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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.

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

MultiScreen$_{HTS}$ 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

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