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Immune Modulation and Prevention of Autoimmune disease by Repeated Sequences from Parasites Linked to Self Antigens

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Short title: Immunomodulatory and therapeutic effect induced by S-Antigen fusion proteins

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ABSTRACT

Parasite proteins containing repeats are essential invasion ligands, important for their ability to evade the host immune system and to induce immunosuppression. Here, the intrinsic suppressive potential of repetitive structures within parasite proteins was exploited to induce immunomodulation in order to establish self-tolerance in an animal model of autoimmune neurological disease.

We tested the tolerogenic potential of fusion proteins containing repeat sequences of parasites linked to self-antigens. The fusion constructs consist of a recombinant protein containing repeat sequences derived from the S-antigen protein (SAg) of *Plasmodium falciparum* linked to a CD4 T cell epitope of myelin. They were tested for their efficacy to control the development of experimental autoimmune encephalomyelitis (EAE), In addition, we used the DO11.10 transgenic mouse model to study the immune mechanisms involved in tolerance induced by SAg fusion proteins.

We found that repeated sequences of *P. falciparum* SAg protein linked to self-epitopes markedly protected mice from EAE. These fusion constructs were powerful tolerizing agents not only in a preventive setting but also in the treatment of ongoing disease. The tolerogenic effect was shown to be antigen-specific and strongly dependent on the physical linkage of the T cell epitope to the parasite structure and on the action of anti-inflammatory cytokines like IL-10 and TGF-β. Other mechanisms include down-regulation of TNF-α accompanied by increased numbers of $FoxP3^+$ cells. This study describes the use of repetitive structures from parasites linked to defined T cell epitopes as an effective method to induce antigen-specific tolerance with potential applicability for the treatment and prevention of autoimmune diseases.

Keywords: Repetitive antigens, Self-antigens, Immunomodulation, Multiple sclerosis, EAE

1. Introduction

Autoimmune disorders are the result of the breakdown of central and peripheral immune tolerance. The onset and progression of autoimmune diseases involve a complex mixture of endogenous and exogenous signals and predisposing factors that result in pathogenic inflammatory reactions mediated by auto-reactive cells (Rashedi et al., 2007). Current first line treatments for autoimmune neurological diseases like multiple sclerosis comprise disease-modifying drugs that moderately control exacerbations of disease and are, however, associated with generalized depression of the immune system.

The main goal of immunotherapy is to target specifically autoreactive T cells while maintaining the immune system's ability to fight foreign antigens avoiding generalized immunosuppression. Immunotherapy aimed to reestablish antigen-specific self-tolerance represents a promising strategy to modulate autoimmunity with potential applicability in translational research. Different methods, such as the application of high or low doses of a specific antigen and defined routes of administration are critical factors that determine the induction of immune tolerance. The doses and concentration of the antigen are often directly correlated with the generation of active and cell-dependent regulatory mechanisms (Turley and Miller, 2010) or with the induction of anergy (Bitar and Whitacre, 1988) or even deletion of antigen-specific T cells (Critchfield et al., 1994; Liblau et al., 1996; Apostolou and von Boehmer, 2004; Judkowski et al., 2004). Likewise, immunological tolerance achieved by oral (Higgins and Weiner, 1988; Weiner, 2000; Weiner et al., 2011) and intranasal (Anderton et al., 1998; Fossati-Jimack et al., 2015) delivery of autoantigens has shown to be mediated by active suppressor mechanisms such as anti-inflammatory cytokines and regulatory cells (Chen et al., 1995; Weiner et al., 2011).

Recently, the use of carriers has shown to improve the stability, half-life and bioavailability of soluble peptides, increasing the efficacy of tolerogenic peptide vaccination by promoting the induction of antigen-specific T regulatory cells (Tregs) (Quintana, 2013; Gupta et al., 2015). Other recent approaches for the induction of specific immune tolerance include the use of synthetic nanoparticles containing specific proteins in combination with immunosuppressive

drugs (Maldonado et al., 2014), stem cell transplantation (Coleman and Steptoe, 2012) and gene transfer (Sack et al., 2014).

We have shown previously that repetitive T cell self-epitopes are effective in the prevention and treatment of ongoing experimental autoimmune encephalomyelitis (EAE) (Falk et al., 2000b; Puentes et al., 2013). This protection was shown to be antigen-specific and mediated by the induction of active suppression involving the action of anti-inflammatory cytokines like IL-10 and TGF-β. Multimerized antigens, initially thought to increase antigenicity by crosslinking MHC class II complexes on the surface of antigen presenting cells (APCs) and thereby leading to an enhanced T cell activation, have shown to suppress the clinical symptoms in experimental animal models of human autoimmune disease, like multiple sclerosis, autoimmune neuritis and type I diabetes (Falk et al., 2000b; Stienekemeier et al., 2001; Piaggio et al., 2007; Puentes et al., 2013).

Despite the abundance of natural repetitive regions in eukaryotic proteins, their function and structure are barely known (Huntley and Golding, 2004). More characterized are the repetitive structures present on the surface of parasites and the frequency and extent of this phenomenon in the parasite taxa suggests that it represents a special feature. It is suggested that natural selection of repeat-arrays in malaria parasites has been exerted as a mechanism of immune evasion (Hughes, 2004). In particular, intracellular parasites have a higher content of repetitive sequences (Bartholomeu et al., 2014) that play important roles such as cell adhesion, invasion and suppression to evade the host immune response (Mendes et al., 2013).

Recently, the significance of the suppression induced by several protozoan parasitic infections has been correlated with the outcome of autoimmune diseases, following concomitant infections. There is ample evidence that parasite infections can ameliorate the course of autoimmune diseases (Gibbon et al., 1997; Mattsson et al., 2000). For example, *Plasmodium* infection has shown to interfere with the EAE autoimmune response through the activation of Tregs (Farias et al., 2011). Similarly, infections with *Trypanosoma* can confer protection against autoimmunity in arthritis models (De Trez et al., 2015) and markedly

suppress the clinical course of experimental EAE, likely due to a modulatory effect of IL-10 on Th1 expansion (Tadokoro et al., 2004). Moreover, certain parasites have been found to have the ability to skew the host's response towards a Th2 phenotype, characterized by increased expression of IL-4, IL-5 and IL-13 (Mohrs et al., 2005). A similar phenomenon is observed in allergies, where helminth infections induce a regulatory CD4+CD25+FoxP3+ T cell population able to suppress allergic responses (Wilson et al., 2005).

In the present study, fusion constructs composed of a recombinant protein containing repeat sequences from the S-Antigen of *P. falciparum* linked to T cell epitopes, were evaluated in the autoimmune EAE model for the ability of inducing tolerance. We chose the SAg protein as it represents a striking model of tandem repeats of amino acid sequences covering nearly two thirds of the parasite protein secreted in large amounts during the erythrocyte stage and it is well known to function as a target for the host immune system (Saint et al., 1987).

Administration of a single dose of these repetitive structures linked to a CD4+ T cell epitope of myelin was sufficient to markedly inhibit the development of EAE. Some of the mechanisms involved in the protective effect induced by the constructs include the production of regulatory cytokines like IL-10 and TGF-β. Tolerance induction seems to be also associated with increased numbers of FoxP3 cells and down-regulation of Th1 response.

Results from this study suggest an immunomodulatory role of parasite repeat sequences anchored to self-antigens, which may have applications to vaccine design and treatment of human autoimmune diseases.

2. Materials and Methods

2.1 Ethics Statement

The animals were maintained and handled according to the Directive 86/609/EEC of the European Community Council and to the institutional, state and federal guidelines. All animal protocols were approved by the ethics committee of the Landesamt für Gesundheit und Soziales (LAGeSo, Berlin, Germany) with registration numbers G0140-06 and G0331-08.

Animals were housed under standard conditions of 12-hour light/dark cycle and given access to food and water *ad libitum*. During periods of active paralysis, animals were provided gel pack and/or moistened food on the cage floor. Animals were sacrificed, by carbon dioxide overdose, when they met endpoint criteria or at study termination.

2.2 Animals

Mice were housed in the animal facility of the Max Delbrück Center under specific pathogen free conditions and handled in accordance with the institutional guidelines. Female C57BL/6 (H-2^b), SJL/J (H-2^s) or JHT$^{-/-}$ (B6.129P2-Igh-Jtm1Cgn/J) mice (Gu et al., 1993), 10 to 12 weeks old were used for EAE studies. SJL/J mice were purchased from Taconic Laboratories. C57BL/6 and Balb/c mice were purchased from Charles River Laboratories. DO11.10 (H-2^d) and OTII (H-2^b) transgenic mice were bred at the animal facility of the Deutsches Rheuma-Forschungszentrum (DRFZ) under SPF conditions (Bundesinstitut für Risikobewertung, Berlin). DO11.10 and OTII mice carry the MHC class II restricted rearranged T cell receptor transgene reactive to $OVA_{323-339}$. JHT$^{-/-}$ mice were kindly provided by Dr. Simon Fillatreau (DRFZ, Berlin). These mice are homozygous for the Igh-Jtm1Cgn targeted mutation and fail to produce functional B-cells as they lack the gene for the heavy chain joining region.

2.3 Peptides and fusion proteins

Proteolipid protein derived $PLP_{139-151}$ (C140S)(HSLGKWLGHPDKF) later referred as $PLP_{139-151}$, myelin oligodendrocyte glycoprotein MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) and ovalbumin $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) peptides (Research Genetics EMC, Tübingen, Germany) were synthesized by standard solid phase F-moc chemistry. All peptides were purified on a C4 -HPLC column (Vydac).

Fusion proteins containing repetitive sequences of the S-antigen (SAg) of *Plasmodium falciparum* were designed as follows: 24 repeats of a 8-mer unit derived from the NF7 isolate (A(L/R)KSDEAE) were linked by a S3 spacer to the N-terminus of the respective CD4+ T cell epitopes: MOG_{38-51} (SAg-MOG_{38-51}), $PLP_{139-151}$ (C140S) (SAg-$PLP_{139-151}$) and $OVA_{323-339}$

(SAg-OVA$_{323-339}$). Since the natural units contained either L or R in their repeats, building blocks with alternating amino acids were used in tandem repeats. The generation and production was carried out by recombinant techniques in E. coli as described previously (Rotzschke et al., 1997). The constructs were isolated using a His-tag located at the C-terminal site of the construct and purified by RP-HPLC to remove endotoxins. Endotoxin levels were determined using the limulus test (Charles River Laboratories).

2.4 Induction of EAE and therapeutic effect of S-antigen fusion proteins

EAE induction was performed as previously described (Puentes et al., 2013). Briefly, female SJL/J and C57BL/6 mice were immunized by subcutaneous injection on the dorsal region with 50 µg of PLP$_{139-151}$ (C140S) or 50 µg of MOG$_{35-55}$ peptide respectively. Peptides were emulsified in incomplete Freund's adjuvant (Sigma), containing 400 µg of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, USA). One day after immunization, 200 ng and 500 ng of *Pertussis* toxin (List Biological Laboratories, Inc UK) were administered intravenously to SJL/J and C57BL/6 mice respectively. Mice were scored daily for clinical signs of disease according to the following scale: 0, no clinical sign; 1, limp tail; 2, limp tail, impaired righting reflex, and paresis of one limb; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, moribund.

The therapeutic effect of SAg fusion proteins was examined before and after disease induction. Concentration, dosing frequency and route of administration were based on our previous studies using repetitive self-peptides (oligomerized peptides), which showed that a single intravenous treatment with a low dosage of oligomers induced protective effect on experimental models of autoimmune diseases (Falk et al., 2000b; Stienekemeier et al., 2001; Puentes et al., 2013). SAg fusion proteins applied on the range of the peptide-concentration used to induce disease (dosages between 50 and 70 µg), were shown to be safe and effective. For vaccination experiments, 7 days before induction of disease, SJL/J and C57BL/6 mice received intravenously 50 µg SAg-PLP$_{139-151}$ or 70 µg SAg-MOG$_{38-51}$ respectively. In a therapeutic setting, mice were treated on day 7 after EAE induction (before

onset). Other group of mice was treated after the appearance of the first clinical signs of disease with the same amount of SAg-constructs as stated above. The effect of SAg-MOG constructs in the suppression of EAE was tested in B cell deficient JHT mice. EAE was induced in $JHT^{-/-}$ and $JHT^{+/-}$ mice by immunization with MOG_{35-55} peptide and further treated with SAg-MOG fusion protein as described above.

2.5 *In vitro* T cell proliferation assay

T cell proliferation assays with primary lymph node cells (LNC) were performed in 96-well round-bottom plates. Briefly, LNC were harvested from DO11.10 mice and after erythrocytes lysis, $5x10^5$ cells/well were cultured in DMEM/10% FCS (Invitrogen- Karlsruhe, Germany) with titrated amounts of $OVA_{323-339}$ peptide or SAg-$OVA_{323-339}$. After 72 hours incubation, (^{3}H)Thymidine (0,65 µCi/ well) (Amersham, Freiburg Germany) was added and 16 hours later the assay was harvested and counted in a Microplate Counter (Wallac 1420 Victor3TM- Turku, Finland). Specific proliferation is indicated as (^{3}H)Thymidine incorporation as counts per minute (cpm).

2.6 Induction of delayed type hypersensitivity reaction (DTH)

The effect of SAg fusion proteins on the modulation of inflammatory response was examined in a DTH assay. For the induction of an OVA-specific DTH reaction $OVA_{323-339}$ -specific T cells were isolated from DO11.10 mice and $3x10^6$ CD4+ T cells were adoptively transferred intravenously into Balb/c recipient mice. One day later, mice were tolerized by intravenous administration of 5 µg free $OVA_{323-339}$, 60 µg SAg-$OVA_{323-339}$ (equimolar amounts, based on OVA-peptide amount) or phosphate-buffered saline (PBS) as a control. OVA-specific Th1 cells were generated *in vitro* by antigen-specific activation of naive CD4+ T cells from DO11.10 (Balb/c background) as previously described (Siegmund et al., 2005). Briefly, T cells from DO11.10 mice were cultured for 6 days in the presence of APCs, 1 µg/ml OVA peptide under Th1 polarizing conditions (5 ng/ml recombinant IFN-γ, 20 ng/ml recombinant IL-12 and 5 µg/ml αIL-4). This protocol results in the generation of an effector cell population containing 50 to 80% cells producing IFN-γ and less than 1% producing IL-4. A total of $5x10^5$

OVA-specific CD4+ Th1 cells were adoptively transferred into treated mice on day 7. 24 hours later, 250 ng of $OVA_{323-339}$ peptide in 5 µl of Incomplete Freund's adjuvant (IFA, Sigma) were injected subcutaneously into the left footpad. PBS/IFA was injected into the right footpad as a control. DTH reaction was measured on the following 8 days by determining footpad swelling using an Oditest micrometergauge (Kroeplin Längen-messtechnik, Schlüchtern, Germany). Footpad swelling relative to day zero was plotted and further analyzed.

2.7 *In vivo* neutralization of cytokines

For *in vivo* neutralization of cytokines, EAE induced mice were treated with SAg fusion proteins and received 0.5 mg of anti-cytokine antibodies administered intraperitoneally on day 7, 9 and 11 after induction of disease. The effect of cytokine blockade on the clinical course of EAE was monitored daily. The following monoclonal antibodies were used: anti-IL-10 receptor (αIL-10R) (clone 1B1.3a) (O'Farrell et al., 1998), anti-transforming growth factor beta (αTGF-β) (clone 1D11) (Dasch et al., 1989) and rat IgG1 isotype control (clone G1-113) (Sigma). αIL-10R and αTGF-β antibodies were produced at the Max Delbrück Center from hybridoma cell lines and purified on Protein G columns (Thermo Scientific-Pierce, Germany) followed by dialysis. The protein concentration was determined by Bradford test and endotoxin levels (<1 EU/mg protein) were detected using the limulus test (Charles River Laboratories). Hybridoma cell lines were kindly provided by Dr. Alexander Scheffold, Deutsches Rheuma-Forschungszentrum, (DRFZ, Berlin, Germany).

2.8 *In vivo* antigen-specific CD4+ T cell response after SAg-OVA administration

To compare the antigen-specific CD4+ T cell proliferation in response to free antigen and SAg fusion constructs, adoptive transfer experiments using TCR transgenic CD4+ T cells specific for the $OVA_{323-339}$ peptide were performed. Briefly, CD4+ T cells were isolated from DO11.10 mice by negative selection using the CD4+ T cell isolation kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) and depleted of CD90+ cells using MACS. Cell purification was assessed by FACS on a LSR II (BD Bioscience). $3x10^6$ purified DO11.10 CD4+ T cells

were labeled according to manufacturer's recommendation with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE (CFSE); Molecular Probes, Eugene, OR) and adoptively transferred into sex-matched Balb/c recipient mice. One day later, animals received 5 µg $OVA_{323-339}$ peptide or 60 µg SAg-$OVA_{323-339}$ (equimolar amounts, based on OVA-peptide amount) administered intravenously. Antigen-specific proliferation was analyzed on day 6 after adoptive transfer. Briefly, lymph nodes and spleens were isolated and further processed to obtain single-cell suspensions. Erythrocytes lysis was performed and cells were stained with the indicated antibodies for further analysis by flow cytometry. Cells were gated on CD4+ KJ1.26+ T cells and the CFSE profile was further analyzed on the gated subset using CellQuest (BD Bioscience) or FlowJo software (Tree Star, San Carlos, CA).

2.9 Analysis of FoxP3+ regulatory CD4+ T cells following SAg treatment

To examine the role of FoxP3+ Tregs on the tolerogenic action of SAg constructs, $3x10^6$ DO11.10 CD4+ CFSE labeled T cells were intravenously transferred into Balb/c mice. One day later, mice received 5 µg of free $OVA_{323-339}$, 60 µg of SAg-OVA (equimolar amounts, based on OVA-peptide amount) or 60 µg of SAg-MOG as control. Lymph node cells were isolated on day 6 and the frequency of Treg cells were determined by intracellular staining of FoxP3 using the mouse FoxP3 staining Kit (eBioscience). Cells were stained with CD25 (clone PC61), CD4 and KJ1.26 antibodies (BD Pharmingen). The KJ1-26 antibody reacts with the DO11.10 clonotypic T cell receptor specific for $OVA_{323-339}$. FoxP3 expression was analyzed on gated CD4+CD25+ KJ1.26+ CFSE+ T cells.

2.10 Antigen-specific suppression of pro-inflammatory TNF-α following SAg treatment

Antigen-specific TNF-α production by CD4+ T cells from animals that received SAg fusion constructs was compared with untreated mice after adoptive transfer experiments using OVA specific CD4+ T cells isolated from DO11.10 mice. $3x10^6$ DO11.10 CD4+ T cells, labeled with CFSE, were transferred into Balb/c mice and one day after, they received intravenously 5 µg

of free $OVA_{323-339}$, 60 µg SAg-OVA (equimolar amounts, based on OVA-peptide amount) or 60 µg of SAg-MOG as a control. 7 days after, mice were primed subcutaneously with $OVA_{323-339}$ peptide in complete Freund's adjuvant (CFA) and sacrificed 6 days later. LNC were isolated and restimulated with 10 µg/ml OVA peptide and control peptide for 6 hours. Antigen-specific cytokine expression was analyzed on KJ1.26+ CD4+ T cells by intracellular staining of TNF-α. Frequencies of TNF-α+ cells were analyzed on gated CD4+ KJ1.26+ T cells.

To measure the production of TNF-α in JHT mice, OTII ($OVA_{323-339}$ specific) cells labeled with CFSE were transferred into $OVA_{323-339}$ primed $JHT^{+/-}$ and $JHT^{-/-}$ mice and treated with SAg-OVA. Lymph node cells were isolated from treated and untreated mice and activated for 6 hours with $OVA_{323-339}$ peptide and α-CD28 (clone 37.51) (Gross et al., 1992) purified at the Max Delbruck Center. The hybridoma cell line was kindly provided by Dr. Alexander Scheffold, Deutsches Rheuma-Forschungszentrum, (DRFZ, Berlin, Germany). 5 µg/ml Brefeldin A (Sigma) was added for the last 4 hours of stimulation for blocking cytokine secretion. Cells were stained for surface CD4 using α-CD4 (clone RM4-5) BD Pharmingen (San Diego, CA), followed by intracellular staining with αTNF-α (clone XT22), BD Pharmingen. For TNF-α detection in antigen-specific activated T cells, combined intracellular α-CD154 (clone MR1) (Miltenyi Biotech, Bergisch-Gladbach, Germany) and TNF-α staining was performed (Frentsch et al., 2005; Puentes et al., 2013). The frequency of CD154+TNF-α+ double positive in OTII CD4+ T cells was determined and analyzed on a LSR II cytometer with a BD Bioscience FACSDivaTM software.

2.11 Statistical analysis

For comparison of clinical EAE scores, significance between groups was determined by nonparametric Mann-Whitney U test. Data represent the mean ±SEM ***P<0.001, **P<0.01, *P<0.05. P values *P<0.05 were considered significant. Calculations were made using Sigmaplot software (San Jose).

3. Results

3.1 Parasite tandem repeats linked to single-encephalitogenic T cell epitopes are sufficient to protect mice from EAE

Our previous studies have shown that repeat antigens consisting of linear copies of T cell epitopes are able to suppress the clinical disease in various autoimmune model systems (Rotzschke et al., 1997; Falk et al., 2000b; Falk et al., 2000a; Mack et al., 2001; Stienekemeier et al., 2001; Piaggio et al., 2007; Puentes et al., 2013). In the present study, we wanted to investigate the suppressive capacity of parasite derived repeat structures linked to encephalitogenic epitopes in an experimental mouse model of multiple sclerosis. New constructs consisting of repeat sequences derived from *Plasmodium* S-Antigen linked to single CD4+ T cell epitopes were designed and tested for their ability to induce suppression. $PLP_{139-151}$ and MOG_{38-51} are encephalitogenic CD4+ T cell epitopes that induce EAE in SJL/J and C57BL/6 mice, respectively. A diagram of the construct consisting of 24 repeats of a 8mer unit (A(L/R)KSDEAE) linked to the N-terminus of different relevant CD4+ T cell epitopes: $PLP_{139-151}$ (SAg-PLP), MOG_{38-51} (SAg-MOG) and $OVA_{323-339}$ (SAg-OVA), is depicted in Fig. S1A. Successful purification of fusion proteins is shown in Fig. S1B.

A single dose of SAg constructs was administered to EAE induced mice with three different regimes: 7 days prior disease induction, 7 days after EAE induction or therapeutically treated soon after disease onset. Fig. 1 shows that a single injection of the SAg fusion constructs in SJL/J mice inhibits significantly the paralysis associated with EAE. SAg-$PLP_{139-151}$ vaccinated mice developed very mild signs of EAE (maximal average clinical score of 0.6±0.6) at the peak of the disease by day 19, in contrast to untreated animals with a maximum score of 2.4±0.4 (Fig. 1A). Moreover, administration of SAg-PLP constructs 7 days after the induction of EAE markedly protected mice from disease development. Treated animals showed only mild clinical signs of disease compared to the control group that was left untreated (Fig. 1B). Likewise, vaccination and treatment of C57BL/6 mice with SAg-MOG_{38-51} clearly inhibited EAE onset compared to their control groups, which developed severe disease showing mean maximal scores of 2.4±0.2 and 2.8±0.2 respectively (Figs. 1C

and 1D). This clearly shows that treatment with the fusion protein containing the residues MOG_{38-55} is sufficient to effectively protect mice from EAE induced with MOG_{35-55}. Parasite derived fusion proteins linked to CD4+ epitopes not only protected animals from autoimmune attack when administered in a preventive manner; in fact, they could also inhibit the evolution of disease when given after the onset of EAE (Fig. S2).

3.2 Antigen-specific effect of SAg fusion proteins

To demonstrate that the suppression induced by SAg constructs is antigen-specific, a fusion protein containing an unrelated epitope was included. For this purpose, SJL/J mice were treated with SAg-PLP or unrelated SAg-MOG. As depicted in Fig. 2, animals that received SAg-MOG developed severe disease (100% incidence) with a maximum clinical score of 2.6±0.2, comparable to untreated mice (maximum score 2.4±0.4). In contrast, the clinical course of EAE in mice treated with SAg-PLP protein, was significantly diminished (maximum score 0.4±0.2). Similarly, SJL/J mice vaccinated with non-specific SAg-OVA constructs developed EAE similar to untreated mice in contrast to SAg-PLP vaccinated mice that were significantly protected against disease development (Fig. S3). These results clearly demonstrate the strong tolerogenic effect of the SAg fusion constructs, which is highly specific for the coupled T cell antigen.

3.3 Tolerogenic effect requires physical linkage of parasite repetitive sequence to T cell epitope

To demonstrate that the parasite derived component does not exhibit tolerogenic effects by itself, we performed experiments using free SAg and CD4+ T cell epitopes administered together but not physically linked. Mice were treated with equimolar amounts of free SAg repeat alone or in combination with free PLP peptide. Results show animals treated with SAg-PLP fusion constructs inhibited EAE development; they showed very mild signs of disease with a maximum clinical score of 0.4±0.2 compared to the control group (maximum clinical score of 2.4±0.4) (Fig. 3). Protection was completely abrogated when the individual components were administered together but not physically linked, reaching similar clinical

score as the untreated group (maximum score of 2.4±0.2). Similarly, in a preventing setting, vaccination with the SAg alone did not result in any significant suppressive effect, in contrast to the SAg fusion protein which inhibited the development of disease (Fig. S4). The results clearly show that the physical link between the T cell epitope and the repeat structure is essential for the effectiveness of the treatment.

3.4 Analysis of the antigen-specific T cell response *in vitro*

We evaluated the capacity of SAg fusion proteins to induce proliferation in comparison to free peptides. T cells purified from lymph nodes of naive DO11.10 mice expressing a transgenic α/β TCR specific for $OVA_{323–339}$ peptide were stimulated with titrated amounts of fusion proteins (SAg-OVA) or free OVA peptide. OVA-specific T cells responded to stimulation with SAg-OVA fusion constructs though to a lesser extend compared to free OVA peptide. Higher concentration of SAg-OVA was required for T-cell activation compared to the peptide. In addition, non-related SAg-MOG did not induce any non-specific proliferation, ruling out any effect of the parasite-derived repeat unit (Fig. S5). The same results were obtained using LNC from OTII mice which also express an $OVA_{323-339}$ specific T cell receptor on CD4+ cells but on the C57BL/6 background (data not shown).

These results indicate that SAg fusion proteins can be efficiently presented to be recognized by naive transgenic T cells to induce proliferation.

3.5 Analysis of the antigen-specific T cell response *in vivo*

As the suppressive effect of SAg fusion constructs was evident in mouse models of autoimmune inflammatory disease, we wanted to further analyze the antigen-specific CD4+ T cell response using the OVA transgenic mouse model. Antigen-specific immunotherapy is often associated with an impairment of antigen-specific response (Melamed and Friedman, 1993; Miller et al., 2007). To test the effect of SAg fusion proteins in the antigen-specific proliferative capacity, $OVA_{323-339}$ specific CD4+ T cells from DO11.10 mice were labeled with CFSE and transferred into sex-matched Balb/c mice, which received equimolar amounts (based on OVA-peptide amount) of free OVA peptide or SAg-OVA fusion constructs. To

evaluate T cell response to the cognate peptide $OVA_{323–339}$, lymph node cells were harvested on day 6 after transfer and antigen-specific proliferation was determined by CFSE dye dilution. The DO11.10 clonotypic KJ1.26 antibody was additionally used to stain OVA specific T cells (Haskins et al., 1983). Results indicate that OVA specific T cells readily proliferated in response to SAg-fusion protein as well as to free OVA peptide. Cells from SAg-OVA and free OVA treated mice underwent a similar number of divisions as shown in Fig. 4. Unrelated SAg-MOG used as a control, did not induce any proliferation (data not shown). This indicates that administration of SAg-OVA did not limit the response to the cognate antigen but induced a CD4+ T cell response highly specific for the coupled T cell epitope.

3.6 SAg fusion proteins treatment increased the number of regulatory FoxP3+ CD4+ T cells

To investigate the role of regulatory FoxP3+ CD4+ T cells in suppression mediated by SAg fusion proteins, we performed adoptive transfer experiments using CFSE labeled $OVA_{323–339}$ specific CD4+ T cells as previously described. LNC from animals that received OVA specific T cells and were treated with SAg-OVA or free OVA were analyzed for the expression of FoxP3+ in antigen-specific cells. We observed about 1.5 fold increase in the Treg frequencies in mice that received SAg-OVA (23-25% of KJ1.26+CD4+CD25+FoxP3+ T cells), compared to animals receiving free OVA peptide (14-19% of KJ1.26+CD4+CD25+FoxP3+ T cells) (Fig. 5). Proliferation of FoxP3 cells was specific for the cognate antigen as cells from mice that received unspecific SAg-MOG did not proliferate,

showing 3-7% of KJ1.26+CD4+FoxP3+ T cells which reflects normal frequencies of regulatory T cells in the mature CD4+CD25+ T cell subpopulation (Sakaguchi, 2004). This also indicates that the parasite-derived repeat unit itself neither induces nor expands regulatory T cells (Treg) in this setting. Together these results show that SAg fusion proteins induced increased proliferative response of regulatory T cells.

3.7 Tolerogenic capacity of SAg constructs depends on IL-10 and TGF-β

Anti-inflammatory cytokines like IL-10 and TGF-β play a critical role in the maintenance of immune tolerance. These cytokines are important in cell mediated suppression and crucial in the generation of regulatory FoxP3+ T cells (Powrie et al., 1996; Asseman et al., 1999; Maloy and Powrie, 2001; Green et al., 2003). The impact of IL-10 and TGF-β on the suppressive function mediated by the SAg fusion constructs in the EAE model was investigated by neutralization of cytokines. Monoclonal antibodies were administered intraperitoneally to SAg-MOG treated mice, blocking the IL-10 receptor or αTFG-β antibody. In each case, neutralizing antibodies were given on days 7, 9, 11 and 13 after induction of EAE. As shown in (Fig. 6), SAg-MOG treated animals were almost fully protected from developing EAE whereas concurrent abrogation of IL-10 signaling completely inhibited the protective effect induced by the treatment. Administration of isotype control did not impair the suppressive effect of the SAg construct. Protection of EAE in SAg-PLP treated SJL/J mice was also abolished by the neutralizing αTGF-β antibody (Fig. S6). The blockage of this specific signaling pathway during an autoimmune response leads to a markedly increased severity of the disease without any apparent signs of remission up to 30 days after EA induction. These results indicate an important role of suppressive cytokines like IL-10 and TGF-β in the induction of an active and cell mediated tolerance established following administration of the SAg constructs.

3.8 S-Antigen treatment reduces antigen-specific expression of pro-inflammatory TNF-α

Our previous studies indicate that protection of autoimmune disease induced by repetitive self-epitopes is associated with the reduction of the pro-inflammatory cytokine TNF-α in antigen-specific T cells. Analysis of the antigen-specific production of the pro-inflammatory cytokine TNF-α was further investigated following SAg administration. To examine the antigen-specific TNF-α production by T cells from animals that received SAg fusion constructs, CFSE labeled OVA specific CD4+ T cells were transferred into Balb/c mice which

also were administered SAg-OVA, SAg-MOG or free OVA. After priming with $OVA_{323-339}$ peptide, cells recovered from lymph nodes were restimulated with the specific peptide and cytokine expression was analyzed on KJ1.26+ CD4+ T cells by intracellular staining of TNF-α (Fig. 7). Results show that treatment with unrelated SAg-MOG protein lead to a high number of OVA specific T cells that produce TNF-α after restimulation with OVA (45% TNF-α+ of total KJ1.26+ CD4+ T cells). TNF-α positive cells were reduced in mice vaccinated with free OVA peptide compared to control mice (29% TNF-α + of total KJ1.26+ CD4+ T cells). This effect was even more pronounced in mice that received SAg-OVA where a reduction of TNF-α secretion by almost 67% was observed (15% TNF-α + of total KJ1.26+ CD4+ T cells) (Fig. 7). No induction of IFN-γ was observed (data not shown). Furthermore, administration of SAg fusion proteins in B cell deficient $JTH^{-/-}$ mice (Fig. S7) was also associated with the down-regulation of TNF-α production by antigen-specific T cells (Fig. S8).

3.9 SAg fusion proteins reduce pro-inflammatory responses

SAg fusion proteins induced suppression of Th1-mediated DTH response. Animals that received SAg-OVA during immunization showed to limit inflammation in comparison to animals that received OVA peptide, as indicated in Fig. S9. These results suggest that SAg fusion proteins contribute to downregulation of the inflammatory response, which is also in line with the observed modulatory effects of these constructs on the DTH response.

4. Discussion

In this study we used a novel approach that makes use of repetitive sequences derived from parasites as a reliable means to induce immune tolerance to specific autoantigens involved in disease pathology. Our previous studies have shown that repeat antigens consisting of linear copies of T cell epitopes are able to induce antigen-specific tolerance and suppress the clinical signs in various experimental autoimmune model systems (Rotzschke et al., 1997; Falk et al., 2000b; Falk et al., 2000a; Mack et al., 2001; Stienekemeier et al., 2001; Piaggio et al., 2007; Puentes et al., 2013). Their suppressive effect has been correlated with

the induction of anergy (Falk et al., 2000a), expansion of regulatory cells (Piaggio et al., 2007) and induction of active suppression (Puentes et al., 2013).

The aim of this study was the determination and characterization of the suppressive capacity of parasite-derived repetitive sequences linked to self-epitopes. The role of parasite tandem repeats in host-parasite interaction and immune evasion has been correlated with an efficient cross-linking to receptors like MHC class molecules and B cell receptors on APCs (Vos et al., 2000; Zinkernagel and Hengartner, 2001). There is evidence that multimeric assembly of repeat units of parasite ligands and host surface molecules form high-avidity complexes necessary for invasion (Paing and Tolia, 2014).

Parasites have developed several mechanisms to escape immune recognition by the host and the remarkably high incidence of conserved repetitive structures within different species seems to be associated with the induction of immune suppression. Recently, growing evidence indicates that concomitant parasitic infections can reduce disease severity in different models of human autoimmune diseases (Tadokoro et al., 2004; Stevenson and Zavala, 2006; Walsh et al., 2009; Farias et al., 2011). For example, regulatory mechanisms induced during *Plasmodium* infection ameliorate the clinical course and immune responses of EAE (Farias et al., 2011) and co-infection with *Trypanosoma* species interferes with the development of EAE through a modulatory effect exerted by IL-10 that limits Th1 cells expansion (Tadokoro et al., 2004). Several models explaining this phenomenon have been proposed, including a shift from a Th1 to a Th2 T cell response (Yazdanbakhsh et al., 2001; Mohrs et al., 2005) as well as the induction of regulatory FoxP3+ T cells (Wilson et al., 2005).

In the present study, the suppressive potential of repeat units in parasite proteins was exploited to induce targeted immunomodulation and to re-establish tolerance to self-antigens in an experimental autoimmune disease model. Fusion proteins consisting of repeat sequences derived from *Plasmodium* S-Antigen were covalently linked to different relevant single CD4+ T cell epitopes and the tolerogenic effect of the constructs was assessed in the EAE model. This study demonstrates that SAg fusion proteins anchored to myelin derived T cell epitopes are potent suppressive molecules that protect animals from developing EAE

when administered before disease induction or soon after the onset of the disease. Importantly, infiltrating T cells were barely found in the CNS cells of mice treated with SAg fusion proteins in comparison with untreated mice (data not shown), suggesting a recruitment of auto-reactive cells in peripheral tissues. Notably, neither the mixture of SAg repeat unit and free peptide epitope nor the parasite-repeat unit alone induced any suppression of disease. The antigen-specific effect of SAg fusion proteins was demonstrated by the fact that treatment with a fusion protein that contained an unrelated epitope did not have any effect in the modulation of EAE. This indicates that the physical linkage of the SAg to the CD4+ T cell epitope is required for the suppressive function and the effect is highly antigen-specific. A similar phenomenon might be occurring in *Plasmodium* infections as it has been reported that antigens containing tandem repeat sequences have T cell epitopes that are not immunodominant; indeed, one of the proposed functions of the repeat sequence is suppression of adjacent epitopes (Targett, 1992). Other preliminary data indicate that administration of fluorescent-labeled SAg constructs bound a distinct subset of B cells (data not shown) which is in line with structural features of repeats antigens that enable crosslinking to B cell receptors. However, SAg constructs were capable of inducing tolerance in B cell deficient mice, indicating that other APCs play an important role in disease suppression induced by these constructs. Future studies are encouraged to determine the role of suppressive cytokines in tolerance induced by SAg fusion proteins in mice lacking B cells.

We used the transgenic DO11.10 mouse model to examine the underlying mechanisms involved in the tolerance induced by the constructs. Advantages of using $OVA_{323–339}$ specific TCR transgenic DO11.10 mice include the ability to track antigen specific CD4 T cells after adoptive transfer. The results suggest that these fusion proteins are efficiently presented for the recognition of antigen-specific cells, inducing proliferation in naive transgenic T cells and that lymph node cells isolated from treated animals proliferate in response to peptides linked to fusion constructs. This indicates that SAg treatment did not lead to complete anergy or depletion of antigen-specific T cells. *In vivo* tracking of proliferation after adoptive cell

transfer rather indicate that treatment led to increased frequencies of FoxP3+CD4+ regulatory T cells, which are known to play a central role in immune modulation (Chen et al., 2003; Fantini et al., 2004; Ng et al., 2013; Mohammadnia-Afrouzi et al., 2015). Similarly, it has been described that *in vitro* incubation of human CD4+ T cells with a random polymer found in the myelin basic protein, copolymer I, can lead to FoxP3 expression, though in a non antigen-specific manner (Hong et al., 2005). Likewise, expansion of FoxP3 regulatory cells was found to be associated with protection against diabetes induced by multimerized epitopes (Piaggio et al., 2007). EAE experiments using FoxP3+ depleted DEREG mice (Lahl et al., 2007) will provide more insights into the role of regulatory T cells in SAg mediated suppression.

On the other hand, neutralization of either IL-10 or TGF-β led to a complete abrogation of the tolerogenic effect of the SAg constructs. The blockade of TGF-β can also have a direct impact in the *de novo* induction of FoxP3+ regulatory T cells as has been previously reported (Horwitz et al., 2008; Shevach et al., 2008). The administration of anti-cytokine antibodies lead to slight increases in the clinical score of treated mice compared to control mice that did not receive the antibody, which can be the result of the neutralization of intrinsic protective cytokines. However, these differences are not as significant as the differences in the clinical score of mice treated with SAg fusion proteins and treated mice receiving anti-cytokine antibodies. In addition, in the DO11.10 transgenic model, SAg fusion proteins downregulate antigen-specific secretion of pro-inflammatory TNF-α, in agreement with our previous study showing TNF-α modulation as one of the mechanisms of oligomers induced suppression (Puentes et al., 2013). Future studies addressing the relevance of TNF-α in the protective effect of EAE induced by the fusion proteins are encouraged. Furthermore, SAg vaccination was also able to reduce antigen-specific DTH response, confirming its tolerogenic effect in a disease model where Th1 effector cell function is inhibited. DTH suppression can also be attributed to the action of suppressive cytokines like IL-10 that is generated during the exposure to SAg fusion proteins (Powrie et al., 1993). Therefore, some of the immunomodulatory mechanisms that may directly contribute to restore tolerance by SAg

constructs involve the production of suppressive cytokines, expansion of regulatory cells and the reduction of pro-inflammatory cytokines.

This study suggests that SAg repeat units can function as carriers to deliver an antigen of interest inducing antigen-specific immunosuppression, likely by targeting APCs rendering them tolerogenic properties. Preliminary results indicate that SAg repeats units increase the bioavailability of the linked epitope-peptide compared to the free-peptide (data not shown). For application, SAg fusion constructs might provide some advantages over the use of adjuvants such as preservation of conformational structures of the peptide antigen in addition to their high solubility, which enable safe systemic administration.

The inherent ability of parasite repetitive antigens represents a promising strategy to promote immune suppression. Thus, the use of the mechanisms involved in parasite immune evasion can be exploited to modulate immune responses to defined target antigens. The present study shows the feasibility of SAg fusion proteins as an effective approach to deliver self-epitopes involved in the generation of autoimmune responses to induce antigen-specific immune suppression for the prevention and treatment of autoimmune diseases.

Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in studies involving animals were in accordance with ethical standards of the Directive 86/609/EEC of the European Community Council and of the institutional, state and federal guidelines. All animal protocols were approved by the ethics committee of the Landesamt für Gesundheit und Soziales (LAGeSo, Berlin, Germany) with registration numbers G0140-06 and G0331-08.

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Conflict of interest

The authors declare that they have no competing interests.

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Figure 1

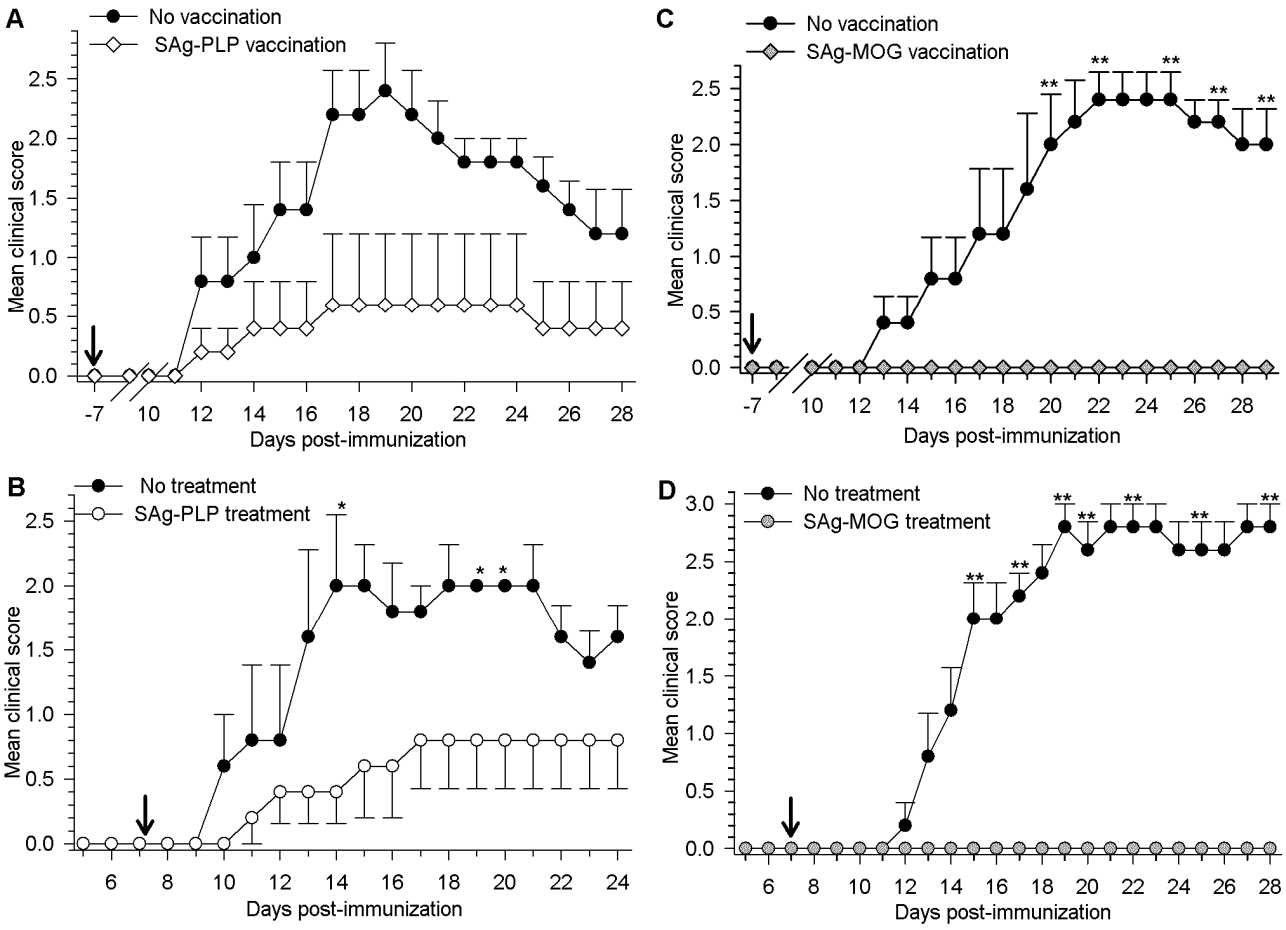

Fig 1. Protective effect of SAg fusion constructs in the EAE model. EAE was induced in SJL/J mice with $PLP_{139-151}$ and in C57BL/6 mice with MOG_{35-55}. Peptides were emulsified in complete Freund's adjuvant and injected subcutaneously. *Pertussis* toxin was injected one day post-immunization. 50 µg of SAg-PLP were intravenously administered to SJL/J mice (**A**) 7 days prior disease induction or (**B**) on day 7 after EAE induction. In the MOG-EAE model, 70 µg of SAg-MOG were intravenously injected to C57BL/6 mice (**C**) 7 days before disease induction or (**D**) on day 7 following EAE induction, as indicated by the arrows. As a control of the experiments a group of mice were left untreated. Mice were scored daily for the development of clinical signs of disease. Mean clinical scores of the distinct groups (n=5) ±SEM are presented. Statistical significance between groups was determined by Mann-Whitney U test. **P<0.01 and *P<0.05 compared with the respective control group.

Figure 2

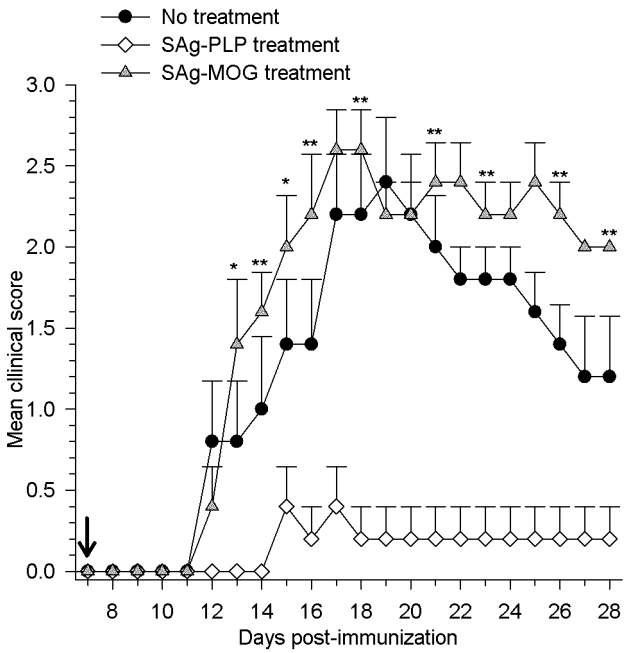

Fig 2. Antigen-specific effect of SAg-PLP in the EAE suppression. EAE was induced in SJL/J mice after subcutaneous injection of $PLP_{139-151}$ peptide emulsified in complete Freund's adjuvant and injection of 200 ng of *Pertussis* toxin one day after priming. Mice were treated on day 7 post-immunization with SAg-PLP fusion protein or unrelated SAg-MOG protein. Animals were monitored daily and the average ±SEM of the clinical score of five mice per group was calculated. Statistical significant difference between different groups was observed, **P<0.01 and *P<0.05.

Figure 3

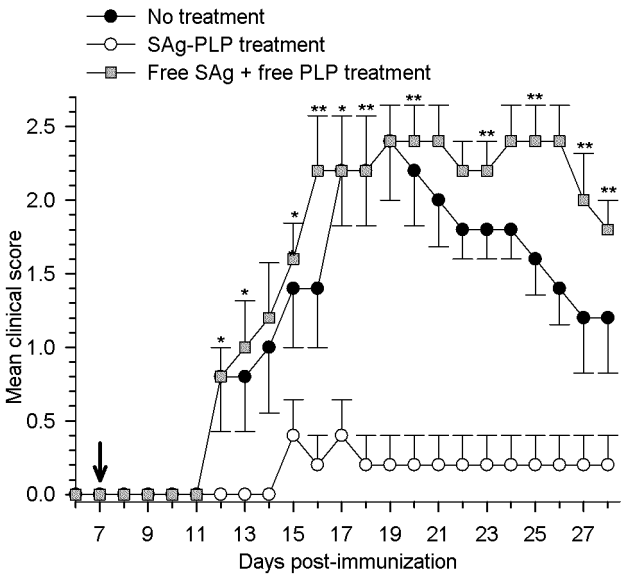

Fig 3. Tolerogenic effect of fusion constructs requires physical linkage. EAE was induced in SJL/J mice with 50 µg of $PLP_{139-151}$ in complete adjuvant containing *Mycobacterium tuberculosis*. Mice were treated intravenously on day 7 post-immunization with 50 µg of SAg-PLP or with equimolar amounts of free SAg together with free $PLP_{139-151}$ peptide. Control animals were left untreated. Animals were scored daily for the development of clinical signs of the disease and the average ±SEM of five mice per group was calculated. Statistical significance between mice treated with SAg-PLP fusion protein and mice receiving a mixture of the S-Ag repeat unit and free PLP peptide was determined by Mann-Whitney U test. **P<0.01 and *P<0.05.

Figure 4

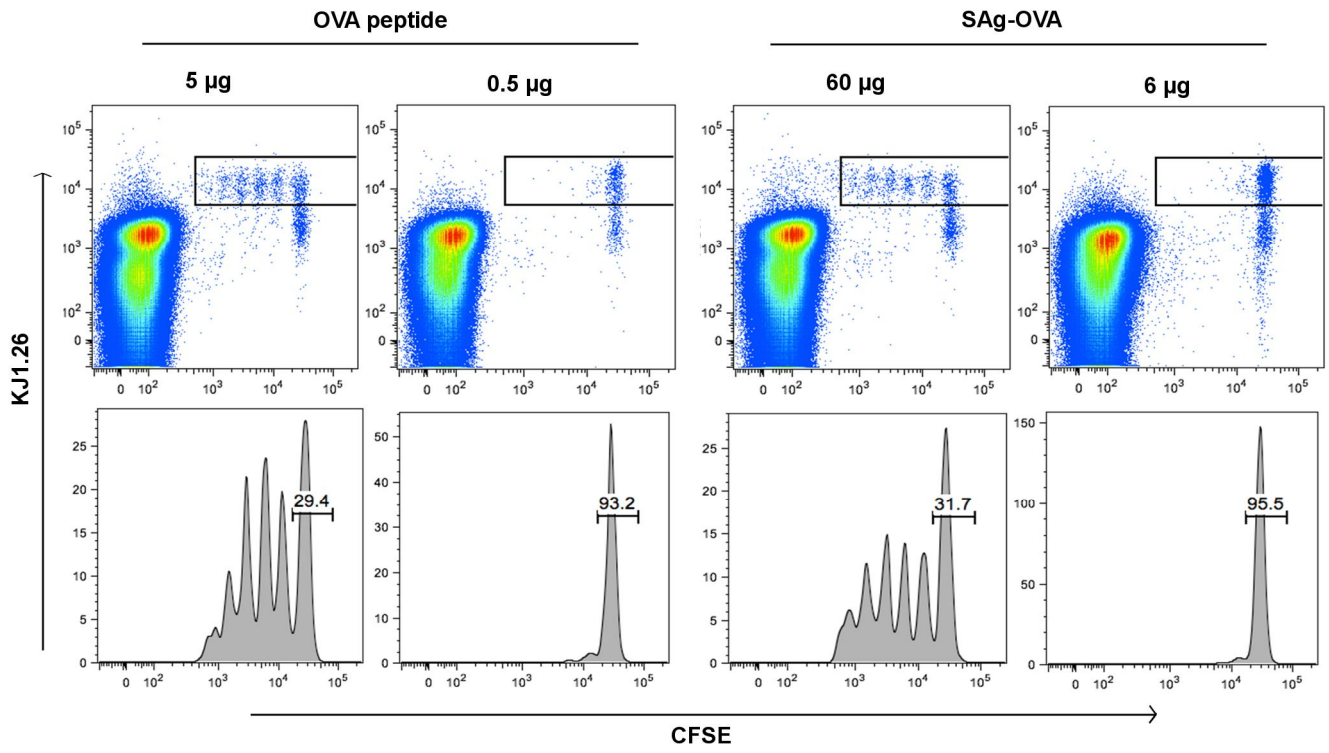

Fig 4. *In vivo* antigen-specific CD4+ T cell response after SAg-OVA administration. CD4+ T cells specific for $OVA_{323-339}$ were isolated from DO11.10 mice and labeled with 5µM CFSE. $3x10^6$ CD4+ T cell were adoptively transferred intravenously into sex-matched recipient Balb/c mice. One day after, mice were given titrated amounts of free $OVA_{323-339}$ or SAg-OVA (equimolar amounts, based on OVA-peptide amount). Mice were sacrificed on day 6 and lymph node cells were isolated. Antigen-specific proliferation was determined by analyzing the CFSE profile gated on CD4+ KJ1.26+ T cells by flow cytometry. A representative plot of 3 independent experiments is shown.

Figure 5

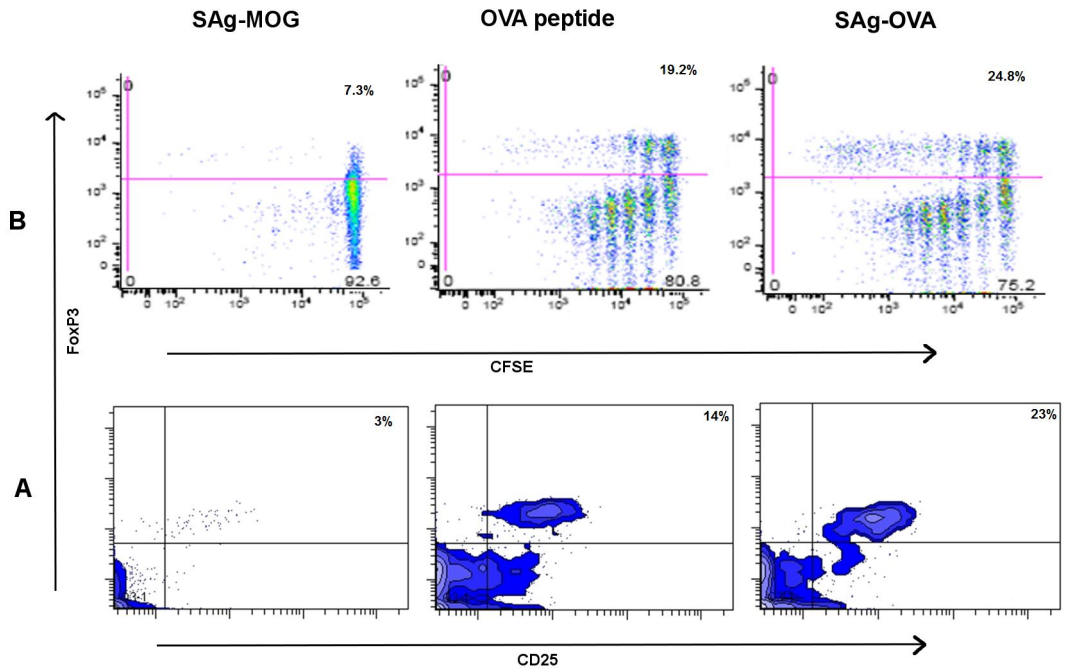

Fig 5. Increased frequency of FoxP3+ regulatory CD4+ T cells following the treatment with SAg constructs. To investigate the impact of FoxP3+ Tregs on the tolerogenic function of SAg constructs in vivo, $3x10^6$ DO11.10 CD4+ CFSE labeled T cells were intravenously transferred into Balb/c mice. One day later, mice received intravenously 5 µg of free $OVA_{323-339}$, 60 µg of SAg-OVA (equimolar amounts, based on OVA-peptide amount) or 60 µg of SAg-MOG as control. Lymph node cells were isolated on day 6. (**A**) Frequency of Treg cells were determined by intracellular staining of FoxP3 in proliferating OVA-specific cells and in (**B**) CD25+ cells in gated KJ1.26+ CD4+ T cells by FACS. Numbers indicate frequencies FoxP3+ Treg cells. Representative plots of 2 independent experiments are shown.

Figure 6

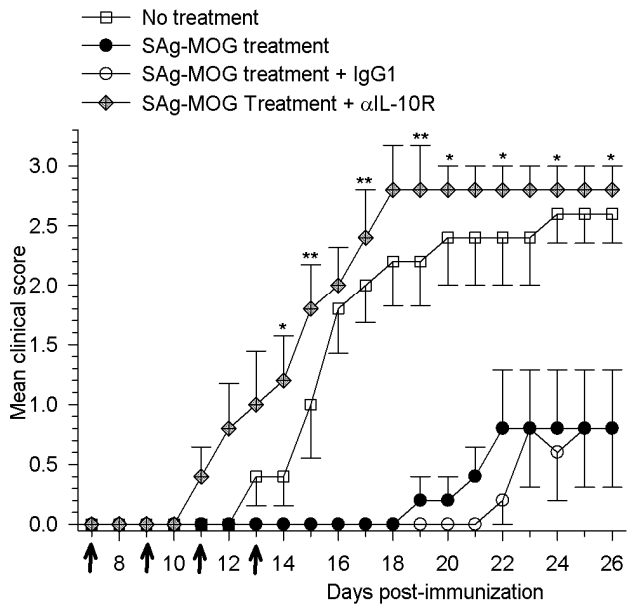

Fig 6. Impact of *in vivo* neutralization of suppressive cytokines on the protective effect induced by SAg fusion proteins. EAE was induced in C57BL/6 mice with MOG_{35-55} peptide and received 70 µg of SAg-MOG administered intravenously on day 7 after priming or were left untreated. To neutralize IL-10, animals received 0,5 mg of αIL-10R antibody (anti-IL-10 receptor) or isotype control IgG1 antibody. Antibodies were administered intraperitoneally on days 7, 9, 11 and 13 as indicated by the arrows. Animals were scored daily for the development of clinical signs of disease. Mean clinical scores ±SEM are presented. Statistical significance between treated mice receiving or not receiving neutralizing αIL-10R antibody was determined by Mann-Whitney U test. **P<0.01 and *P<0.05.

Figure 7

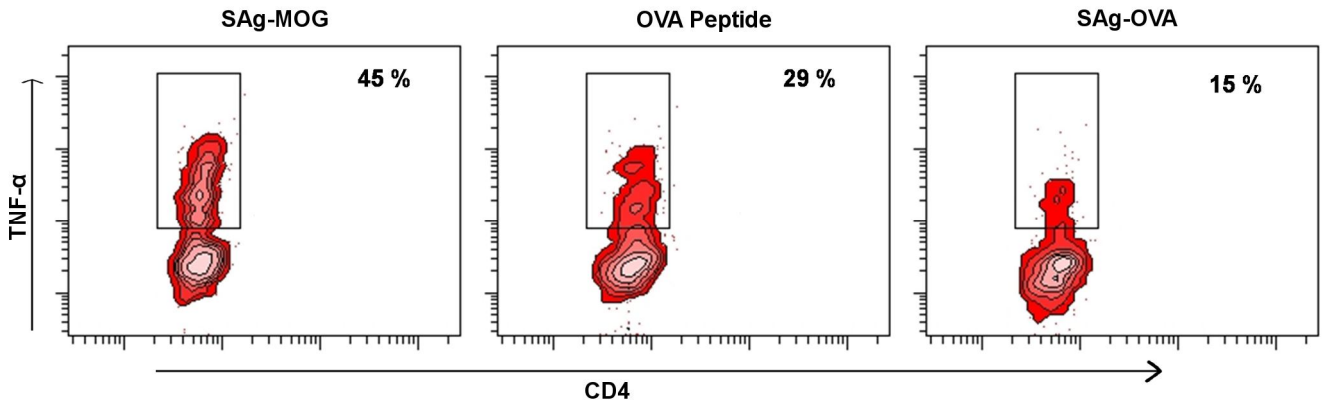

Fig 7. Antigen-specific suppression of pro-inflammatory TNF-α following treatment with SAg constructs. To investigate the impact of tolerogenic constructs on antigen-specific CD4+ effector T cell responses in vivo, $3x10^6$ DO11.10 CD4+ T cells were adoptively transferred intravenously into Balb/c mice. Mice received 5 µg of free $OVA_{323-339}$, 60 µg of SAg-OVA (equimolar amounts, based on OVA-peptide amount) or 60 µg of SAg-MOG as control one day after transfer. Mice were primed on day 7 by subcutaneous administration of 5 µg $OVA_{323-339}$ peptide in CFA. Mice were sacrificed 6 days later and lymph node cells were collected and restimulated with 10 µg/ml OVA peptide and control peptide for 6 hours. Antigen-specific cytokine expression was analyzed on KJ1.26+ CD4+ T cells by intracellular staining of TNF-α. The plots show the frequencies of TNF-α+ cells analyzed on gated CD4+ KJ1.26+ T cells.

Supporting information

Figure S1

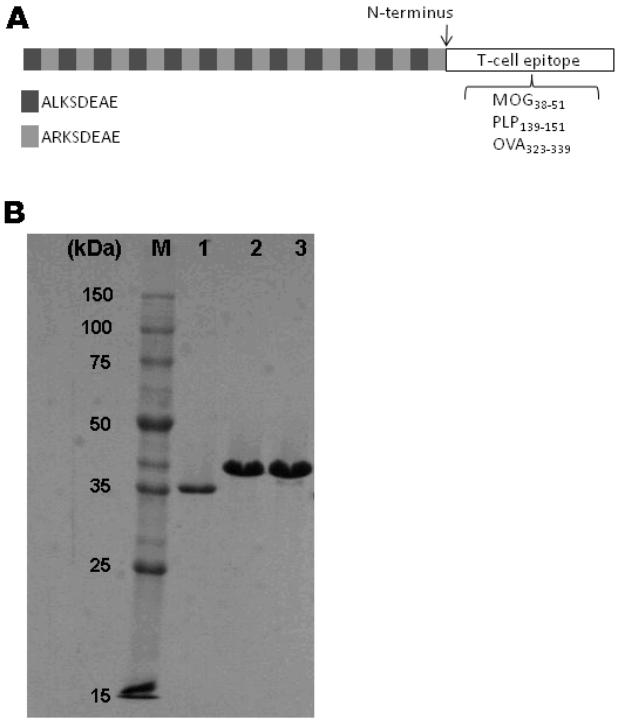

Fig S1. Generation of parasite-derived fusion proteins. (**A**) Schematic diagram of fusion proteins. Parasite repetitive structures were fused through a linker to CD4+ T cell antigens and expressed in E.coli. Fusion proteins containing repetitive sequences of the S-antigen (SAg) of *P. falciparum* were designed: 24 repeats of a 8-mer unit derived from the NF7 isolate (A(L/R)KSDEAE) were linked to the N-terminus of the respective CD4+ T cell epitopes: MOG_{38-51} (SAg-MOG_{38-51}), $PLP_{139-151}$ (C140S) (SAg-$PLP_{139-151}$) and $OVA_{323-339}$ (SAg-$OVA_{323-339}$). Since the natural units contained either L or R in their repeats, building blocks with alternating amino acids were used in tandem repeats. (**B**) Analysis of the expression of epitope fusion-constructs containing S-Antigen repeats fused to CD4 T cell epitopes in E. coli. Lane 1: SAg 12-mer (22 KDa); lane 2: SAg 12-mer MOG_{38-51} S3 (24Kda); lane 3: SAg 12-mer $PLP_{139-151}$ S3 (24 KDa) and lane M: molecular weight markers. The weights calculated from the DNA sequence are lower compared to those estimated from the SDS gel due to abnormal SDS binding (SAg sequence is 38% ASP and GLU) (Matagne et al.,1991).

Figure S2

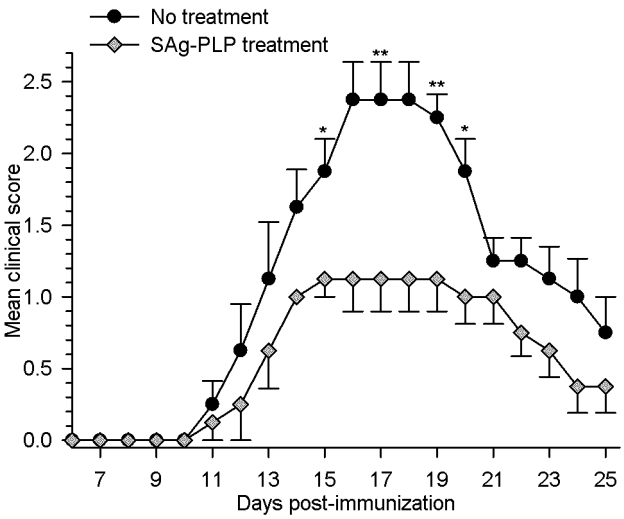

Fig S2. Therapeutic effect of SAg fusion proteins. SJL/J mice (n=8) were individually treated once the first clinical signs of EAE appeared. 50 μg of SAg-PLP fusion protein was given intravenously to diseased mice. Administration of fusion protein inhibits the evolution of EAE. The curve shows the mean ±SEM daily clinical score. Statistical significance between groups was determined by Mann-Whitney U test. **P<0.01 and *P<0.05.

Figure S3

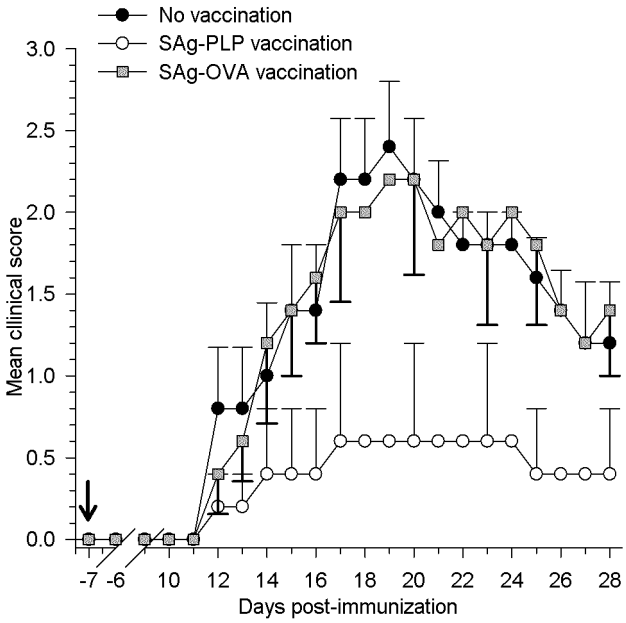

Fig S3. Antigen-specific EAE protection after vaccination with SAg-PLP fusion proteins. EAE was induced in SJL/J mice using 50 μg of the encephalitogenic $PLP_{139-151}$ peptide in CFA. The following day, animals received 200 ng of *Pertussis* toxin. Mice were vaccinated 7 days prior disease induction with 50 μg of SAg-$PLP_{139-151}$ or unrelated SAg-$OVA_{323-339}$ fusion proteins. Mice were monitored daily and the mean clinical score of five mice per group was plotted. One representative experiment of two performed is shown.

Figure S4

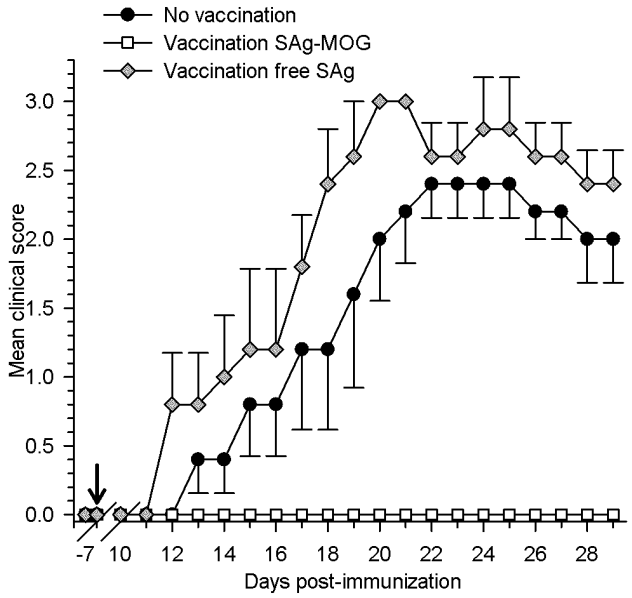

Fig S4. SAg repeat unit alone does not induce protection in the MOG-EAE model. EAE was induced in C57BL/6 mice by priming with 50 µg of MOG_{35-55} peptide and injection of 500 ng of *Pertussis* toxin one day after priming. Mice were vaccinated 7 days before disease induction with 70 µg of SAg-MOG fusion protein or SAg only. Mice were monitored daily and the average ±SEM of the clinical score of five mice per group was calculated.

Figure S5

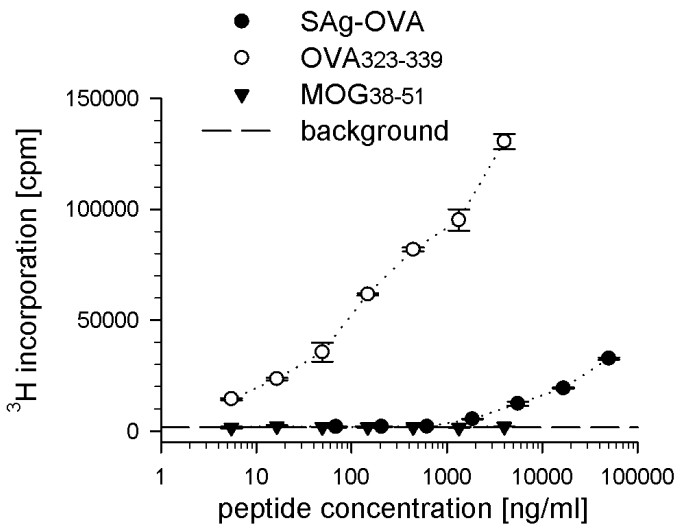

Fig S5. T cell proliferation induced by SAg fusion protein. To compare the proliferative capacity of free peptide and SAg fusion constructs *in vitro*, [^{3}H]Thymidine proliferation assay was performed. Lymph node cells were harvested from DO11.10 mice and 5x10^5 cells were incubated for 96 hours with titrated amounts of SAg-OVA, free OVA$_{323-339}$ and MOG$_{38-51}$ as negative control. [^{3}H]Thymidine was added after 72 hours. Counts per minute (cpm) mean values and standard deviation of triplicates are shown.

Figure S6

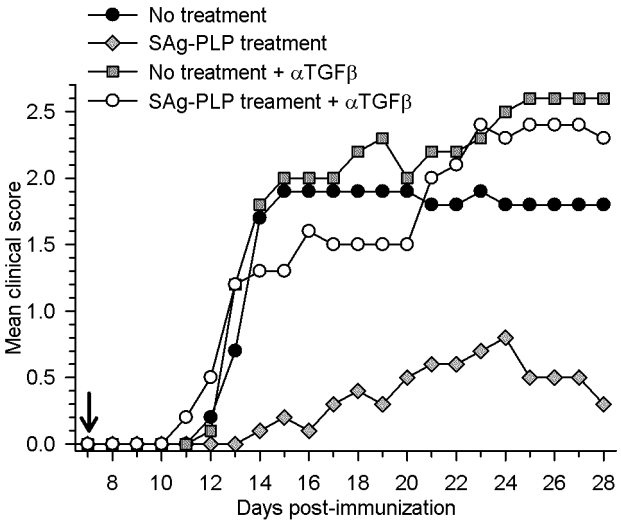

Fig S6. Impact of *in vivo* neutralization of TGF-β on the protective effect induced by SAg fusion proteins. EAE was induced in SJL/J mice with $PLP_{139-151}$ peptide. On day 7 after EAE induction, mice received intravenously 50 µg of SAg-PLP or in combination with 0.5 mg of αTGF-β antibody. Control groups were left untreated with or without αTGF-β antibody treatment. Neutralizing antibodies were administered intraperitoneally on days 7, 9 and 11. Animals were scored daily for the development of clinical signs of the disease. Mean clinical score from the groups (n=5) are presented.

Figure S7

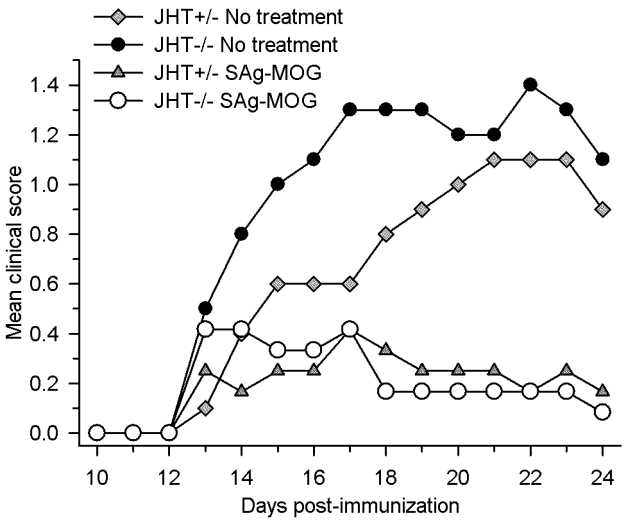

Fig S7. Suppressive capacity of SAg constructs is independent of B cells. EAE was induced in $JHT^{+/-}$ and $JHT^{-/-}$ mice after subcutaneous immunization with MOG_{35-55} peptide emulsified in adjuvant containing *Mycobacterium tuberculosis* and injection of *Pertussis* toxin. Mice were treated with SAg-MOG fusion protein and monitored daily. Averages of the clinical scores were calculated. The plot shows that SAg constructs can also induce tolerogenic effect in B cell deficient $JHT^{-/-}$ mice.

Figure S8

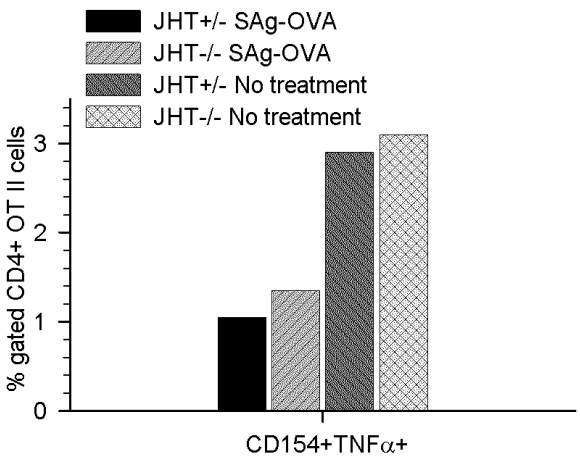

Fig S8. Antigen-specific suppression of pro-inflammatory TNF-α following treatment with SAg fusion proteins in B cell deficient mice. To measure the production of TNF-α in $JHT^{-/-}$ mice, OTII ($OVA_{323-339}$ specific) cells labeled with CFSE were transferred into $OVA_{323-339}$ primed $JHT^{+/-}$ and $JHT^{-/-}$ mice and treated with SAg-OVA. Lymph node cells were isolated from treated and untreated mice and activated for 6 hours with $OVA_{323-339}$ peptide and α-CD28. Cytokine secretion was blocked by the addition of Brefeldin A for the last 4 hours of stimulation. Cells were stained for surface CD4 followed by intracellular staining with αTNF-α. For analysis of TNF-α in antigen-specific activated T cells; combined intracellular α-CD154 staining was performed. The frequency of CD154+TNF-α+ double positive in OTII CD4+ T cells was determined and analyzed by FACSDivaTM software (BD Bioscience).

Figure S9

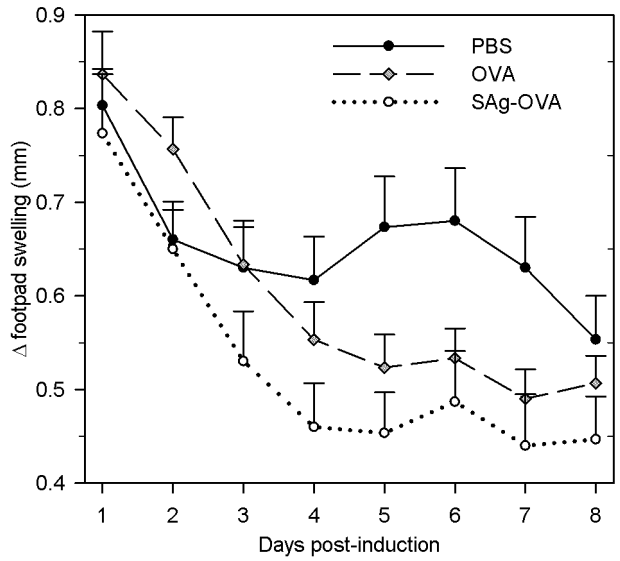

Fig S9. Suppressive capacity of SAg-OVA in a DTH model. $OVA_{323-339}$ specific T cells were isolated from DO11.10 mice and $3x10^6$ CD4+ T cell were adoptively transferred intravenously into Balb/c mice. One day later, mice were tolerized by intravenous administration of 5µg free $OVA_{323-339}$ or 60µg SAg-OVA (equimolar amounts, based on OVA-peptide amount). DTH reaction was induced on day 7 by adoptive transfer of OVA-specific cells that were cultured for 6 days under Th1 polarizing conditions. One day later mice were immunized into the footpad by intradermal injection of 250 ng $OVA_{323-339}$ in IFA or PBS/ IFA. DTH reaction was measured 8 days post immunization by determining footpad swelling compared to days zero.