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Co-inoculation of DNA and protein vaccines induces antigen-specific T cell suppression

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Abstract

Immunization can sometimes lead to antigen-specific immune suppression. In this study, we investigated this phenomenon by testing several combinations of DNA and protein vaccines directed against various viruses. We find that co-inoculation of mice with combined DNA and protein vaccines induces immune suppression if the two vaccines are "matched" by targeting the same antigen. Conversely, vaccine combinations never lead to immune suppression if they are derived from different viruses and, thus, mismatched antigenically. We have further identified $CD4^+CD25^-$ T cells as the type of regulatory T cells induced by and are responsible for suppressing T cell activities in an antigen-specific manner in immunized animals. These regulatory T cells are phenotypically unique in their expression of Foxp3, IL-10, and IFN-γ. Our study thus shows for the first time that co-administration of antigen-matched DNA and protein vaccines can generate this type of adaptive regulatory T cells.

Keywords: Co-immunization; Antigen-specific suppression; Regulatory T cells; $CD4^+CD25^-$

Although immunization is commonly used for eliciting immune responses against pathogen, it can also be used to dampen untoward immunity, via the induction of T cells of immune suppressive function. Such adaptive regulatory T cells (Treg cells), as opposed to naturally arising Treg cells [1–3] are phenotypically different from the latter. A well-documented case is the $CD8a^+$ DC-induced adaptive Treg cells that inhibit the development of airway hyper-reactivity [4]. These Treg cells are developed from naive $CD4^+CD25^-$ T cells during a Th1-polarized immune response and are composed of heterogeneous cells, including TH1-like as well as TH2-like Treg cells [5]. The expression of transcription factors Foxp3 and T-bet and cytokines IL-10 and IFN-γ plays a critical role in the function of these Treg cells. Despite this example, in general, deliberate induction of Treg cells has been difficult to achieve in vivo. Consequently, there is a pressing need to develop additional methods useful for generating adaptive Treg cells in a predictable way.

In this study, we investigated whether co-immunizing animals with a combination of DNA and protein vaccines may constitute one of such methods. To this end, we have tested several DNA and protein vaccine combinations derived from several different viruses, including foot-and-mouth disease virus (FMDV), porcine reproductive and

Abbreviations: FMDV, foot-and-mouth disease virus; 146S, purified inactivated foot-and-mouth disease virus; PRRSV, porcine reproductive and respiratory syndrome virus; CSFV, classical swine fever virus; HBV, hepatitis B virus; SI, stimulation index; RT-PCR, reverse transcription-polymerase chain reaction; HPRT, hypoxanthine phosphoribosyl transferase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PMS, phenazine methosulfate.

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respiratory symptom virus (PRRSV), the classic swine fever virus (CSFV), and hepatitis B virus (HBV). We have found that such combinations consistently induce antigen-specific immune suppression in immunized mice if the DNA and protein vaccines are matched by the targeted antigen. Furthermore, the immune suppression is due to the induction of Treg cells belonging to the $CD4^+CD25^-$ subset and expressing Foxp3, IL-10, and IFN-γ. These data suggest that this kind of adaptive Treg cells can be deliberately induced via immunization.

Materials and Methods

Animals and immunization. Adult female BALB/c and C57BL/6 mice (8–10 week of age) were from Beijing Vital Laboratory Animal Technology Company, Ltd., (Beijing, China) and received pathogen-free water and food.

The mice were immunized with protein (50 μg/mouse), plasmid (50 μg/mouse) or their mixture (50 μg protein and 50 μg plasmid/mouse) into tibialis anterior muscle. All mice were immunized on day 0 and boosted on day 14. All experiments were reproduced in three independent repeats using three mice per group.

Antigens. The 146S antigen was obtained as previously reported [6] and the PRRS and CSFV antigens were obtained from killed vaccine formulations by centrifugation to remove oil. The resulting preparations were stored at 4 °C. Yeast-expressed HBV surface antigen (HBsAg) was purchased from Beijing Tiantan Biological Products Co., Ltd (Beijing, China). The concentrations of the 146S, PRRS, and CSFV antigens were determined by SDS–PAGE and the Bradford assay. Plasmid pcD-VP1 coding for the FMDV VP1 gene was constructed as previously described [6]. Plasmid pcD-S2 was constructed by inserting the coding sequence for the surface antigen S2 from the *adr* strain of HBV genome into the pcDNA3 vector. Plasmid provax-E2 containing the CSFV E2 gene was constructed as previously described [7].

T cell proliferation. Spleens were removed from immunized mice on day 7 after the second immunization and used to prepare single T cell suspensions. The cells were stimulated in vitro by 5 μg/ml of each following antigen, 146S, PRRS, HBV peptide or E2 peptide, and BSA as a non-specific antigen, or ConA as a positive control. T cell proliferation was determined by the MTS/PMS colorimetric method and expressed as stimulation index. The cells were incubated at 37 °C with 5% CO_2 for 48 h. A Cell Titer 96® Aueous-based non-radioactive cell proliferation assay (Promega, USA) was used to quantify cell proliferation. A mixture of MTS/PMS (20 μl each well) were added to wells based on the protocol and allowed to develop for 4 h. OD values of wells were immediately read at 490 nm by a plate reader (Magellan, Tecan Austria GmbH). Data were expressed as stimulation index (SI), calculated as the mean reading of triplicate wells stimulated with an antigen, divided by the mean reading of triplicate wells stimulated with medium.

Cell isolation. Seven days after boost immunization, splenocytes were prepared from immunized mice by isotonic lysis and nylon wool enrichment. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were purified, respectively, using the MagCellect mouse $CD4^+CD25^+$ regulatory T cell isolation kit as per the manufacturer's protocol (R&D Systems, Inc., Huntingdon Valley, USA). Briefly, $CD4^+$ T cells were first isolated by negative selection and $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were further separated by positive selection for CD25 using immuno-magnetic beads and a magnetic separation rack (New England Biolabs Inc. MA, USA). The resulting $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^-$ T cells were >90% pure, respectively, as determined by flow cytometry (FACSCalibur, BD Bioscience, San Jose, USA). $CD8^+$ T cells were isolated by positive selection using the MagCellect mouse $CD8^+$ T cell isolation kit (R&D Systems). The purity of $CD8^+$ T cells was >90% by flow cytometric analysis.

Mixed lymphocyte reaction. The $CD4^+$, $CD8^+$, or $CD4^+CD25^-$ T cell populations were purified from C57BL/6 mice as described under cell Isolation above and used as the responder cells. Stimulator cells were isolated from the spleen of naïve BALB/c mice by panning using anti-CD3 monoclonal antibody (eBioscience, USA) to delete T cells and were further treated with mitomycin C before use. The responder and stimulator cells were mixed at various ratios, from 10:1, 3:1; 1:1, 1:3 to 1:10 (C57BL/6 respondor: BALB/c stimulator), seeded at 1×10^5 total cells per well in triplicate wells, and cultured for 48 h, followed by the MTS/PMS colorimetric [8]. The OD values were read at 490 nm with the plate reader (Magellan).

Flow cytometry and intracellular staining. For $CD4^+CD25^+$ T cells staining, total T cells from immunized mice were isolated and stained with anti-CD4-FITC and anti-CD25-PE mAbs (BD Phamingen, San Diego, USA) in PBS/1% BSA for 30 min at 4 °C. The cells were analyzed with a FACScalibur and the Cell Quest Pro Software (BD Bioscience). For intracellular staining of IL-10, Foxp3, IL-4, and IFN-γ, purified $CD4^+CD25^-$ T cells were stimulated in culture for 24 h with the 146S antigen and anti-CD28 mAb (eBioscience). The cells were then treated with monensin (100 μg/ml) for 2 h and blocked with Fc-Block (BD Phamingen). The cells were fixed with 4% paraformaldehyde, permeabilized with 1% saponin, and then stained with PE-conjugated anti-IL-10, anti-IL-4, anti-Foxp3, or anti-IFN-γ mAbs (BD Phamingen) for 30 min at 4 °C, according to the manufacturer's procedure. The cells were washed and analyzed by FACS.

RT-PCR analysis for TGF-β. Total RNA was isolated and used for cDNA synthesis, using the MMLV reverse transcriptase (Promega, USA) and oligo$(dT)_{18}$ primers. After the normalization against the housekeeping gene HPRT, all cDNA samples were PCR-amplified with optimized primers specific for TGF-β, as described previously [9,10]. PCR products were analyzed by agarose gels and stained with ethidium bromide.

Statistics. Data were subjected to Student's *t* test. Difference is deemed significant if $P < 0.05$. Particularly, * indicates $P < 0.05$ and **, $P < 0.01$.

Results and Discussion

Co-inoculation of antigen-matched DNA and protein vaccines impairs T cell responses to the immunizing antigen

Previously, we showed that mice and cattle developed impaired T cell responses to FMDV after being immunized with an anti-FMDV DNA vaccine in combination with an FMDV-derived protein vaccine [11]. To determine the molecular requirement for this form of immunization-induced immune suppression, we studied the effects of combining DNA and protein vaccines derived from other viruses, namely HBV, PRRSV, and CSFV, in addition to FMDV. C57BL/6 mice were immunized twice with various vaccine combinations; spleen T cells were then isolated from the mice on day 7 after the second vaccination and tested by a T cell proliferation assay for reactivity against a recall antigen. The result showed that when the DNA and protein vaccines were derived from the same virus and, therefore, matched at the antigen level, they impaired recall responses by T cells to the specific antigen. Thus, as depicted in Fig. 1A and B, T cells recovered from mice that had been co-immunized with a pair of antigen-matched DNA and protein vaccines failed to proliferate vigorously in response to recall antigens, in contrast to the control T cells from mice immunized with a DNA vaccine alone. Conversely, when the DNA and protein vaccines were derived from different viruses and, thus, mismatched antigenically, they did not impair T cells (Fig. 1C). We therefore conclude that the induction of antigen-specific T cell