail: h.schellekens@uu.nl)

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on the product, rhIFNβ may induce ADA in up to 90% of patients and these Abs interfere with rhIFNβ efficacy.[12–15] The differences in frequency of ADA induced by distinct rhIFNβ products originates most likely from different aggregate content of these products. Betaferon® containing the highest number of aggregates has been found to be the most immunogenic in several clinical studies as well as in immune tolerant mice.[13,16,17] Nevertheless, the production of ADA against different rhIFNβ products shares common features between these products suggesting that the same immunological processes are involved in ADA production against all rhIFNβ drugs. Moreover, similar characteristics of ADA formation have been found in both clinical studies and in experiments with immune tolerant mice. For example, IgG has been identified as the main ADA isotype in both patients and mice.[16,18–20] Also, in immune tolerant mice $CD4^+$ T cells were shown to be vital for ADA response, suggesting a TD mechanism underlying immunogenicity of rhIFNβ.[19] However, other clinical and mouse data have shown features typical for a TI response such as an apparent lack of immunological memory and the involvement of MZ B cells.[17,19,20]

We used immune tolerant mice to study the immune mechanisms underlying immunogenicity of recombinant human interferon beta (rhIFNβ). These mice are, like patients, immune tolerant for rhIFNβ. Therefore, administration of rhIFNβ could to certain extent mimic the processes leading to immunogenicity in patients. As both patients and mice receive injections of a self-like protein, the basic immunological processes that take place (e.g., activation of $CD4^+$ T cells, GCs, immunological memory formation, etc.), should reflect the ADA production in both patients and mice.[21]

In this report, we describe a series of experiments designed to improve our understanding of the mechanisms underlying immunogenicity of rhIFNβ using immune tolerant mice. We aimed to determine at which time point during ADA formation $CD4^+$ T cells are most crucial and to assess the formation of GCs to explain the apparent lack of immunological memory found in our previous studies. Additionally, we studied the effect of adjuvants on immunogenicity of rhIFNβ in immune tolerant mice.

MATERIAL AND METHODS

Animals

Heterozygous transgenic (tg) C57Bl/6 mice carrying the human interferon beta (*hIFN*β) gene, originally developed in our institute, were bred at the Central Laboratory Animal Institute (Utrecht University, The Netherlands).[22] tg C57Bl/6 males were crossbred with non-transgenic (non-tg) FVB/N mice (Janvier; BioServices, Uden, The Netherlands).[16] The hIFNβ genotype of the offspring (F1) was determined by PCR of chromosomal DNA isolated from ear tissue. Both PCR positive (tg) and PCR negative (non-tg) littermates (males and females) of 6–10 weeks of age were used for the experiments. The non-tg mice were included in experiments as reference mice that develop an immune response via a TD mechanism. Mice were housed in standard perspex cages and given access to food (Hope Farms, Woerden, The Netherlands) and water (acidified) *ad libitum*. All experiments were performed according to Institutional Ethical Committee Regulations of the Utrecht University, the Netherlands.

Recombinant hIFNβ

Betaferon® (recombinant human IFNβ-1b, rhIFNβ-1b drug product) was obtained from Schering (Berlin, Germany). Lyophilised powder containing 300 μg of rhIFNβ, 15 mg of human serum albumin (HSA), and 15 mg of mannitol was reconstituted in 1 mL of 10 mM sodium phosphate pH 7.4, 137 mM sodium chloride (phosphate-buffered saline, PBS). Because of the lack of glycosylation, rhIFNβ-1b forms significant amounts of aggregates upon reconstitution.[23] Before injection, Betaferon® was further diluted to the desired concentration in PBS. RhIFNβ-1a drug substance was supplied by Biogen Idec (Cambridge, Massachusetts) and was formulated to a concentration of 270 μg/mL in 100 mM sodium phosphate pH 7.2, 200 mM sodium chloride. Before injections rhIFNβ-1a was diluted with PBS.

Animal Experiments

Anti $CD4^+$ T-Cell Treatment

Both tg and non-tg animals received intraperitoneal (i.p.) injections of 5 μg of Betaferon® in 100 μL of PBS for 3 weeks (on days 1–5, 8–12, and 15–19).

To study the involvement of $CD4^+$ T cells, mice received i.p. injections of 100 μg of anti-CD4 Ab GK1.5 (Bioceros, Utrecht, The Netherlands) in 100 μL of PBS on three consecutive days. Depletion was maintained by administration of an additional 100 μg of GK1.5 every 3–4 days until the end of the experiment. One group of tg and non-tg mice started GK1.5 treatment before Betaferon® treatment (day −4, $n = 13$), whereas other groups started GK 1.5 treatment after 2, 4, or 6 injections of Betaferon® (days 2, 4, or 8, $n = 10$). A control group without $CD4^+$ T-cell depletion received a corresponding volume of saline (100 μL) on the same days as the group that was depleted before the start of Betaferon® treatment (from day −4, $n = 6$). Depletion efficiency was assessed for each group on two or three days after initial GK 1.5 injections and every 6–7 days until the end of the experiment. At each time point, three mice per group were sacrificed, spleens isolated and checked for $CD4^+$ T-cell depletion by an PE anti $CD4^+$ Ab (clone RM4.4 eBiosciences, Vienna, Austria), which recognizes a different epitope than GK1.5, using a FACS Canto II flow cytometer (BD Biosciences, Breda, The Netherlands). The depletion efficiency was on average 80%.

Blood was collected via cheek puncture at different time points before the start of Betaferon® treatment (day 1), during the three treatment weeks (days 8, 12, and 19) and 1 week after treatment (day 26) in lithium heparin tubes (Greiner-bio-One, Alphen aan den Rijn, The Netherlands). On day 26, animals were sacrificed by decapitation after which blood was collected.

Plasma was isolated by spinning down the blood for 10 min at 3000*g* at 4°C and stored at −20°C until further assessment for anti-rhIFNβ Abs.

Formation of GCs

In order to study, the presence of GCs after treatment with therapeutic interferon beta, mice received rhIFNβ-1a drug substance. This rhIFNβ product does not contain HSA (in contrast to Betaferon®) and allows assessment of GCs specific for rhIFNβ.

tg and non-tg mice ($n = 16$) were treated with 5 μg of rhIFNβ-1a drug substance in 100 μL of PBS on days 1–5, 8–12, and 15–18. As controls tg and non-tg mice ($n = 8$) were treated with

Pneumovax 23® (Sanofi Pasteur MSD, Hoofddorp, Belgium) on days 1 and 11 of the experiment as described previously.[24] Pneumovax 23® is a TI antigen and should not evoke GC formation. To enhance the immune response and therefore facilitate formation of GCs, another two groups of tg and non-tg mice received either rhIFNβ-1a drug substance ($n = 16$) or Pneumovax 23® ($n = 8$) adsorbed onto aluminium hydroxide gel (alum; Sigma–Aldrich, Zwijndrecht, The Netherlands) in the same doses and on the same days as described above. The preformed colloidal suspension of alum was mixed with rhIFNβ-1a or Pneumovax 23® to the final concentration of 9% (vol/vol). The mixtures were incubated on a roller bench for 30 min at RT in order to allow the rhIFNβ-1a and Pneumovax 23® to adsorb onto the alum. Negative control groups of tg and non-tg mice receiving saline on days 1–5, 8–12, and 15–18 were also included ($n = 4$). Half of animals from each group were sacrificed by decapitation on day 9 and the other half of the mice on day 19 of the experiment. Spleens were isolated, snap-frozen in liquid nitrogen and stored at −80°C for immunohistochemical analysis of GC formation (description below). To assess Ab formation, blood was collected, plasma was isolated by spinning down the blood for 10 min at 3000*g* at 4°C, and stored at −20°C until further analysis.

Impact of Adjuvants on the Immunogenicity of rhIFNβ in Immune Tolerant Mice

Preparation of rhIFNβ-1a/Adjuvant Solutions. Complete and incomplete Freund's adjuvant (CFA/IFA), alum, Saponin from *Quillaja saponaria Molina*, $CuCl_2$ and Cholera Toxin Subunit B (CTB) were purchased from Sigma–Aldrich. Montanide ISA 50 V2 was obtained from SEPPIC (Puteaux, France).

Complete and incomplete Freund's adjuvant or Montanide ISA 50 V2 was mixed with rhIFNβ-1a solution in a 1:1 ratio and vortexed for 30 s to obtain a homogenous emulsion. Alum was mixed with the rhIFNβ-1a solution to a final concentration of 9% (vol/vol). The mixture was incubated on the roller bench for 30 min at RT in order to allow rhIFNβ-1a adsorbing onto the alum. Saponin was added to rhIFNβ-1a to a final concentration of 0.2%. $CuCl_2$ was dissolved in PBS to a final concentration of 0.08 M (stock solution). To avoid metal-induced oxidation of rhIFNβ-1a and therefore formation of protein aggregates, the stock solution of $CuCl_2$ was mixed with rhIFNβ-1a in a 1:1 ratio directly before injection. The final concentration of $CuCl_2$ of 0.04 M was chosen. Identical to the concentration used in the protocol of metal-induced oxidation of rhIFNα described by Hermeling et al.[25] CTB was conjugated to rhIFNβ-1a using the 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (Sigma–Aldrich) as a linker (see *Supporting Materials* for details). All solutions were stored at 4°C before applying them to the mice.

Animal Treatment. In all animal experiments performed to study impact of adjuvants on the immunogenicity of rhIFNβ-1a mice were treated with rhIFNβ-1a according to a modified regimen described by Hermeling et al.[22] Briefly, tg and non-tg mice (unless indicated elsewhere $n = 8$ per group) received 15 i.p. injections of rhIFNβ-1a, 5 μg/100 μL of PBS per injection, over a time period of three weeks (days 1–5, 8–12, and 15–19).

Experiment I was divided into two parts. In the initial part, the following adjuvants were tested: CFA/IFA, Saponin, alum and Montanide. As follow up, CTB and $CuCl_2$ were tested additionally. CFA was administered together with rhIFNβ-1a on day 1 of the experiment and IFA together with rhIFNβ-1a on days 3 and 5. On the other days, mice from this group received rhIFNβ-1a treatment only. Mice coinjected with Montanide received the rhIFNβ-1a/Montanide emulsion on days 1–5. On days 8–12 and 15–19, these mice were injected with rhIFNβ-1a only. Saponin and alum were coinjected with rhIFNβ-1a during the whole period of the experiment (days 1–5, 8–12, and 15–19). A control group was treated with rhIFNβ-1a without the addition of an adjuvant. In the follow up study groups consisted of tg mice ($n = 8$) and non-tg controls ($n = 7$ in groups treated with CTB and $CuCl_2$ and $n = 6$ in group injected with rhIFNβ-1a alone). Mice received rhIFNβ-1a with or without adjuvant (control) on days 1–5, 8–12, and 15–19.

Mice were anesthetized by isoflurane inhalation and euthanized by decapitation 3 days after the last injection (day 22). The development of ADA was monitored in blood samples collected submandibularly on days −2, 10 (before the rhIFNβ-1a injection of that day), and 22 (during euthanasia). Plasma was isolated by spinning down the blood for 10 min at 3000*g* at 4°C and was stored at −20°C until further assessment for anti-rhIFNβ Abs.

Because in *Experiment I* CTB was found to be a potent enhancer of ADA, production in tg mice *Experiment II* was performed to determine if this enhanced immunogenicity could lead to the formation of immunological memory. tg ($n = 8$) and non-tg ($n = 7$) mice were treated with rhIFNβ-1a with or without (controls) CTB for 3 weeks as described in the previous paragraph. After the initial treatment, a 6-week washout phase was applied after which the mice were rechallenged with two injections of 5 μg of rhIFNβ-1a on days 64 and 65. On day 73, the animals were euthanized by decapitation under anesthesia. The anti-rhIFNβ Abs were determined in blood samples collected submandibularly on days −2, 10 (before rhIFNβ-1a injection of that day), 22, 50, 61, 68, and 73 (during euthanasia). Plasma was isolated by spinning down the blood for 10 min at 3000*g* at 4°C and stored at −20°C until further assessment for anti-rhIFNβ Abs.

Ab Assays

Titers of anti-rhIFNβ Abs were measured by a direct sandwich ELISA as described before.[22] Briefly, 96 wells microtiter plates (Greiner-Bio-One, Alphen aan den Rijn, The Netherlands) were coated with 0.1 μg of rhIFNβ-1a per well. After an overnight incubation, plates were washed with washing buffer (PBST: PBS + 0.05% Tween 20) and subsequently blocked with 4% milk in PBST. For the screening assay, plasma was diluted 1:100 in blocking solution and the plate incubated for 1 h at RT with constant orbital shaking (400 rpm). Anti-rhIFNβ Abs were detected by HRP conjugated goat anti-mouse total IgG Ab (Thermo Fisher Scientific, Landsmeer, the Netherlands). The color reaction was initiated by addition of 3,3′,5,5′-tetramethylbenzidine (TMB; Thermo Fisher Scientific) and stopped by 0.18 M of H_2SO_4. The optical density values were measured with a BMG Spectrostar microplate reader (Isogen Life Sciences, De Meern, The Netherlands) at a wavelength of 450 nm (OD450).

Plasma samples were defined positive if the background corrected absorbance values were ten times higher than that of the pre-treatment sera. Titers of positive samples were determined by subsequent ELISAs, using a twofold serial dilution

series with a starting dilution of 1:10 (Study 2.3.1. day 8, Study 2.3.2 and Study 2.3.3) or 1:100 (Study 2.3.1 day 19).

Titers of anti-Pneumovax 23® Abs were measured by a modified method as described by Zysk et al.[26]. Briefly, pneumococcal polysaccharides were conjugated with poly-L-lysine and then coated on the Nunc™ Maxisorp™ microtiter plate (Thermo Fisher Scientific). Plates were washed twice with distilled water and incubated for 10 min with PBS containing 0.05% Tween 20. Serial 5-fold dilutions of plasma samples in PBS were then incubated on the plates for 2 hrs at 37°C. HRP conjugated goat anti mouse IgG Ab (Thermo Fisher Scientific) diluted 1:4000 in PBST was added on the plate and incubated at 4°C overnight. The colour reaction was initiated by adding TMB and terminated by 0.18 M H_2SO_4. OD450 was measured with BMG SpectroStart microplate reader (Isogen Life Sciences).

The titers of anti-rhIFNβ Abs and anti-Pneumovax 23® Abs were calculated by plotting the absorbance values of the serial dilutions against log dilution. The plots were fitted to a sigmoidal dose–response curve using GraphPad Prism version 4.00 (GraphPad Software, La Jolla, California). The reciprocal of the dilution of the EC50 value was considered the titer of the plasma. ADA levels of the tg mice in experiment 2.3.2, "*Formation of GCs*," were considered positive using the described cut off point. However, they were too low to calculate titers as described above. Therefore OD450 values determined for a dilution of 1:100 were used for data analyses and figures.

Immunohistochemistry of GCs

Five micrometer sections of frozen spleens embedded in OCT medium (CellPath, Newtown, UK) were cut on a cryostat (Leica, Rijswijk, The Netherlands). Sections were air dried and fixed in 2% paraformaldehyde (4°C, 2 min) followed by 10 min of blocking with 3% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 2% Tween 20. Endogenous peroxidases were inactivated by 10 min incubation with 3% H_2O_2 in MetOH. GC-specific cells were stained with biotin conjugated peanut agglutinin (PNA; Sigma–Aldrich) diluted 1:200 in blocking solution (4°C, O/N). Then sections were incubated with Alkaline Phosphatase conjugated ExtrAvidin® (Sigma–Aldrich) (1:100, 30 min, RT), washed and the visualization was initialized by adding nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt substrate (Sigma–Aldrich). Naïve B cells were visualized by rat antiCD45R Ab (eBiosciences, Vienna, Austria) (1:200, 1 h, 37°C) followed by anti-rat-HRP conjugate (eBiosciences) (1:200, 1 h, 37°C). The B cells were visualized by 3-amino-9-ethylcarbazole substrate according to the manufacturer's protocol (Sigma–Aldrich). Sections were analysed by Nikon Eclipse 2000-U microscope (Nikon Corporation, Amsterdam, The Netherlands). The density of GCs, expressed as the number of PNA positive regions on the section per surface of the section was calculated using ImageJ software (National Institute of Health, Bethesda, Maryland). The number of GCs was normalized by dividing the number of GCs in spleens from treatment groups by the basal number of GCs in the control spleens from saline treated animals.

Statistical Analysis

All obtained data were tested for normal distribution by a Shapiro–Wilk test. Because obtained data were not normally distributed non-parametric tests were used to determine statistical differences between groups. A Kruskal–Wallis test was used to determine the overall effect of treatments between experimental groups. A one tailed Mann–Whitney test was used to assess if treatment with GK1.5 (anti $CD4^+$ T-cell Ab) significantly decreased ADA titers compared with the saline treated mice. In order to compare GCs number between tg and non-tg animals as well as to assess the effect of adjuvant within the tg or non-tg mouse groups, two tailed Mann–Whitney test was used. To determine if the adjuvants increased the Ab formation in treatment groups, when compared with controls, a one tailed Mann–Whitney test was used. All calculations were performed using IBM® SPSS® 20.0 (IBM, Amsterdam, The Netherlands) software and a p value equal or lower than 0.05 was considered significant.

RESULTS AND DISCUSSION

Timing of $CD4^+$ T-Cell Involvement in the ADA Response Against Betaferon®

To investigate when $CD4^+$ T cells are important for ADA production, they were depleted on different time points before and during Betaferon® treatment. As shown in Figure 1, $CD4^+$ T-cell depletion in tg and non-tg mice either before or directly after (day 2) the start of Betaferon® treatment significantly inhibited ADA responses compared with control mice on both days 8 and 19. When $CD4^+$ T-cell depletion was initiated after the fourth injection of Betaferon® (day 4) tg animals developed comparable ADA titers to non-depleted controls on day 8. In contrast, in corresponding non-tg animals ADA titers on day 8 were significantly lower when compared with the non-depleted control group. On day 19, both tg and non-tg animals, in which depletion was started after the 4th injection of Betaferon®, had comparable ADA levels as control mice. When $CD4^+$ T-cell depletion started on day 8, no significant effect on ADA formation was observed for either the tg or non-tg animals on day 19.

Our results confirm the importance of $CD4^+$ T cells for ADA formation in tg mice. They show that $CD4^+$ T cells are recruited in anti rhIFNβ Ab responses shortly after the first administration of Betaferon® and are vital during subsequent ADA production. Moreover, obtained results show that presence of $CD4^+$ T cells within the first 4 days after the start of Betaferon® treatment was sufficient for ADA production similarly to non-depleted controls. Therefore, the presented data suggest a similar role of $CD4^+$ T cells in the production of ADA in tg immune tolerant mice and non-tg mice, which develop an immune response via the TD pathway. Sauerborn et al.[27] proposed that production of ADA against Betaferon® starts with the TI activation of MZ B cells, which subsequently may attract and activate $CD4^+$ T cells. Our data suggest that such MZ B cells' driven activation of $CD4^+$ T cells occurs rapidly after administration of the immunogen (Betaferon®) as no clear delay in the ADA production was found in tg mice, when compared with non-tg controls. However, one cannot exclude the possibility that in tg mice $CD4^+$ T cells and MZ B cells are activated simultaneously.

Formation of GCs

The aim of this experiment was to determine if GCs are formed during the response against therapeutic rhIFNβ. RhIFNβ-1a drug substance resulted in a significant ADA response in non-tg mice and much lower ADA response in the tg mice (Figs. 2a and

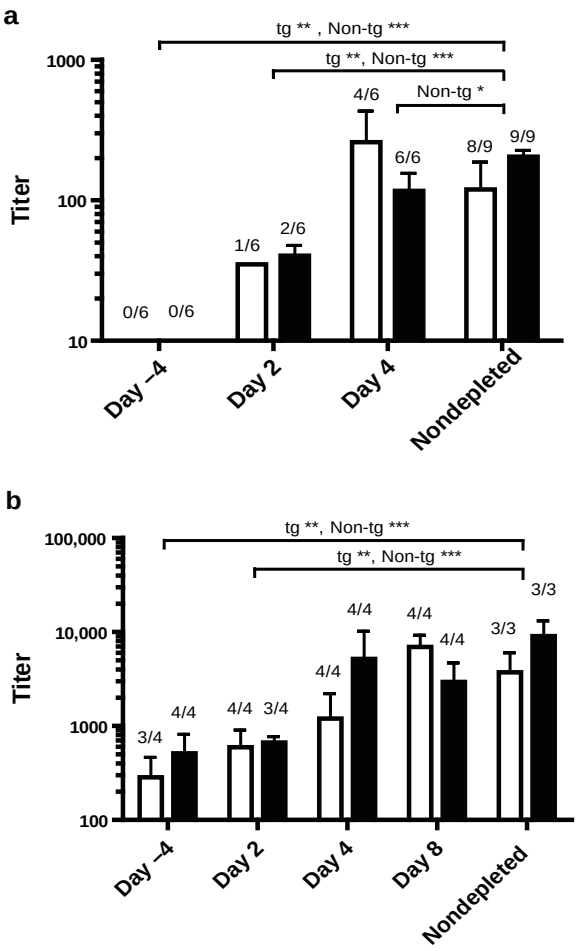

Figure 1. Formation of total anti-rhIFNβ IgG in $CD4^+$ T-cell depleted animals on day 8 (a) and 19 (b) of Betaferon® treatment. The *x* axis shows on which day of experiment the depletion of $CD4^+$ T cells was initiated. White bars show average titer of tg ADA positive animals with corresponding SD. Black bars show average titer of non-tg ADA positive animals with corresponding SD. Numbers above bars indicate the number of ADA positive animals out of total amount of animals in group. The lines above the bars indicate significant differences between ADA titers between treatment group and nondepleted control group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2b). Alum led to an increase of ADA levels in non-tg animals, but not in the tg animals. Administration of Pneumovax 23® induced the formation of similar Ab titers in both tg and non-tg mice (Fig. 2c). No effect of the adjuvant on the Ab titer was seen in animals receiving Pneumovax 23®.

Non-tg animals treated with rhIFNβ-1a drug substance had a similar number of GCs as tg mice on days 9 and 19 (Figs. 3a and 3b). The number of GCs in the tg and non-tg mice was not significantly different between rhIFNβ-1a drug substance and Pneumovax 23® treatment. However, the GCs in mice treated with rhIFNβ-1a seemed to have a higher area of PNA^+ than GCs in mice treated with Pneumovax® (Fig. 3a). For the tg mice, adsorption of rhIFNβ-1a drug substance to the adjuvant did not increase the number of GCs either on day 9 or on day 19. In contrast, in non-tg mice the adjuvant significantly increased the number of GCs by a factor of 4 on day 9 ($p = 0.009$). On day 19, this increase was just failing to reach statistical significance ($p = 0.051$). Aluminum hydroxide did not have an effect on the number of GCs formed after Pneumowax 23® treatment in the tg and non-tg mice.

Our results show that although GCs are present in spleens of the tg mice and their number is similar to non-tg controls the ADA production significantly differs between tg and non-tg mice. Despite the low number of GCs ADA titers in non-tg animals were significantly higher when compared with tg mice. This observation suggests a different dynamic of the maturation process of B cells within GCs of non-tg mice. It might be possible that in non-tg animals more mature plasma B cells are released from the GCs compared with tg animals, or that there is a suppressing mechanism within GCs of tg mice, which decreases the production of ADA. To stimulate ADA production and therefore facilitate the formation of GCs, rhIFNβ-1a drug substance was adsorbed on alum. This adjuvant is known to activate a Th 2 type of immune response.[28,29] Indeed, it was able to enhance ADA formation and increase the number of GCs in the non-tg mice. However, it failed to increase Ab production against rhIFNβ-1a drug substance in tg animals. The number of GCs in the tg animals also remained unchanged after the addition of adjuvant. The lack of aluminum hydroxide effect in tg mice suggests the existence of a down regulating mechanism inhibiting the production of ADA to therapeutic proteins in tg animals or a less-efficient maturation of B cells during stimulation with a "self-like" antigen.

Pneumovax 23® has been previously used as a TI antigen control in experiments on rhIFNβ immunogenicity published by Sauerborn et al.[19] They showed that $CD4^+$ T cells are necessary for effective ADA production against Betaferon®, but not for response against Pneumovax 23®. Therefore Pneumovax 23® was considered to be an adequate control for a TI response in the current experiment. However, we did observe some GCs in mice injected with Pneumovax 23®, but their size (area of PNA positive B cells) seemed to be smaller than the size of GCs induced by rhIFNβ-1a (Fig. 3a). Our observation is in accordance with reported data showing that although GCs might be formed after injection of certain TI type II antigens, their appearance is different than of GCs induced by TD antigens.[30,31] Therefore, the different size of GCs induced by rhIFNβ-1a and Pneumovax 23® in tg mice suggests that the mechanism of GC formation in tg animals treated with rhIFNβ-1a is different from a classical TI response, and possibly a TD response.

Impact of Adjuvants on the Immunogenicity of rhIFNβ in Immune Tolerant Mice

The lack of increased ADA production by alum in tg mice raised the question if the observed effect is an intrinsic feature of alum or if it is common for more adjuvants. Therefore, follow-up studies, referred to as *Experiment I* and *Experiment II*, were performed, in which more commonly used adjuvants have been tested. In *Experiment I*, we first evaluated several adjuvants known to be effective in induction of immune response against TD antigens: CFA/IFA, Montanide ISA 50 V2, saponin and alum. The ADA titers measured on the day 22 of experiment are shown in Figure 4a. None of the tested adjuvants was able to trigger ADA production in tg mice. Montanide ISA 50 V2, saponin and alum significantly increased the ADA titers on day 22 in non-tg mice. Among all tested adjuvants saponin seemed to be the most potent as the average ADA titer in non-tg mice

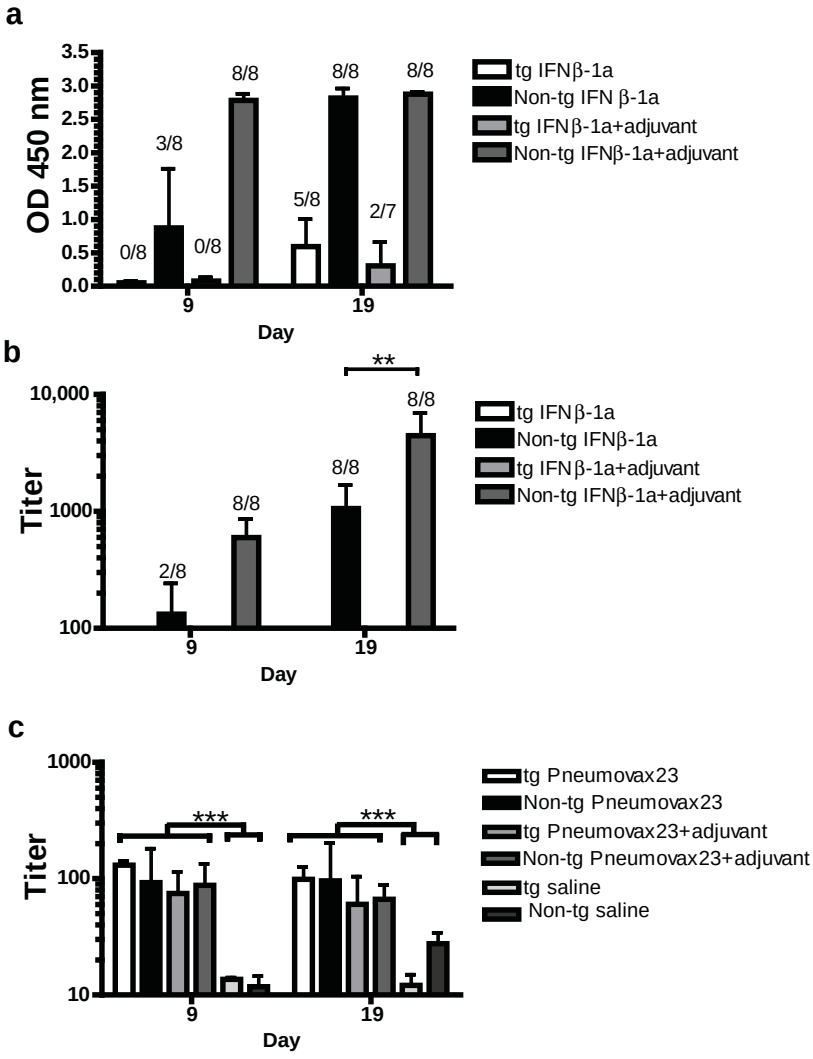

Figure 2. (a) Effect of aluminum hydroxide on the production of total IgG anti rhIFNβ-1a Abs in tg and non-tg animals showed as the change of OD450 values. The Ab levels in tg animals, although in part of them exceeded the positivity threshold (see definition in *Materials and Methods* section), were too low to calculate titers according to described method. The bars show average OD450 of all animals in group with corresponding SD. The numbers above bars indicate the number of animals classified as positive out of total amount of mice per group. (b) Effect of aluminum hydroxide on the titer of total IgG anti rhIFNβ-1a Abs in tg and non-tg animals. The bars show the average titer of ADA positive animals with corresponding SD. Adjuvant significantly increased ADA titers of non-tg animals on day 19. (c) Production of anti Pneumovax23® total IgG. The treatment with Pneumovax 23® whether with or without adjuvant significantly increased total IgG titers when compared with control group. Bars show the average titer from all animals in group with corresponding SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

was 60-fold higher than in control non-tg mice receiving only rhIFNβ-1a ($p < 0.0001$). Alum induced an 8.7-fold increase and Montanide ISA 50 V2 elevated the titers by twofold ($p = 0.002$ and $p = 0.021$, respectively). No impact of CFA/IFA on Ab titers was observed in the non-tg mice.

In the follow-up study, mice were treated with rhIFNβ-1a conjugated to CTB or mixed with $CuCl_2$. RhIFNβ-1a/CTB conjugates did induce measurable ADA titers in three out of eight tg mice (Fig. 4b). $CuCl_2$-induced ADA in 1 tg mouse. In non-tg mice, administration of rhIFNβ-1a–CTB conjugates resulted in a significant increase of ADA titers, when compared with control mice receiving only rhIFNβ-1a ($p = 0.034$). The average ADA titer in non-tg mice receiving rhIFNβ-1a with or without $CuCl_2$ did not differ significantly.

CTB was found in *Experiment I* to be an effective adjuvant for rhIFNβ-1a in part of tg mice; therefore, an additional study (*Experiment II*) was performed to determine if CTB may lead to a complete breaking of tolerance and, in consequence, to the formation of immunological memory against rhIFNβ. Animals were administered with rhIFNβ-1a conjugated with CTB according to the same regime as in *Experiment I*. As shown in Figure 5, all tg and non-tg mice treated with rhIFNβ-1a and CTB developed ADA on day 22 of the experiment. This increased immunogenicity of CTB–rhIFNβ-1a conjugates in *Experiment II* originated most likely from usage of two different batches of conjugates in these experiments. Moreover, ADA titers in mice treated with CTB–rhIFNβ conjugates were significantly higher than in control mice ($p < 0.001$ and $p = 0.017$

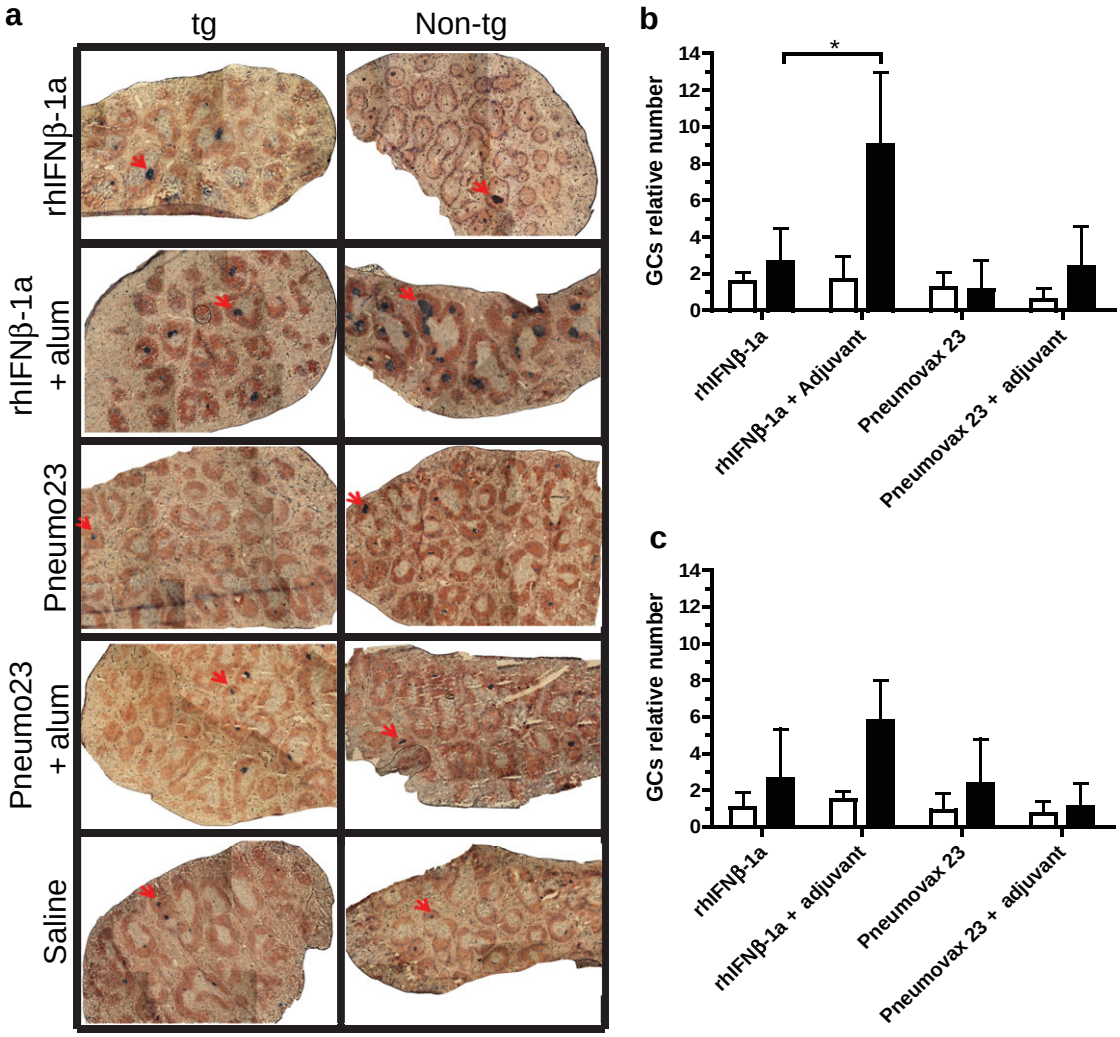

Figure 3. (a) Formation of GCs in spleens of tg and non-tg animals 9 days after the beginning of rhIFNβ-1a or Pneumovax23® treatment. The red arrows indicate the GC specific, PNA positive B cells (dark blue) surrounded by naive, B220 positive cells (brown). (b) The relative number of GCs in the spleens 9 days after rhIFNβ-1a or Pneumovax23® treatment. (c) The relative number of GCs in the spleens 19 days after rhIFNβ-1a or Pneumovax23® treatment. The bars show relative number of GCs per section surface of the tg (white) and non-tg (black) animals' spleen with corresponding SD. Adsorption of rhIFNβ-1a on aluminum hydroxide increased number of GCs in non-tg animals on day 10 of Betaferon® treatment. $^{*}p < 0.05$.

for tg and non-tg mice, respectively). Interestingly, injections of CTB resulted in a faster ADA onset in both tg and non-tg mice compared with non-tg mice receiving only rhIFNβ-1a. The initial treatment was followed by a 6 weeks washout phase, after which mice were rechallenged with rhIFNβ-1a without CTB. At the end of the washout phase (day 61), the ADA were found only in three out of seven tg animals. In contrast, ADA was still detected in all non-tg mice irrespective of the primary treatment they received. On days 63 and 64, mice received two injections with rhIFNβ-1a. As shown in Figure 6, rechallenge resulted in a fast and robust increase of ADA titers in both groups of non-tg mice, regardless of the initial treatment ($p < 0.001$ and $p = 0.008$ for mice treated initially with or without CTB, respectively). In tg mice, however, ADA titers did not increase after rechallenge.

Many factors have been suggested to contribute to ADA development. Some of them might act as adjuvants, such as host cells impurities, leachates from storage containers and formulation's excipients.[32,33] However, in these reports an impact of the leachates and bacterial compounds had been tested using foreign, TD antigens, which are known to be susceptible to the effect of adjuvants. However, there are no data that confirm that adjuvants boost immune responses against non-TD, self-like antigens such as therapeutic proteins. In fact, recombinant human insulin was shown to be non immunogenic in immune tolerant mice even when administered with FA.[34] Direct proof showing the capability of these compounds to enhance the production of ADA against a "self" protein in immune tolerant animals or humans is, however, lacking.

The most commonly used adjuvants are mineral salts, water/oil emulsions and bacterial compounds. In this study, we evaluated several adjuvants, some of which are commonly used in animal and human vaccines. In addition, we tested the adjuvant properties of copper salt, which was used as a catalyst for the oxidation and aggregation of rhIFNα. These studies showed that aggregates induced by copper oxidation are indeed immunogenic in the immune tolerant mice, suggesting that copper might also have adjuvant properties.[35] The mode

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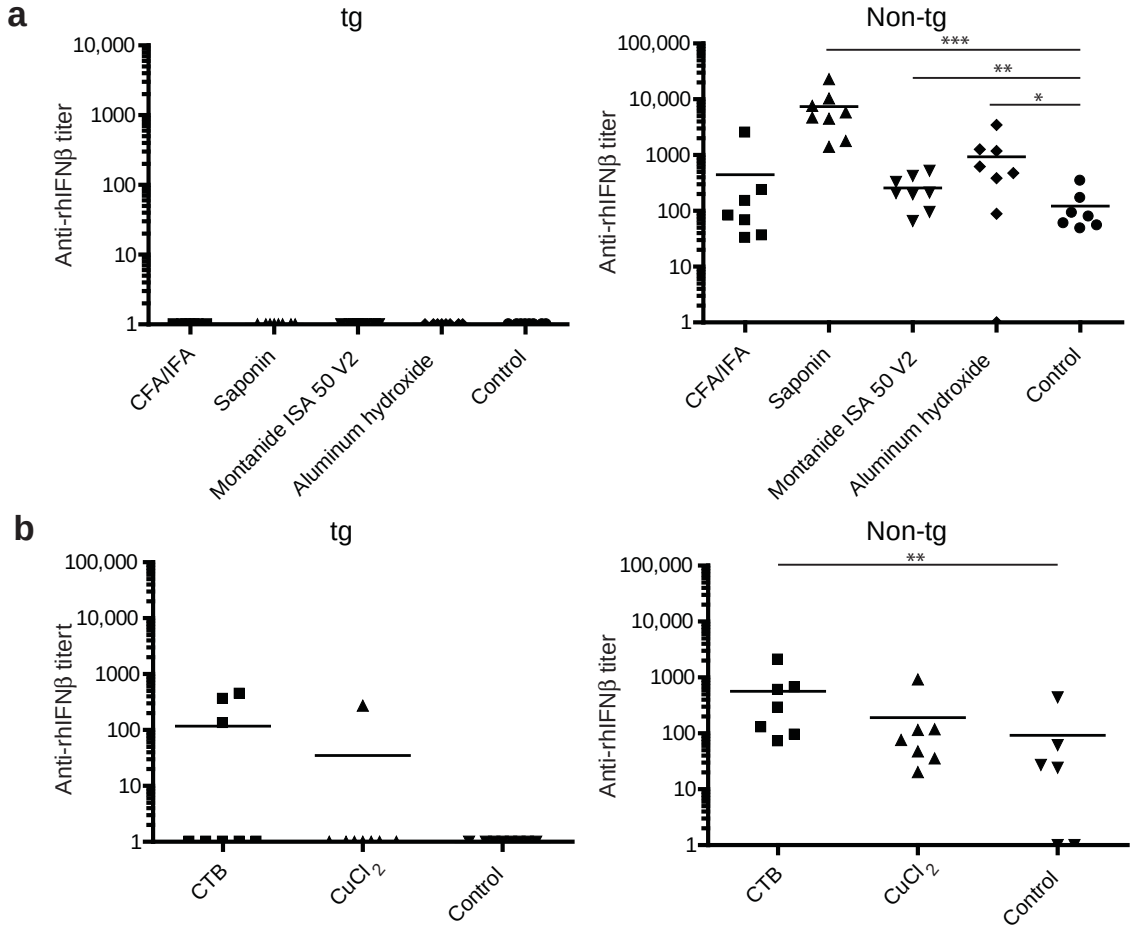

Figure 4. Production of anti rhIFNβ-1a ADA on Day 22 in tg and non-tg mice in *Experiment I* (a) initial study, (b) follow-up study. Each symbol represents the ADA titer in a single mouse. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

of action of tested adjuvants remains unclear, but may be explained by the formation of a depot at the site of injection and enhanced antigen uptake by dendritic cells,[36–39] the stimulation of pattern recognition receptors,[40] induction of cell membrane permeabilization attracting T cells[41] and increased uptake by antigen presenting cells by binding to the GM1 ganglioside.[42]

Our results clearly show that most adjuvants used to boost immune response against TD antigens fail to induce ADA formation towards the "self" antigen rhIFNβ-1a in immune tolerant mice. Although some of them, that is saponin, were extremely potent in enhancing the immune response in non-tg mice (TD antigen) only CTB, when conjugated with rhIFNβ-1a, significantly increased both the number of tg mice with detectable ADA and ADA titers. This might be caused by several potential mechanisms: (i) CTB may enhance the uptake of rhIFNβ-1a by antigen presenting cells, (ii) CTB might attract T helper cells to the immune response and as a foreign protein conjugated to rhIFNβ-1a may by-pass T-cell tolerance, (iii) CTB–rhIFNβ conjugates might form aggregate-like oligomers, which enhance ADA production. Especially the last explanation is attractive because it has been shown that protein therapeutics, when aggregated, are considerably more immunogenic than monomeric proteins.[23,35,43] RhIFNβ-1a, when conjugated to CTB, formed oligomers of molecular weight exceeding 170 kDa (Supplementary Data). However, the exact mechanisms causing this adjuvant effect of CTB has to be elucidated.

Carrier, bacterial proteins have been shown to be very potent adjuvants enhancing the immune response against both TD and TI antigens.[39,44] Moreover, they have been also shown to trigger memory formation, specific to TI counterpart of conjugates.[45,46] However, the rechallenge of tg mice with rhIFNβ-1a did not result in enhanced ADA production in contrast to non-tg mice. That strongly suggests that the production of ADA during primary treatment with CTB–rhIFNβ conjugates did not lead to the formation of rhIFNβ specific immunological memory. It indicates that tolerance of tg mice towards hIFNβ was not broken by adjuvant usage. These results are in accordance with our previous observations showing that aggregated rhIFNβ-1b, although very potent in triggering ADA production, failed to induce a clear immunological memory response after rechallenge in tg mice.[20] Our data suggest possible existence of suppressing mechanism, which prevents memory formation. For example, it could be hypothesized that regulatory T cells (T_{regs}), which are known to prevent autoimmunity by down regulation of effector T cells,[47] might inhibit formation of memory. However, further studies are needed to determine the mechanism responsible for apparent lack of memory.

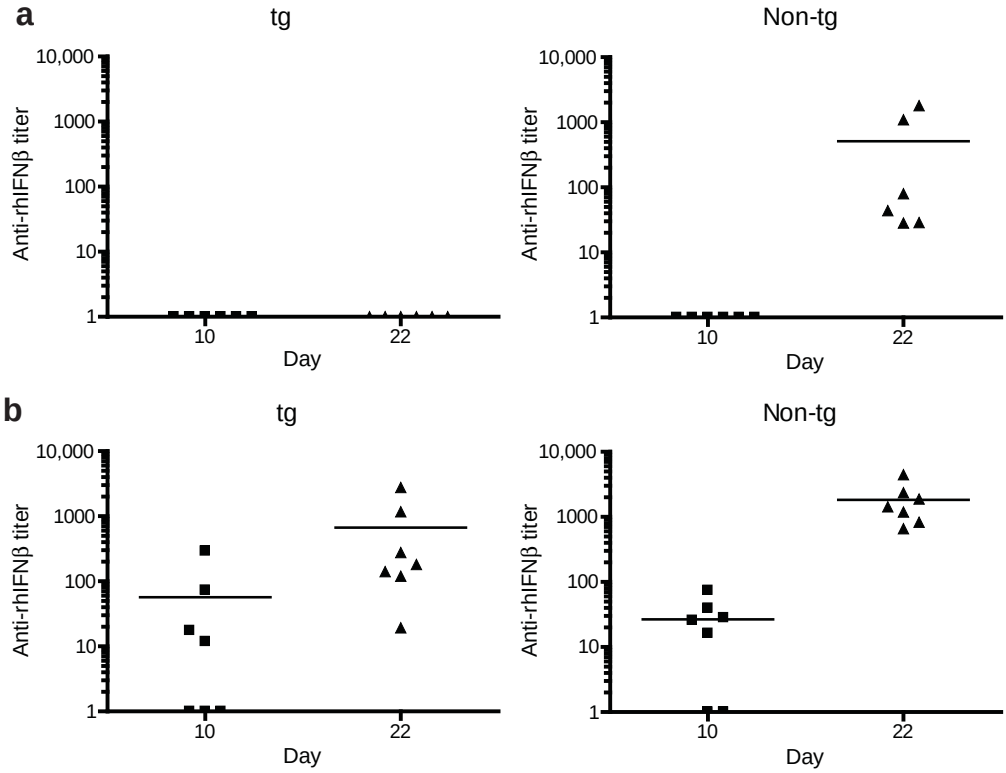

Figure 5. Production of anti rhIFNβ-1a ADA in tg and non-tg mice on days 15 and 22 (primary treatment) of *Experiment II*. (a) Mice treated with rhIFNβ-1a, (b) mice treated with CTB–rhIFNβ-1a conjugates. Each symbol represents the ADA titer in a single mouse.

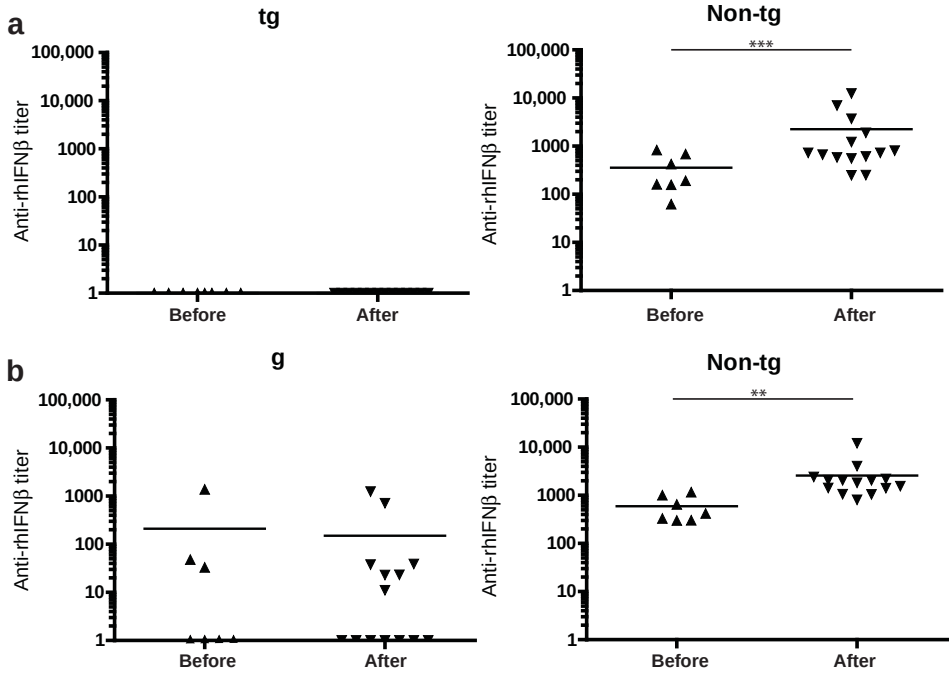

Figure 6. Titers of anti rhIFNβ-1a ADA in tg and non-tg mice before (day 61) and after (days 68 and 73) rechallenge with rhIFNβ-1a in *Experiment II*. (a) Mice primarily treated with rhIFNβ-1a, (b) mice primarily treated with CTB–rhIFNβ-1a conjugates. Each symbol represents the ADA titer in a single mouse. $^{**}p < 0.01$, $^{***}p < 0.001$.

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CONCLUSIONS

The Ab response to rhIFNβ in tg immune tolerant animals is not a "classical" immune response. The early stages of ADA production display features typical for the TD mechanism: formation of GCs and dependency on $CD4^+$ T cells. However, adjuvants known to increase Ab response to classical TD antigens, were not effective in enhancing the ADA production toward rh protein in immune tolerant mice. Moreover, we showed that ADA production triggered by CTB, the only effective adjuvant in tg mice, was not accompanied by the development of rhIFNβ specific immunological memory. Our results strongly emphasize that immunogenicity might be a novel type of immune response distinct from the well-known classical mechanism. Therefore, immunogenicity predictions cannot be based on the current knowledge of classical immune responses.

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