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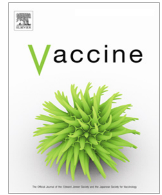

Low antigen dose formulated in CAF09 adjuvant Favours a cytotoxic T-cell response following intraperitoneal immunization in Göttingen minipigs

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ABSTRACT

The relationship between the antigen dose and the quality of an immune response generated upon immunization is poorly understood. However, findings show that the immune system is indeed influenced by the antigen dose; hence underlining the importance of correctly determining which dose to use in order to generate a certain type of immune response.

To investigate this area further, we used Göttingen minipigs as an animal model especially due to the similar body size and high degree of immunome similarity between humans and pigs. In this study, we show that both a humoral and a cell-mediated immune (CMI) response can be generated following intraperitoneal immunization with tetanus toxoid (TT) formulated in the CAF09 liposomal adjuvant. Importantly, a low antigen dose induced more TT-specific polyfunctional T cells, whereas antigen-specific IgG production was observed upon high-dose immunization. Independent of antigen dose, intraperitoneal administration of antigen increased the amount of TT-specific cytotoxic CD8β^+ T cells within the cytokine-producing T-cell pool when compared to the non-cytokine producing T-cell compartment.

Taken together, these results demonstrate that a full protein formulated in the CAF09 adjuvant and administered to pigs via the intraperitoneal route effectively generates a cytotoxic T-cell response. Moreover, we confirm the inverse relationship between the antigen dose and the induction of polyfunctional T cells in a large animal model. These finding can have implications for the design of upcoming vaccine trials aiming at establishing a cytotoxic T-cell response.

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1. Introduction

Vaccines can contain different amounts of target antigen; however, it is not well known how the antigen dose influences the quality of a resulting immune response. Relatively few studies directly investigate this, although an inverse relationship between antigen dose and the duration of delayed type hypersensitivity has been proposed [1]. Also, it has been hypothesized that more T cells and antigen are required for Th2 than Th1 responses [2]. Recent findings further support an inverse relationship between the antigen dose and the induction of CD4$^+$ T-cell polyfunctionality and functional avidity in both mice and humans [3–5].

Given that the antigen dose can influence the immune response, correctly determining the first-in-human dose based on preclinical animal studies becomes even more crucial, and translating findings from preclinical vaccine research is dependent on animal models reliably mimicking human patients. Previously, the body weight of the animal alone has been used for extrapolation; but due to resulting unsuccessful clinical trials, using the body surface area (BSA) of the animal has been a suggested approach [6]. However, the BSA method still shows extreme inaccuracy [7]; suggesting the need for further improvement in strategies converting animal

Abbreviations: BSA, body surface area; Cat, catalogue number; CMI, cell-mediated immune; CTL, cytotoxic T lymphocyte; DC, dendritic cell; i.m., intramuscular; i.p., intraperitoneal; s.c., subcutaneous; SEB, *staphylococcal enterotoxin B*; SFC, spot forming cells; TT, tetanus toxoid.

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doses to human equivalents in order to reliably study the effect of antigen dose on the immune response.

In contrast to rodents; the porcine metabolic rate, important metabolic enzymes, and the immunome closely resemble the human counterparts [8–11]. Moreover, pigs are fully immune competent and display high MHC-allelic diversity with the number of known porcine MHC class I alleles continuously expanding due to an improved detection method [12]. For vaccine research to be reliably translated to humans, it is crucial to perform the preclinical tests in an animal model with a fully competent immune system [13,14]; further supporting the potential in using pigs as a large animal model in the interphase from early rodent work to clinical trials in humans.

In this study, we hypothesised that a cytotoxic immune response can be generated in pigs following intraperitoneal (i.p.) immunization. Moreover, we hypothesized that the quality of the resulting immune response is influenced by the antigen dose. Tetanus toxoid (TT) was used as a model antigen and formulated in CAF09; a dimethyldioctadecylammonium bromide liposomal adjuvant with synthetic monomycolyl glycerol and the TLR3 agonist poly I:C as immune modulators [15]. We i.p. administered 10-fold titrations of the full TT protein to Göttingen minipigs and investigated effects of antigen dose on the humoral and cell-mediated immune (CMI) response to further evaluate the potential of pigs for translational vaccine research.

2. Materials and methods

2.1. Pigs

Fifteen Göttingen minipigs aged ~2 to 4.5 months and derived from four different litters were purchased from Ellegaard A/S (Sorø, Denmark), housed at the National Veterinary Institute, Technical University of Denmark (Frederiksberg C, Denmark) and randomized into three groups based on sex, litter, and weight ($n = 5$). Animal procedures were carried out in accordance with both national and international guidelines, and all procedures comply with the ARRIVE guidelines. The institutional committee as well as the Danish Animal Experiments' Inspectorate (Ethical approval ID: 2012−15−2934−00557) approved all procedures.

2.2. Immunizations

Animals received either 1 μg, 10 μg, or 100 μg of purified TT (State Serum Institute, batch: T 262-01) formulated in the CAF09 adjuvant as previously described [15]. The CAF09 adjuvant was kindly provided by Dennis Christensen (Statens Serum Institut, Copenhagen, Denmark). Each immunization was comprised of 1 ml CAF09 and 1 ml TT diluted in 10 mM Tris buffer. Immunizations were delivered via the intraperitoneal (i.p.) route using an 18G × 2″ needle; no anaesthesia was used. Animals were primed and subsequently boosted twice with two week intervals (Supp. Table 1).

2.3. Cell isolation

Blood was collected into sodium heparinized vacutainer tubes (BD Diagnostics, catalogue number (cat.): 362753) and purified using SepMate tubes (StemCell Technologies, cat.: 85450) according to manufacturer's protocol. In brief, the blood was diluted in PBS/2%FBS (ThermoFischer Scientific, cat.: 10082147) and separated using Lymphoprep (StemCell Technologies, cat.: 07851). Following separation, the cells were counted using the Nucleocounter NC-200 (Chemometec, Allerød, Denmark).

2.4. IFN-γ ELISpot

$\text{MultiScreen}_{\text{HTS}}$ IP Filter Plates (Merck Millipore, cat.: MSIPS4510) were pre-wet in 35% ethanol (v/v in sterile milliQ water) and coated with 5 μg/ml mouse anti-swine IFN-γ antibody (ThermoFischer Scientific, cat.: MP700) overnight at 4 °C. The plates were blocked with AIM V™ media (ThermoFischer Scientific, cat.: 12055091), no serum, for at least one hour at 37 °C. To each well, 2×10^5 freshly isolated PBMCs were added and incubated for 20 h at 37 °C in the presence of 1.5 μg/ml TT, 1.5 μg/ml *staphylococcal enterotoxin B* (SEB) (Sigma Aldrich, cat.: S4881) as positive control, or media alone. Biotin Mouse Anti-Pig IFN-γ (BD Biosciences, cat.: 559958) was used at 1 μg/ml for detection with incubation for 1 h at room temperature (RT). Streptavidine-Alkaline Phophatase conjugate (Sigma Aldrich, cat.: 11 089 161 001) was diluted 1:2000 and added to the plates with incubation on a shaking table for 1 h at RT. Finally, 100 μl/well of BCIP®/NBT Liquid Substrate System (Sigma Aldrich, cat.: B1911) was added and spot development was terminated after five minutes. The plates were allowed to air-dry in the dark. The AID EliSpot Reader version 6.0 (Autoimmun Diagnostika GmbH, Strassberg, Germany) was used for analysis. Data is shown with subtraction of the background levels of spot forming cells (SFCs) from culturing with media alone.

2.5. IgG ELISA

The 96-well polysorp plate (ThermoFischer Scientific, cat.: 475094) was coated with 0.125 μg/ml TT and incubated overnight at 4 °C. Serum samples, diluted 1:10,000, were added to the plate with incubation on a shaking table for 1 h at RT. Biotinylated goat anti-pig IgG (Bio-Rad, cat.: AAI41), was diluted 1:20,000 and used as secondary antibody with incubation on a shaking table for 1 h at RT. HRP-conjugated streptavidin (ThermoFischer Scientific, cat.: N100) diluted 1:8000 was added; the plate was incubated on a shaking table for 1 h at RT. Finally, tetramethylbenzidine (Kem-En-Tec, cat.: 4380 L) was added and the reaction was terminated with 0.5 M sulfuric acid after five min at RT. A microplate reader (ThermoFischer Scientific) was used to determine the absorbance at 450 nm; corrections for unspecific background were done by subtraction of the signal at 650 nm.

2.6. Flow cytometry

Antibodies were used at pre-determined concentrations (details in Supp. Table 2). PBMCs were stimulated for 16 h with 2 μg/ml TT, media alone, or 1 μg/ml SEB as a positive control, followed by 6 h culturing in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich, cat.: B7651-5MG). Cells were surface stained for 30 min at 4 °C with antibodies against CD3 and CD8β in combination with a live/dead stain. Fixation/Permeabilization Solution Kit (BD Biosciences, cat.: 554714) was used according to manufacturer's protocol. Intracellular cytokine staining was conducted using antibodies against IFN-γ, TNF-α, and perforin for 30 min at 4 °C. Samples were acquired on an LSRFortessa (BD Bioscience) flow cytometer, and 200,000 viable $CD3^+$ cells were recorded for analysis. Data was analysed using FlowJo Data Analysis Software version 10.

2.7. Statistical analysis

Despite low numbers of animals, the data were analysed by parametric analyses as non-baseline data passed the Shapiro-Wilk normality test and presumably represent normally distributed populations. Results are thus shown as the mean or the mean ± SEM and statistical comparisons were performed using either paired or unpaired Student's *t*-test. GraphPad Prism version

7.00 for Windows (California, United States) was used for statistical analysis. $P < 0.05$ (*) was considered significant, and $P < 0.005$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) are indicated.

3. Results

3.1. Immunization with a low antigen dose drives a CMI response

We firstly evaluated the amount of IFN-γ produced during the immunization trial. Prior to immunization, all animals were TT naïve as demonstrated by the lack of IFN-γ SFCs at day 0 (Fig. 1A). A quantification of the IFN-γ SFCs in response to CAF09-formulated TT revealed that a CMI response was generated in all the groups already at day 27 (Fig. 1B). The immune response in each group was enhanced by an additional immunization as indicated by the presence of more IFN-γ SFCs at day 41; most pronounced in the 1 μg and 10 μg group (Fig. 1B). Additionally, we investigated whether several rounds of i.p. immunization induced a humoral immune response. No TT-specific IgG antibodies were detected in serum samples prior to the first immunization in any of the groups (Fig. 1C). Two immunizations were sufficient to generate TT-specific IgG antibodies only in the 100 μg dose group; however, all groups displayed a humoral response to TT following three injections (Fig. 1C). A comparison of the three immunization groups revealed that animals receiving 1 μg TT produced a stronger TT-specific IFN-γ response when compared to animals receiving 100 μg TT (Fig. 1D). In contrast, immunization with a high antigen dose induced a stronger humoral immune response (Fig. 1E).

3.2. T-cell-derived IFN-γ is enhanced by immunization with a low antigen dose

Having established that the dose of immunizing antigen affected the subsequent IFN-γ responses detected by *ex vivo* IFN-γ ELISpot, we further investigated the effect of antigen dose directly on T cells. The capacity of T cells to produce IFN-γ against TT following *in vitro* re-stimulation at day 41 was determined by flow cytometry; a representative gating strategy is depicted in Supp. Fig. 1.

Although numbers of IFN-γ^+ TT-specific T cells were small, the flow cytometric plots clearly indicated that T cells derived from the 1 μg, and somewhat also the 10 μg group, were IFN-γ^+ while animals receiving 100 μg of CAF09-formulated TT did not seem to respond (Fig. 2A). This was substantiated by a statistically significant higher percentage of T cells producing IFN-γ against TT in animals receiving 1 μg of antigen compared to 100 μg immunized pigs (Fig. 2B). Interestingly, a titration effect could be observed across the groups (Fig. 2B); thus suggesting an inverse relationship between the percentage of IFN-γ^+ T cells and the CAF09-formulated antigen dose. Analysis of the $CD3^-$ population did not reveal IFN-γ producing cells in response to TT (data not shown).

3.3. TT-specific cytotoxic CD8β^+ T cells are increased within the IFN-γ^+ T-cell population

Given that the antigen dose when formulated in CAF09 is inversely correlated with the amount of IFN-γ responsive T cells, we further investigated whether the phenotype of the T cells was also affected by the antigen dose. The CD8β marker was used to distinguish between cytotoxic and helper T cells as previously described [16]. The ratio between CD8β^+ and CD8β^- T cells was evaluated in both the IFN-γ^- and the IFN-γ^+ T-cell population for all groups (Fig. 3A–C). When quantifying the ratios, a significant increase in CD8β^+ T cells was detected in the IFN-γ^+ T-cell population for both the 1 μg (Fig. 3D) and the 10 μg group (Fig. 3E). In the high dose group, four out of five animals also showed a tendency towards an increase in CD8β^+ T cells within the IFN-γ^+ T-cell population (Fig. 3F). Taken together, these results demonstrate that the TT-specific CTLs are increased within the IFN-γ^+ T-cell population independently of the antigen dose formulated in CAF09.

3.4. TNF-α^+ T cells are slightly increased when immunizing with a low antigen dose

In addition to IFN-γ, TNF-α is an important effector molecule produced by cytotoxic $CD8^+$ T cells [17]. For this reason, we investigated whether TNF-α was also affected by the antigen dose. The ability of T cells to produce TNF-α in response to TT was again evaluated using flow cytometry; a representative gating strategy is outlined in Supp. Fig. 1. Across all groups and in all individual animals, TNF-α-producing T cells were readily detectable (Fig. 4A). When comparing the percentage of TNF-α^+ T cells, no difference could be observed between the 1 μg and the 10 μg groups, while four out of five pigs in the 100 μg group were non-responders (Fig. 4B). Although non-significant, a trend towards an inverse relationship between CAF09-formulated antigen dose and the ability of T cells to produce TNF-α could thus be observed (Fig. 4B).

3.5. TT-specific cytotoxic CD8β^+ T cells are increased within the TNF-α^+ T-cell population

Since the cytokine-producing T-cell population was shifted towards a cytotoxic phenotype when measuring IFN-γ (Fig. 3), we speculated whether this would also be the case for TNF-α. The relationship between cytotoxic and helper T cells, as determined by the expression of the CD8β molecule, was determined within the TNF-α-producing and TNF-α^- T-cell population (Fig. 5A–C). An increase in the amount of CD8β^+ T cells in the TNF-α^+ population was observed for all groups, when comparing to the TNF-α^- population (Fig. 5A–C). This observation was clearly supported by a statistical analysis of the CD8β^+/CD8β^- ratio in the TNF-α-producing and non-producing T-cell population. Here, a significant increase in cytotoxic CD8β^+ T cells within the TNF-α^+ T-cell population was demonstrated for all the groups (Fig. 5D–F). Together, these results show a specific increase in CTLs within TNF-α^+ T-cell population independent of the CAF09-formulated antigen dose.

Moreover, perforin has been reported to be an important effector molecule for CTLs [18]. Therefore, we also investigated the effect of antigen dose on the ability of CTLs to produce perforin in response to TT. A substantial population of perforin$^+$CD8β^+ T cells was detected in all animals (Supp. Fig. 2A). Despite this, no difference was observed when comparing the percentage of perforin$^+$CD8β^+ T cells across the groups (Supp. Fig. 2B); hence showing that the production of perforin is independent of the antigen dose when administered in CAF09 adjuvant.

3.6. Low antigen dose induces more TT-specific polyfunctional T cells

The ability to induce polyfunctional $CD4^+$ T cells in humans has been shown to be inversely correlated with antigen dose following intramuscular (i.m.) immunization [4]. Therefore, we investigated whether an i.p. administration route had similar effect on the ability to induce polyfunctional T cells in response to CAF09-adjuvanted TT. Flow cytometric analysis of re-stimulated PBMCs harvested at day 41 was performed using a gating strategy as depicted in Supp. Fig. 1. T cells producing both TNF-α and IFN-γ were detected in both the 1 μg and the 10 μg group; however, this population of double-cytokine-positive T cells appeared to be mostly absent in the high dose group (Fig. 6A). When quantifying the percentage of TNF-α^+IFN-γ^+ T cells across the three groups, a

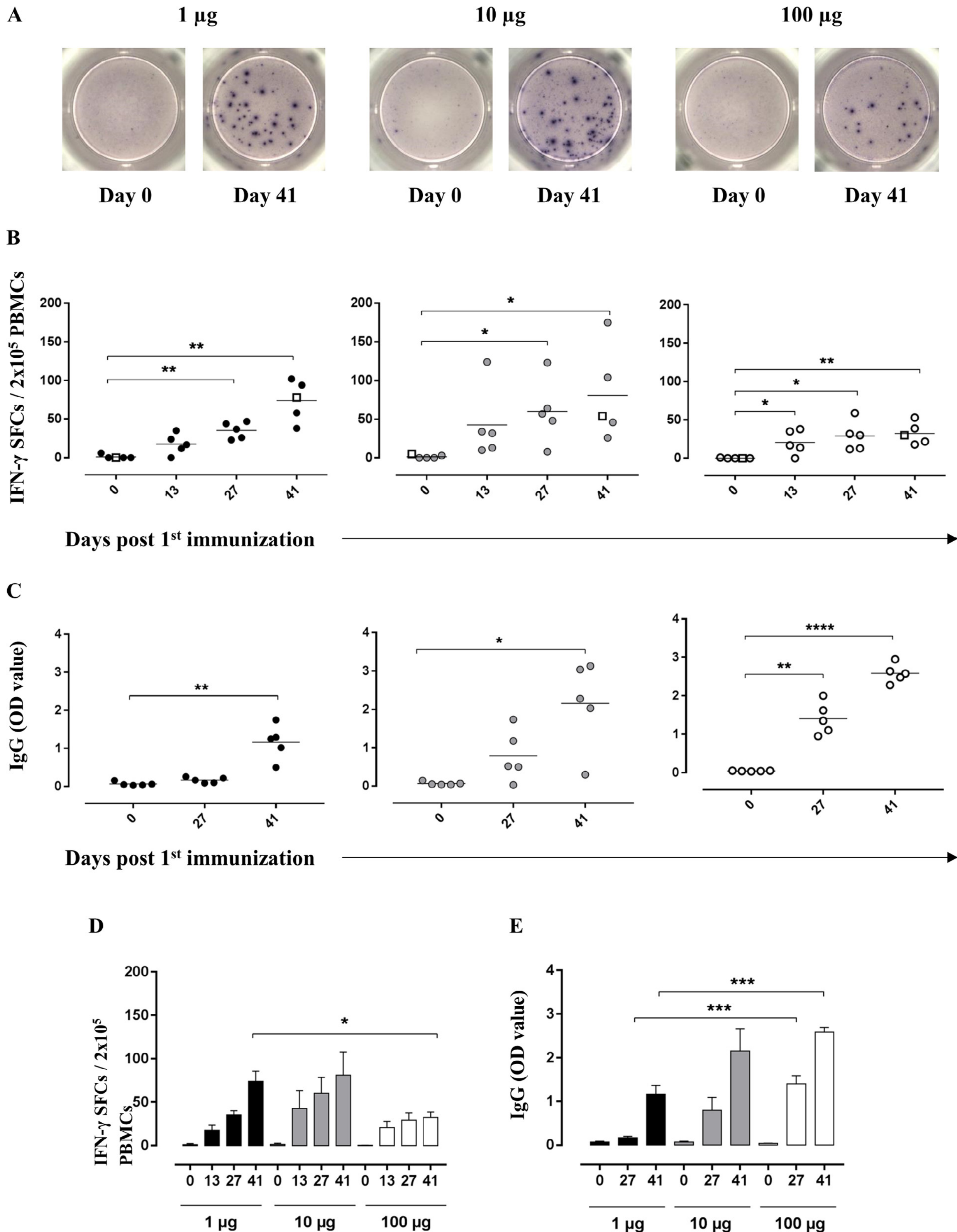

Fig. 1. Immunization with a low antigen dose preferentially drives a CMI response. Göttingen minipigs were intraperitoneally immunized with either 1 µg, 10 µg, or 100 µg of tetanus toxoid formulated in the CAF09 adjuvant. Immunizations were administered three times with two weeks in between. All animals were blood sampled prior to each immunization and two weeks post the last injection. (A) IFN-γ ELISpot images at day 0 and 41 from one representative animal in each group in response to tetanus toxoid. (B) Quantification of IFN-γ ELISpot responses against tetanus toxoid from animals receiving 1 µg (black circles), 10 µg (grey circles), or 100 µg (white circles). Open squares indicate the representative animal shown in (A). Data is presented as spot forming cells (SFCs) per 2×10^5 PBMCs with indication of the mean. (C) ELISA-based detection of anti-tetanus IgG in serum samples from animals immunized with 1 µg (black circles), 10 µg (grey circles), or 100 µg (white circles). Data is shown as OD values with indication of the mean. (D) Comparison of IFN-γ SFCs in response to tetanus toxoid across all groups and for each time point. Data is shown as mean ± SEM. (E) Comparison of the anti-tetanus IgG production across all groups and for each time point. Data is shown as mean ± SEM. Statistical evaluation by paired student's *t*-test (B and C) or unpaired student's *t*-test (D and E), ($n = 5$).

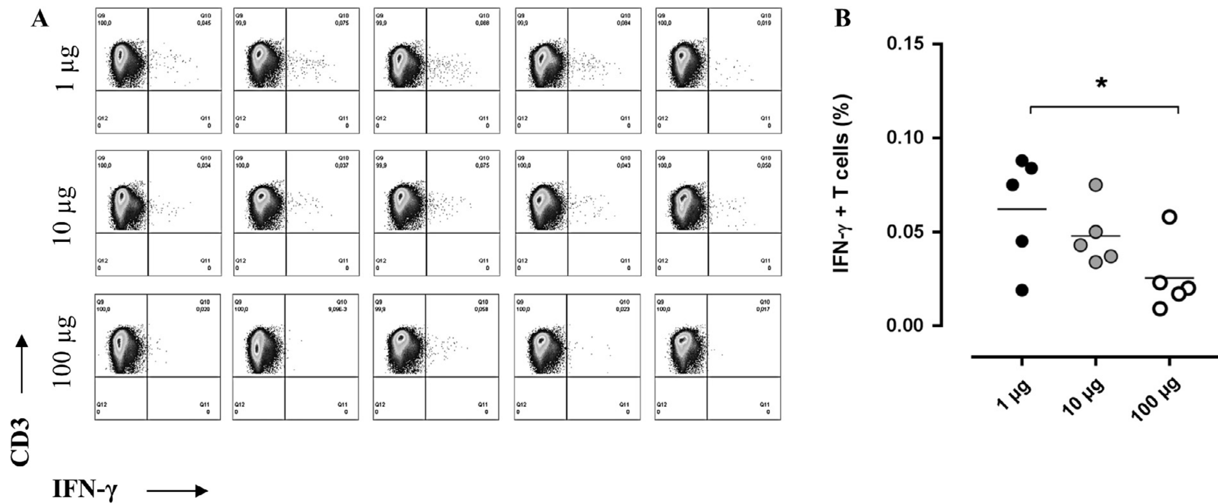

Fig. 2. Flow cytometry corroborates the inverse relationship between antigen dose and the percentage of IFN-γ$^+$ T cells. PBMCs purified at day 41 were stimulated *in vitro* with tetanus toxoid and IFN-γ production was determined by flow cytometry. Analysis included pre-gating on single, viable CD3$^+$ cells. (A) Flow cytometric plots showing IFN-γ$^+$CD3$^+$ cells in the 1 μg (upper panel), 10 μg (middle panel), and 100 μg group (lower panel). Individual animals in each group are shown and horizontally aligned. (B) Percentage of IFN-γ-producing T cells across all groups with indication of the mean. Numbers indicate the percentage of IFN-γ$^+$ T cells as a proportion of total T cells. The background level of IFN-γ-producing T cells in response to media alone were at least 2-fold lower when compared to stimulation with TT or ≤0.03%, while in average 0.36% of the T cells produced IFN-γ in response to the positive SEB stimulation. Statistical evaluation in (B) by unpaired student's *t*-test, ($n = 5$).

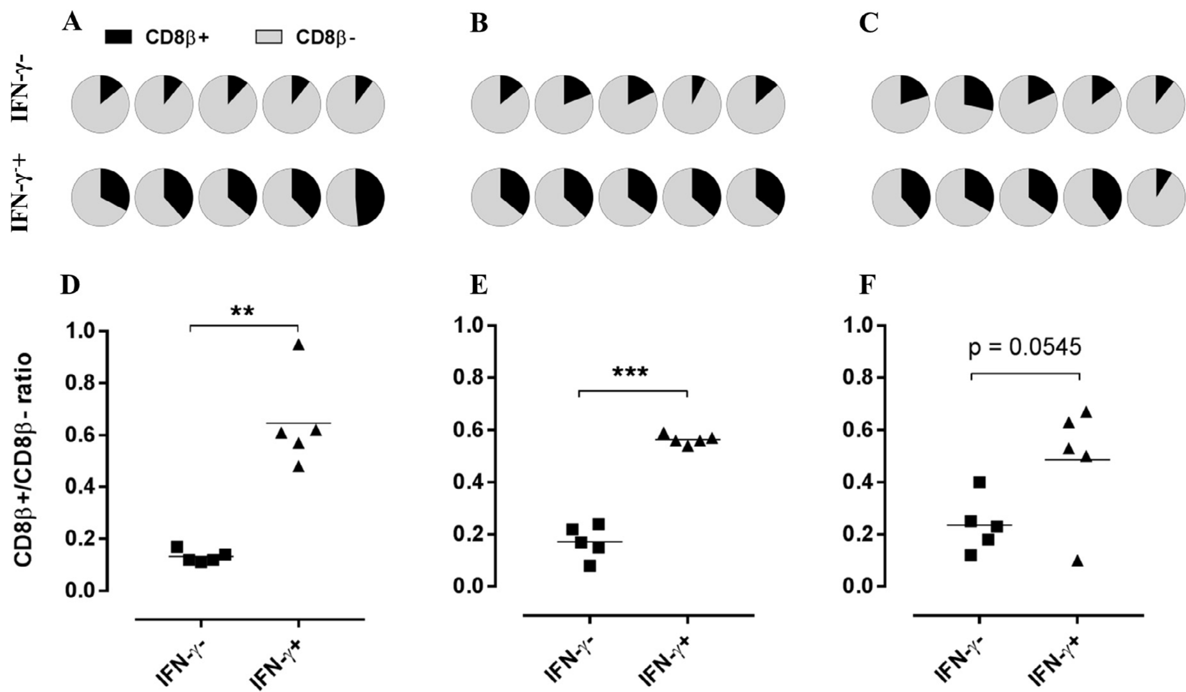

Fig. 3. Tetanus-specific cytotoxic CD8β$^+$ T cells are increased within the IFN-γ$^+$ T-cell population. PBMCs were harvested at day 41 stimulated *in vitro* with tetanus toxoid. By flow cytometry, CD8β expression was individually determined in both the IFN-γ$^-$ and the IFN-γ$^+$ T-cell population. Pie charts from animals immunized with either 1 μg (A), 10 μg (B), or 100 μg (C) tetanus toxoid showing the distribution of CD8β$^-$ (grey) and CD8β$^+$ T cells (black) in both the IFN-γ$^-$ (upper panel) and the IFN-γ$^+$ (lower panel) T-cell population. Individual animals in each group are shown. The CD8β$^+$/CD8β$^-$ ratio in both the IFN-γ$^-$ (squares) and the IFN-γ$^+$ T-cell subsets (triangles) of animals immunized with 1 μg (D), 10 μg (E), or 100 μg (F) of tetanus toxoid are shown with indication of the mean. Statistical evaluation in D, E, and F by paired student's *t*-test ($n = 5$).

clear titration effect could be observed with a low dose specifically inducing more polyfunctional T cells (Fig. 6B). It should be noted that only the 1 μg group clearly demonstrated a population comprising IFN-γ$^+$ single-producing T cells (Fig. 6A).

4. Discussion and conclusions

During this study, we showed the induction of a CTL response when administrating CAF09-formulated TT via the i.p. route in Göttingen minipigs. A low antigen dose resulted in a predominant CMI response, whereas a high dose favoured TT-specific IgG production. Previously, TT has been used as a model antigen in pigs [19], and a study reported the animals to be antigen naïve prior to immunization [20]. Our data confirmed this; hence showing that the anti-TT response was indeed vaccine-induced.

Our observed cell- and antibody-mediated responses are not surprising, as the anti-TT response has been reported to be a mixture between Th1 and Th2 [21,22]. Humans i.m. immunized against alum-adjuvanted TT showed a strong CD4$^+$ T-cell response [23], whereas we demonstrated an increased amount of CTLs

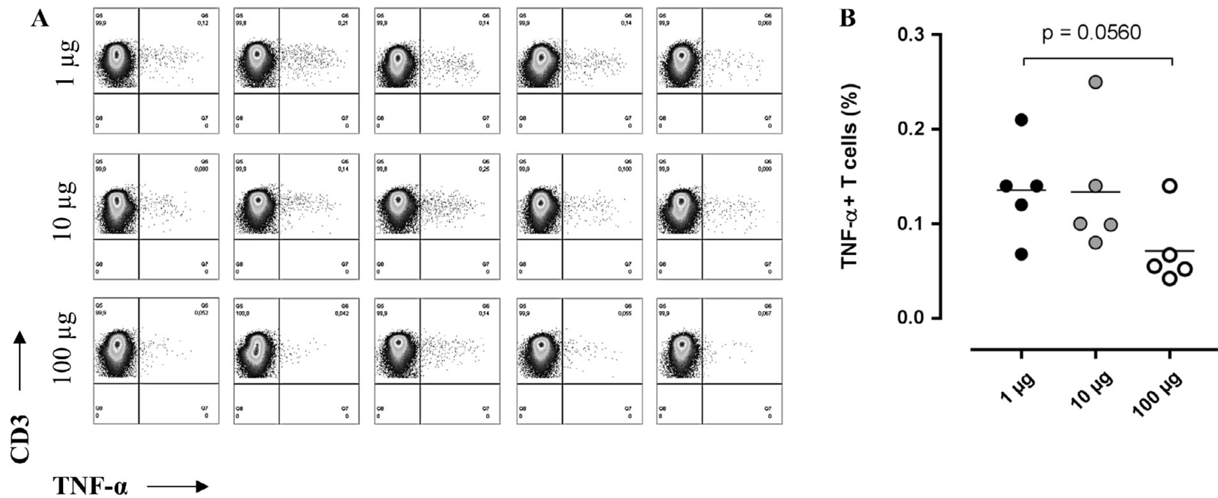

Fig. 4. Lowering the antigen dose tends to trigger a higher percentage of TNF-α⁺ T cells. PBMCs were purified at day 41 and stimulated *in vitro* with tetanus toxoid. Production of TNF-α was determined by flow cytometry, and pre-gating included selection of single, viable $CD3^+$ cells. (A) Flow cytometric plots showing TNF-α^+CD3^+ cells in the 1 µg (upper panel), 10 µg (middle panel), and 100 µg group (lower panel). Individual animals in each group are shown and horizontally aligned. (B) Amount of TNF-α-producing T cells across all groups with indication of the mean. Numbers indicate percentage of TNF-α^+ T cells as a proportion of total T cells. The background level of TNF-α-producing T cells in response to media alone were at least 2-fold lower when compared to stimulation with TT or ≤0.06%, while in average 0.98% of the T cells produced TNF-α in response to the positive SEB stimulation. Statistical evaluation in (B) by unpaired student's *t*-test, ($n = 5$).

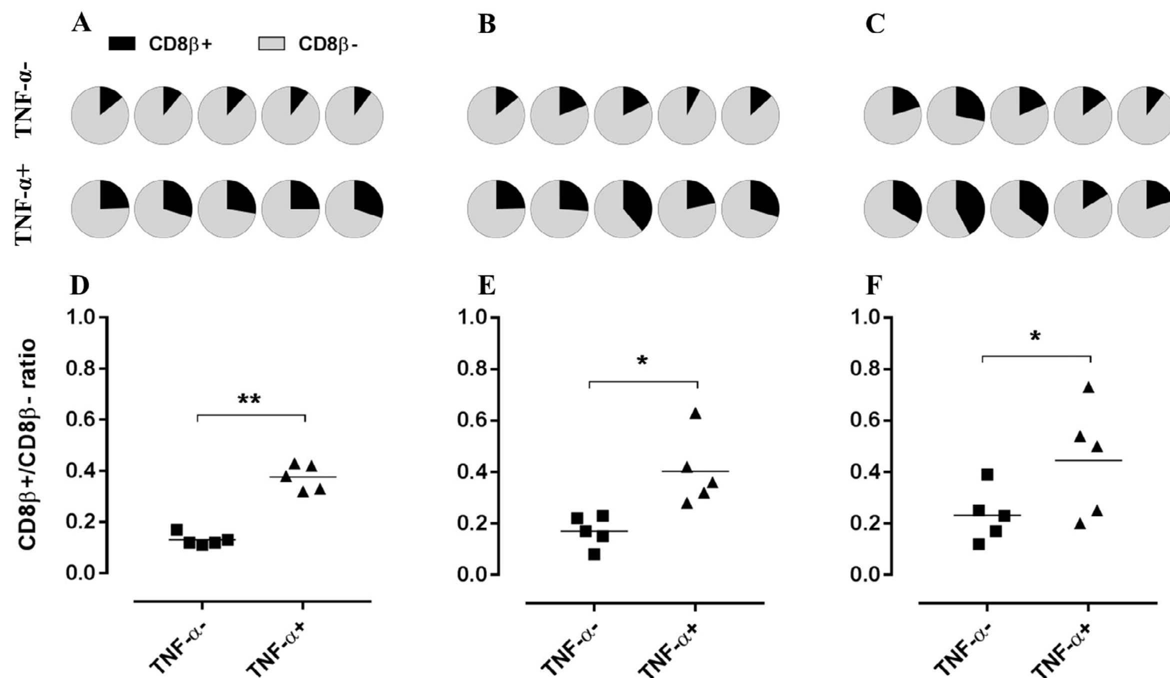

Fig. 5. The TNF-α^+ T-cell population comprises an increased representation of cytotoxic CD8β^+ T cells. PBMCs were purified at day 41 and stimulated *in vitro* with tetanus toxoid. Flow cytometry analysis included pre-gating on single, viable $CD3^+$ cells, and the CD8β expression was then individually determined in both the TNF-α^- and the TNF-α^+ T-cell subset. Pie charts from animals immunized with either 1 µg (A), 10 µg (B), or 100 µg (C) of tetanus toxoid showing the distribution of CD8β^- (grey) and CD8β^+ T cells (black) in both the TNF-α^- (upper panel) and the TNF-α^+ (lower panel) T-cell population. Individual animals in each group are shown. The CD8β^+/CD8β^- ratio in both the TNF-α^- and the TNF-α^+ T-cell subset from animals immunized with 1 µg (D), 10 µg (E), or 100 µg (F) of tetanus toxoid are shown with indication of the mean. Statistical evaluation in D, E, and F by paired student's *t*-test ($n = 5$).

within the pools of IFN-γ and TNF-α producing T-cells. This discrepancy likely reflects the differences in adjuvants and delivery route. It is well known that the immune response generated upon vaccination differs depending on which TLR is activated [24,25] and i.p. administration of cationic liposomes like CAF09 is superior in generating strong CTL responses when compared to subcutaneous (s.c.) and i.m. injection in mice [26]. Establishment of a CTL response against a full protein is dependent on cross-presentation by dendritic cells (DCs); the process by which extracellular antigen is taken up and presented in the context of MHC class I [27,28]. Specifically for i.p. immunizations in mice, vaccine self-drainage to lymphoid organs was shown to efficiently provide antigen to cross-presenting DCs [26]. Upon i.p. immunization in pigs, self-drainage might also play an important role; thus enabling DCs to effectively prime naïve $CD8^+$ T cells and induce a strong CTL response. Hence, the observed inverse relationship between antigen dose and the induction of a polyfunctional CMI response might be differently affected with the use of a different adjuvant system

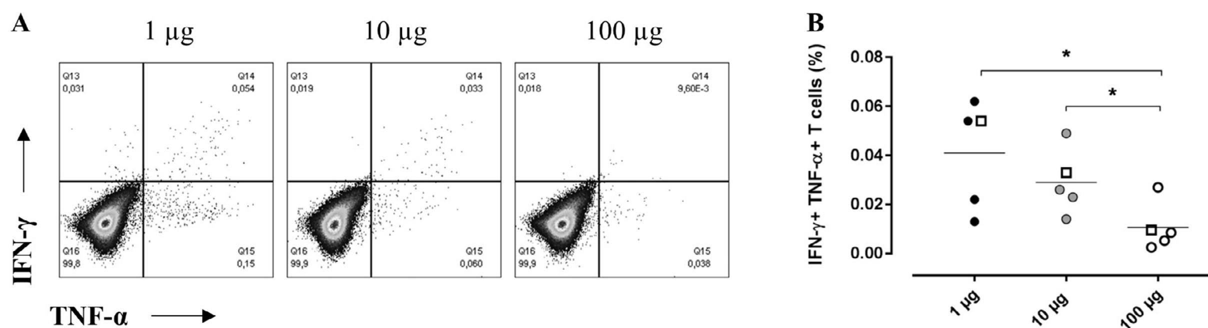

Fig. 6. A lower antigen dose increases the percentage of IFN-γ^{+}TNF-α^{+} T cells. PBMCs were purified at day 41 and stimulated *in vitro* with tetanus toxoid. Cytokine production was determined by flow cytometry, and the analysis included pre-gating on single, viable CD3^{+} cells. (A) Representative flow cytometric plots showing IFN-γ^{+}TNF-α^{+} T cells in the 1 µg (left), 10 µg (middle), and the 100 µg (right) group. (B) Percentage of IFN-γ^{+}TNF-α^{+} T cells as a proportion of total T cells across all groups. Open squares indicate the representative animal shown in (A). Statistical evaluation in (B) by unpaired student's *t*-test, ($n = 5$).

comprising other TLR agonists than poly I:C or the use of other delivery routes.

The antigen dose has previously been shown to influence the immune response following immunization [29,30]. In both mice and humans, immunization with a low dose protein induced high frequencies of CD4^{+} T cells producing IL-2, IFN-γ, and TNF-α [3,4]. In contrast, our data showed a specific increase in CTLs within the cytokine-producing T-cell pool. Notably, the studies reporting a specific induction of polyfunctional CD4^{+} T cells were in response to *Mycobacterium tuberculosis*-derived antigens [3,4], and protection against this bacteria is known to be dependent on a CD4^{+} T-cell response [31–34]. Overall, these studies and our data all support an inverse relationship between CAF09-formulated antigen dose and the induction of polyfunctional T cells.

Moreover, the antigen dose has been reported to influence the avidity and quality of CTLs [35–37]. In addition, the expression level of inhibitory receptors like PD-1 and CTLA-4 on CD4^{+} T cells was found to be decreased, when mice were immunized with a low antigen dose [5]; Future studies should evaluate the effect of antigen dose on both the quality and the activation/memory stage of the TT-reactive T cells in pigs in order to select the optimal strategy for establishment of a vaccine-induced cytotoxic immune response. In conclusion, our results showed that it is possible to induce a CTL response by i.p. delivering a CAF09-formulated protein in pigs. Moreover, we confirmed the inverse relationship between the antigen dose and the induction of polyfunctional T cells previously demonstrated in mice and humans. The T-cell subsets affected might differ depending on the antigen in question; however, the antigen dose clearly affects the immune response induced by immunization. Therefore, correctly determining the first-in-human dose becomes even more important. Due to its similarities in both metabolism and immunome with humans, we believe that pigs can serve as an important animal model for preclinical optimization of vaccine doses.

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Conflict of interest statement

The authors declare no conflicts of interest.

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Experimental design: NHO, TMF, and GJ. Performed the experiments: NHO, JTJ, and TMF. Data analysis and interpretation: NHO, JTJ, and GJ. Drafted the manuscript and figures: NHO. Manuscript revision: NHO, TMF, JTJ, SB, MHA, and GJ. All the authors approved the final manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.08.057.

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