

A DNA adjuvant encoding a fusion protein between anti-CD3 single-chain Fv and AIMP1 enhances T helper type 1 cell-mediated immune responses in antigen-sensitized mice

Byeong Cheol Lee,¹ Insug O'Sullivan,² Eugene Kim,¹ Sang Gyu Park,³ Seung Yong Hwang,⁴ Daeho Cho⁵ and Tae Sung Kim¹

¹School of Life Sciences and Biotechnology, Korea University, Seoul, Korea, ²Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL, USA, ³Functional Expansion Research Laboratory, Clinical Research Institute, Seoul National University Hospital, Seoul, ⁴Division of Molecular and Life Sciences, Hanyang University, Ansan, and ⁵Department of Life Science, Sookmyung Women's University, Seoul, Korea

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Correspondence: Dr T. S. Kim, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea.

Email: tskim@korea.ac.kr

Senior author: Tae Sung Kim

Summary

T helper type 1 (Th1) cell-mediated immune responses contribute to host defences against intracellular pathogen infections and cancer. Previously, we found that aminoacyl tRNA synthetase-interacting multifunctional protein 1 (AIMP1) activated macrophages and dendritic cells to enhance Th1 responses. Herein, we manipulated this property to improve Th1 immune responses *in vivo* by constructing a mammalian expression plasmid (pAnti-CD3sFv/AIMP1) encoding AIMP1 fused to the anti-CD3 single-chain Fv (sFv), the smallest unit of the antibody that interacts with the CD3 ϵ region of the T-cell receptor. Intramuscular injection of ovalbumin (OVA)-sensitized BALB/c mice with pAnti-CD3sFv/AIMP1 DNA adjuvant increased the OVA-specific, interferon- γ production by their CD4⁺ T cells and the levels of anti-OVA immunoglobulin G2a (IgG2a) isotype in their sera. Furthermore, the pAnti-CD3sFv/AIMP1 DNA adjuvant decreased interleukin-4 production and anti-OVA IgE levels in the OVA-injected mice. Importantly, the pAnti-CD3sFv/AIMP1 was more efficient than a mixture of pAnti-CD3sFv and pAIMP1 in inducing OVA-specific Th1 immune responses and also in inhibiting OVA-specific Th2 responses during antigen priming. These studies indicated that the pAnti-CD3sFv/AIMP1 fusion DNA adjuvant enhanced Th1 immune responses in antigen-sensitized mice.

Keywords: aminoacyl t-RNA synthetase-interacting multifunctional protein 1; anti-CD3; DNA adjuvant; T cell; T helper cell response

Introduction

Characterization of the polarized subsets of T helper cells, type 1 (Th1) and Th2, has increased our understanding of how these cell-types coordinate a variety of immune responses to control human diseases.^{1,2} Th1 cells secrete interferon- γ (IFN- γ) and interleukin-2 (IL-2), and regulate cell-mediated immunity. The Th1 immune responses mediate host defences against a variety of intracellular pathogens and are crucial in cancer immunotherapy.^{3–5} In contrast, Th2 cells produce IL-4, IL-5, IL-6 and IL-10, which are involved in the development of humoral immune responses. The Th2 responses induce immune protection against extracellular microbes and are involved in allergic pathogenesis. These two Th subsets regulate

each others' function through the antagonistic activity of their respective cytokines.

A number of factors determine which Th subset predominates during an immune response. These include the physical form of the antigen, the density and affinity of the peptide ligand, the nature of the costimulatory signals provided by antigen-presenting cells (APCs), the hormones released into the microenvironment, the genetic background of the T cells, and changes in histone remodelling.^{6–8} The cytokine microenvironment in which the initial antigen priming occurs is especially crucial for influencing whether an immune response favours Th1 or Th2 polarization. Both IFN- γ and IL-12 induce Th1-mediated responses, but IL-4 drives the response to favour the Th2 subsets. Recent work revealed that

Abbreviations: AIMP1, aminoacyl t-RNA synthetase interacting-multifunctional protein 1; Anti-CD3sFv, anti-CD3 single chain Fv; APC, antigen-presenting cell; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IgG2a, immunoglobulin G2a; IL, interleukin; i.m., intramuscularly; i.p., intraperitoneally; mAb, monoclonal antibody; OVA, ovalbumin; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; sFv, single-chain Fv; Th1, T helper type 1.

transforming growth factor- β negatively regulates Th1 polarization.⁹

Aminoacyl-tRNA synthetase-interacting multifunctional protein 1 (AIMP1) was initially identified as an auxiliary factor associated with the macromolecular aminoacyl-tRNA synthetase complex.¹⁰ Various stimuli, including tumour necrosis factor- α , heat shock and hypoxia, promote AIMP1 secretion, and it acts as a multifunctional cytokine on endothelial and immune cells.^{11,12} Our previous studies demonstrated that AIMP1 stimulated macrophages and dendritic cells to secrete Th1 cytokines, leading to enhanced Th1 responses and IFN- γ production from antigen-primed Th1 cells.^{13,14}

Nucleic acids encoding antigens or therapeutic proteins can be used as vaccines. For example, DNA plasmids inoculated into muscle are effectively internalized by myofibres, resulting in expression of the encoded genes.¹⁵ By this mechanism, DNA vaccines can stimulate both cell-mediated and humoral immunity simultaneously, without the safety risks associated with live vaccines.¹⁶ In addition, bacterial DNA and oligonucleotides (CpG DNA) can enhance immune responses and therefore show promise as novel adjuvants of DNA-based immunotherapy.¹⁷ Potential applications for DNA vaccines range from prophylactic vaccines to immunotherapy for infections, cancer, and autoimmune and allergic diseases.^{18–20} In this study, as a strategy to efficiently induce and polarize Th cell-mediated immunity toward the Th1 response by DNA-based immunization, we constructed a mammalian expression plasmid that harboured a fusion of complementary DNAs (cDNAs) encoding anti-CD3 single-chain Fv (sFv) and AIMP1, linked by a nucleotide spacer encoding six amino acids (pAnti-CD3sFv/AIMP1).

Previously, we reported that murine immunization with covalently linked IL-2 and anti-CD3sFv proteins confined the effect of IL-2 to CD3⁺ T cells, thereby selectively protecting CD3⁺ T cells from dexamethasone-induced apoptosis. Furthermore, this anti-CD3sFv/IL-2 protein conjugate strongly increased the CD3⁺ T-cell population in spleen cells in both *in vitro* and *in vivo* models.²¹

Herein, we report that injection with pAnti-CD3sFv/AIMP1 DNA increased the production of ovalbumin (OVA)-specific IFN- γ and anti-OVA immunoglobulin G2a (IgG2a) antibody in OVA-sensitized mice, leading to efficient induction of Th1 immune responses during antigen priming.

Materials and methods

Mice, cells and reagents

Female 6- to 8-week-old BALB/c (H-2^d) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were maintained under specific pathogen-free conditions and treated according to the National Institutes of

Health Guidelines for the Care and Use of Laboratory Animals. The HeLa cell line was obtained from the American Type Culture Collection (Rockville, MD), and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (Gibco BRL, Grand Island, NY). Murine recombinant IFN- γ and recombinant IL-4 were purchased from Genzyme (Cambridge, MA). The OVA was obtained from ICN Biomedicals (Montreal, PQ). Anti-OVA IgG1 and IgG2a monoclonal antibodies (mAbs) were used as standards for each isotype-specific enzyme-linked immunosorbent assay (ELISA). Rabbit anti-OVA serum was purchased from Cappel (Durham, NC), and anti-AIMP1 was purchased from Imagen (Seoul, Korea). Polyclonal mouse anti-OVA were obtained from sera of immunized mice following serial injections of OVA in complete and incomplete Freund's adjuvant. Horseradish peroxidase-labelled goat anti-mouse IgG1 and IgG2a were purchased from Southern Biotechnology Associates (Birmingham, AL).

Construction of an expression plasmid harbouring the gene for anti-CD3sFv/AIMP1

A mammalian expression plasmid (donated from Dr M.E. Reff)²² containing a simian virus 40 origin of replication, designed to express immunoglobulin genes, was modified to eliminate the immunoglobulin-coding region and the neomycin-resistance gene. The pAnti-CD3sFv/AIMP1 was constructed by first inserting anti-CD3sFv cDNA in frame with a human immunoglobulin κ leader sequence, to allow for secretion of the translated protein. Anti-CD3sFv cDNA was cloned by polymerase chain reaction (PCR) from an anti-CD3sFv-containing plasmid (obtained from Dr B.R. Blazar, University of Minnesota, Minneapolis, MN) using primers carrying the desired restriction sites. Subsequently, AIMP1 cDNA was inserted downstream of the anti-CD3sFv cDNA, separated by a spacer encoding six amino acid residues (SSGGGG) (Fig. 1a). As controls, pAnti-CD3sFv and pAIMP1 were constructed by PCR using primers harbouring stop codons at the 3' primer of each gene. The constructed plasmids were, respectively, electroporated into *Escherichia coli*, and purified from large-scale cultures by alkaline lysis and caesium chloride density gradient centrifugation. The endotoxin levels of the purified plasmids were < 15 EU/mg DNA, as determined by a *Limulus* amoebocyte lysate assay kit (BioWhittaker, Walkersville, MD).

Transfection, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

HeLa cells were transfected with pAnti-CD3sFv/AIMP1 or pAnti-CD3sFv DNA using Superfect transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The culture supernatants from the

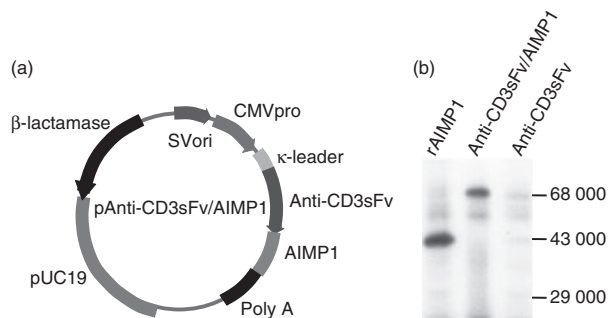


Figure 1. Expression of a recombinant anti-CD3sFv/AIMP1 protein by HeLa cells transfected with pAnti-CD3sFv/AIMP1 DNA. (a) Schematic drawing of the expression plasmid harbouring the anti-CD3sFv and AIMP1 fusion gene (pAnti-CD3sFv/AIMP1). (b) Western blot analysis of recombinant proteins expressed by the transfected HeLa cells. Recombinant proteins, purified by immunoprecipitation using CD3-conjugated Sepharose 4B resin or anti-AIMP1, were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted by anti-AIMP1.

transfected cells were harvested after 3 days, and secreted proteins were immunoprecipitated using CD3-conjugated Sepharose 4B resin (Sigma, St Louis, MO). The resins were washed three times with 0.1% Tween-20 in Tris–HCl buffer (pH 8.0); the precipitated proteins were analysed on SDS–PAGE in a Mini-Protein II gel apparatus (Bio-Rad, Richmond, CA) and transferred onto nitrocellulose membrane by semi-dry electroblotting. The blots were probed with anti-AIMP1. To detect immunoreactive bands, membranes were washed, exposed to horseradish peroxidase-labelled anti-mouse IgG, and developed with an enhanced chemiluminescence system (Amersham, Arlington Heights, IL), according to the manufacturer's protocol.

Sensitization of mice with OVA and intramuscular injection of plasmid DNA

Mice were injected intraperitoneally (i.p.) with 200 µg OVA adsorbed on 5 mg Al(OH)₃ adjuvant (200 µl), followed by one additional injection with 200 µg OVA alone 1 week later. One week after the OVA injection, mice were injected intramuscularly (i.m.) with the indicated amounts of plasmid DNA in 100 µl of 0.85% saline. The injection depth of the needle was adjusted to 2 mm with a steel collar, as previously described.²³

In vitro stimulation of splenic CD4⁺ T cells

Spleens from mice were aseptically removed and single-cell suspensions were prepared. CD4⁺ T cells were purified by negative selection using magnetic activated cell sorting with a cocktail of biotinylated anti-mouse CD8, I-A, B220 and Mac-1 antibodies (Miltenyi, Sunnyvale, CA). More than 98% of the isolated cells were verified as CD4⁺ T cells by cytofluorometry. For the cytokine assay, the purified CD4⁺

T cells (5×10^5 cells/well in 96-well plates) were incubated *in vitro* with 100 µg OVA for 4 days, after which IFN-γ and IL-4 levels in culture supernatants were determined by ELISA, as described below.

Cytokine assay

Levels of IFN-γ and IL-4 in the culture supernatants were quantified by sandwich ELISA, using mAbs specific for mouse IFN-γ and IL-4 (Genzyme), as previously described.²⁴ The mAbs used for coating the plates and detecting the cytokines were as follows: for IFN-γ, rat anti-mouse IFN-γ (HB170) and biotinylated rat anti-mouse IFN-γ (XMG1.2); for IL-4, rat anti-mouse IL-4 (BVD4) and biotinylated anti-mouse IL-4 (BVD6). The lower limit of detection was 125 pg/ml for IFN-γ and 3 pg/ml for IL-4. Recombinant mouse IFN-γ and IL-4 (Genzyme) were used as standards.

Determination of anti-OVA antibodies IgG2a, IgG1 and IgE

Mice were bled during the course of the experiments and the amounts of OVA-specific IgG2a, IgG1 and IgE in the sera were measured by ELISA, as previously described.²⁵ Briefly, to determine levels of anti-OVA IgG2a and anti-OVA IgG1, ELISA plates were coated with 100 µl of 5 µg/ml OVA. After coating, serial dilutions of the sera were added to the plates and incubated overnight at 4°. After washing, horseradish peroxidase-labelled anti-mouse IgG2a or anti-mouse IgG1 was added, and the plates were incubated for 2 hr at room temperature. After additional washing, *o*-phenylenediamine substrate was added; the plates were incubated for 10 min and the optical density was determined at 492 nm using a Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). Ovalbumin-specific IgE was measured by ELISA using rat anti-mouse IgE for coating and biotinylated OVA/streptavidin-peroxidase for detection.

Statistical analyses

Student's *t*-tests and one-way analysis of variance followed by the Bonferroni method were used to determine the statistical significance of differences between the values of experimental and control groups. *P*-values < 0.05 were considered statistically significant.

Results

Characterization of the anti-CD3sFv/AIMP1 fusion protein expressed in HeLa cells

We constructed an expression plasmid harbouring an anti-CD3sFv/AIMP1 fusion gene (pAnti-CD3sFv/AIMP1,

Fig. 1a), as described in the *Materials and methods* section. We then transiently transfected HeLa cells with the plasmid to produce the recombinant anti-CD3sFv/AIMP1 fusion protein. As a control, we also constructed a plasmid harbouring the anti-CD3sFv gene (pAnti-CD3sFv), which produced recombinant anti-CD3sFv protein when expressed in HeLa cells. We characterized these recombinant anti-CD3sFv/AIMP1 and anti-CD3sFv proteins by purifying them with CD3 immunoprecipitation and subjecting them to Western blot analysis using anti-AIMP1. As shown in Fig. 1b, the resulting immunoreactive bands corresponded to the expected size (approximately 72 000 molecular weight) of the anti-CD3sFv/AIMP1 protein. The anti-CD3sFv/AIMP1 protein expressed in HeLa cells was immunoblotted with anti-AIMP1, indicating that both the anti-CD3sFv and AIMP1 parts of the recombinant anti-CD3sFv/AIMP1 fusion protein were immunologically reactive for CD3 and anti-AIMP1, respectively.

Intramuscular injection with pAnti-CD3sFv/AIMP1 DNA increased the production of OVA-specific IgG2a isotype in OVA-sensitized mice

To determine whether injection with pAnti-CD3sFv/AIMP1 DNA efficiently induced Th1 cell-mediated immune responses, BALB/c mice were serially injected i.p. with OVA/alum and i.m. with various amounts (0–500 µg) of pAnti-CD3sFv/AIMP1, pAnti-CD3sFv, or an empty vector as a control, as described in the *Materials and methods* section. The mice were bled 2 weeks after injection, and the levels of anti-OVA IgG2a isotype in the sera were determined. As shown in Fig. 2, mice immunized with pAnti-CD3sFv/AIMP1 produced significant amounts of anti-OVA IgG2a. Importantly, the levels of anti-OVA IgG2a in the mice injected with pAnti-CD3sFv/AIMP1 DNA were significantly higher than those in the mice injected with pAnti-CD3sFv DNA.

Injection with pAnti-CD3sFv/AIMP1 DNA induced and maintained Th1 responses in OVA-sensitized mice

We next measured anti-OVA IgG2a production over time in response to injection with pAnti-CD3sFv/AIMP1 DNA. The mice were serially injected i.p. with OVA/alum and i.m. with 150 µg pAnti-CD3sFv/AIMP1 or pAnti-CD3sFv, and they were bled at 1, 2, 4 or 8 weeks after injection. The levels of anti-OVA IgG2a in the sera were determined. As shown in Fig. 3, the anti-OVA IgG2a isotype was detected at 1 week after the injection and the serum levels were sustained throughout the entire 8-week observation period. Mice injected with pAnti-CD3sFv/AIMP1 DNA maintained higher levels of anti-OVA IgG2a during the 8-week observation period than the control mice injected with pAnti-CD3sFv DNA.

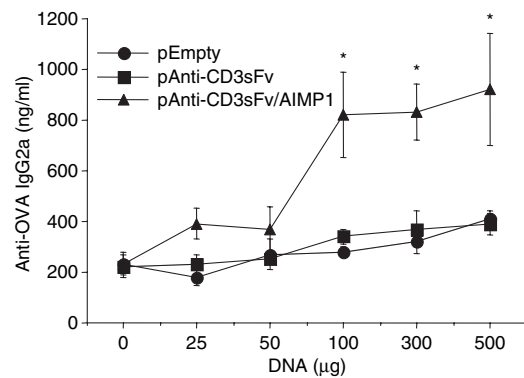


Figure 2. Increased production of anti-ovalbumin (OVA) immunoglobulin G2a (IgG2a) in OVA-sensitized mice injected with pAnti-CD3sFv/AIMP1 DNA. BALB/c mice (seven per group) were serially injected intraperitoneally with OVA/alum and intramuscularly with the indicated amounts of pAnti-CD3sFv/AIMP1, pAnti-CD3sFv DNA, or an empty vector (pEmpty) as a control. The mice were bled 2 weeks after injection, and the levels of anti-OVA IgG2a were determined by enzyme-linked immunosorbent assay. The values represent the mean $\pm$ SE of seven mice. The experiment was repeated twice with similar results. * $P < 0.01$, relative to the group injected with pAnti-CD3sFv DNA.

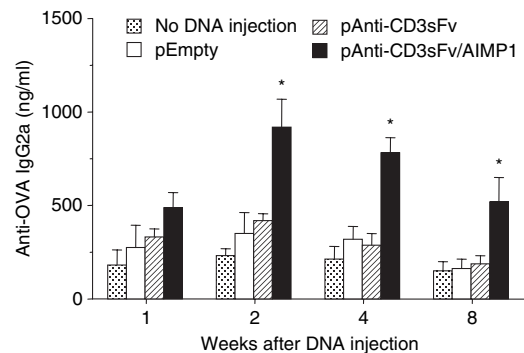


Figure 3. Sustained effect of pAnti-CD3sFv/AIMP1 DNA injection on increasing anti-ovalbumin (OVA) immunoglobulin G2a (IgG2a) levels in OVA-sensitized mice. BALB/c mice (five per group) were serially injected intraperitoneally with OVA/alum and intramuscularly with 150 µg pAnti-CD3sFv/AIMP1 DNA, pAnti-CD3sFv DNA, or the pEmpty DNA, or they were left uninjected. The mice were bled at 1, 2, 4, or 8 weeks after injection, and the levels of anti-OVA IgG2a in the sera were determined by enzyme-linked immunosorbent assay. The values represent the mean $\pm$ SE of five mice. The experiment was repeated three times with similar results. * $P < 0.01$, relative to the group injected with pAnti-CD3sFv DNA.

We also determined whether injection with pAnti-CD3sFv/AIMP1 DNA increased the production of IFN- γ , a Th1 cytokine, in OVA-sensitized BALB/c mice. As before, mice were serially injected i.p. with OVA/alum and i.m. with 150 µg of pAnti-CD3sFv/AIMP1 DNA or pAnti-CD3sFv DNA. The spleens from the injected mice were removed at 1, 2, 4 or 8 weeks after injection, and

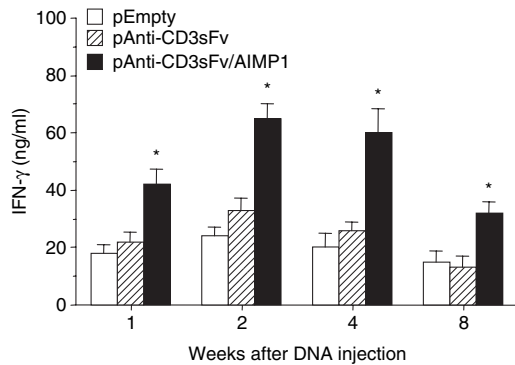


Figure 4. Increased production of ovalbumin (OVA)-specific, interferon- γ (IFN- γ) in CD4⁺ T cells from OVA-sensitized mice injected with pAnti-CD3sFv/AIMP1 DNA. BALB/c mice (five per group) were serially injected intraperitoneally with OVA/alum and intramuscularly with 150 μ g pAnti-CD3sFv/AIMP1, pAnti-CD3sFv DNA, or the empty vector as a control. The mice were killed at 1, 2, 4, or 8 weeks after injection, and splenic CD4⁺ T cells were prepared. The cells were stimulated *in vitro* with 100 μ g/ml OVA for 4 days, and the levels of IFN- γ in the culture supernatants were analysed by enzyme-linked immunosorbent assay. The values represent the mean $\pm$ SE ($n = 5$). * $P < 0.01$, relative to the group injected with pAnti-CD3sFv DNA.

purified splenic CD4⁺ T cells from each treatment condition were stimulated *in vitro* with 100 μ g OVA. The levels of IFN- γ in the cell culture supernatants were determined by an IFN- γ -specific ELISA. As shown in Fig. 4, splenic CD4⁺ T cells from the mice injected with pAnti-CD3sFv/AIMP1 DNA produced significantly higher levels of IFN- γ than those from the mice injected with pAnti-CD3sFv

DNA, indicating that the fusion DNA induced a Th1-type response in these cells more efficiently than the anti-CD3sFv DNA alone.

The pAnti-CD3sFv/AIMP1 DNA was more efficient in inducing Th1 immune responses than a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA

To investigate the importance of the direct linkage between anti-CD3sFv and AIMP1 in the pAnti-CD3sFv/AIMP1 plasmid, we included a group of mice injected with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA. BALB/c mice were serially injected i.p. with OVA/alum and i.m. with 150 μ g pAnti-CD3sFv or pAnti-CD3sFv/AIMP1 DNA, or with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA (150 μ g each). Two weeks after injection, the levels of IFN- γ produced by splenic CD4⁺ T cells were determined after *in vitro* stimulation with 100 μ g OVA. Five mice from each group were individually bled at 2 weeks after the injection and the levels of anti-OVA-IgG2a in the sera were analysed. As shown in Fig. 5, anti-OVA IgG2a isotype levels were significantly higher in sera from mice injected with pAnti-CD3sFv/AIMP1 DNA than in sera from the control mice injected with pAnti-CD3sFv DNA, or from those injected with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA. Furthermore, injection with pAnti-CD3sFv/AIMP1 DNA induced higher levels of OVA-specific IFN- γ production in CD4⁺ T cells than did injection with pAnti-CD3sFv DNA, or with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA.

Therefore, these results clearly indicated that pAnti-CD3sFv/AIMP1 DNA more efficiently induced

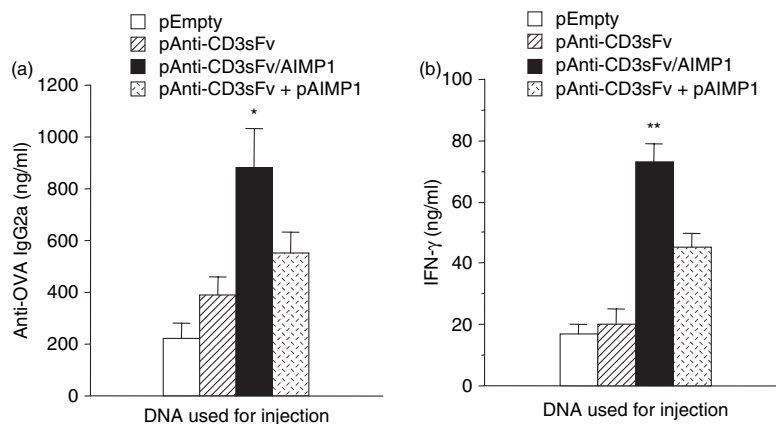


Figure 5. The pAnti-CD3sFv/AIMP1 DNA was more efficient in inducing T helper type 1 (Th1) immune responses than a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA. BALB/c mice (five per group) were serially injected intraperitoneally with ovalbumin (OVA)/alum and intramuscularly with 150 μ g pAnti-CD3sFv/AIMP1 or pAnti-CD3sFv DNA, or with a mixture of 150 μ g pAnti-CD3sFv DNA and 150 μ g pAIMP1 DNA. (a) Two weeks after injection, the levels of anti-OVA IgG2a in the sera were determined by enzyme-linked immunosorbent assay. The values represent the mean $\pm$ SE of five mice. The data are representative of two separate experiments. * $P < 0.05$, relative to the group injected with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA. (b) The levels of IFN- γ produced from splenic CD4⁺ T cells were determined after *in vitro* stimulation with 100 μ g/ml OVA. The values represent the mean $\pm$ SE ($n = 3$). ** $P < 0.01$, relative to the group injected with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA.

antigen-specific, Th1 immune responses than did a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA.

Injection with pAnti-CD3sFv/AIMP1 DNA inhibited OVA-specific IL-4 production from CD4⁺ T cells in OVA-sensitized mice

Since injection with pAnti-CD3sFv/AIMP1 DNA promoted OVA-specific Th1 cell-mediated immune responses, we investigated whether it concomitantly inhibited OVA-specific Th2 cell-mediated immune responses. In these experiments, BALB/c mice were serially immunized i.p. with OVA/alum and i.m. with varying amounts of pAnti-CD3sFv/AIMP1 DNA, pAnti-CD3sFv DNA, or the mixture of pAnti-CD3sFv DNA and pAIMP1 DNA. Two weeks after injection, the levels of IL-4 produced by splenic CD4⁺ T cells were determined after *in vitro* stimulation with 100 µg OVA. Serum levels of anti-OVA IgE were also determined at 2 weeks after injection. As shown in Fig. 6, the levels of IL-4 production and anti-OVA IgE in mice immunized with pAnti-CD3sFv/AIMP1 DNA were significantly lower than those from the mice injected with pAnti-CD3sFv DNA or with the pAnti-CD3sFv DNA and pAIMP1 DNA mixture. However, the levels of anti-OVA IgG1 did not significantly differ among any of the groups of mice. These findings revealed that pAnti-CD3sFv/AIMP1 DNA more efficiently inhibited Th2 cell-mediated responses than did a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA.

Discussion

In this study, injection with pAnti-CD3sFv/AIMP1 fusion DNA induced significantly higher levels of antigen-specific Th1 immune responses in OVA-sensitized mice than did a mixture of pAIMP1 DNA and pAnti-CD3sFv DNA. These enhanced Th1 responses were characterized by high OVA-specific IgG2a isotype titres in sera and high levels of IFN-γ production from the OVA-specific splenic CD4⁺ T cells. Injection with a mixture of pAIMP1 and pAnti-CD3sFv DNA also increased the production of OVA-specific IFN-γ and anti-OVA IgG2a, but the response was significantly lower than those induced by the pAnti-CD3sFv/AIMP1 DNA fusion construct. It appeared that the linkage of AIMP1 to the anti-CD3sFv efficiently confined the Th1-polarizing effects of AIMP1 to the OVA-specific T cells. The pAnti-CD3sFv/AIMP1 DNA also efficiently induced OVA-specific Th1-mediated immune responses in C57BL/6 mice (H-2^b), indicating that these phenomena were not strain-specific (data not shown). Furthermore, anti-CD3sFv/AIMP1 DNA inhibited OVA-specific IL-4 production from CD4⁺ T cells in OVA-sensitized mice (Fig. 6). The serum levels of anti-OVA IgE were significantly lower in mice injected with

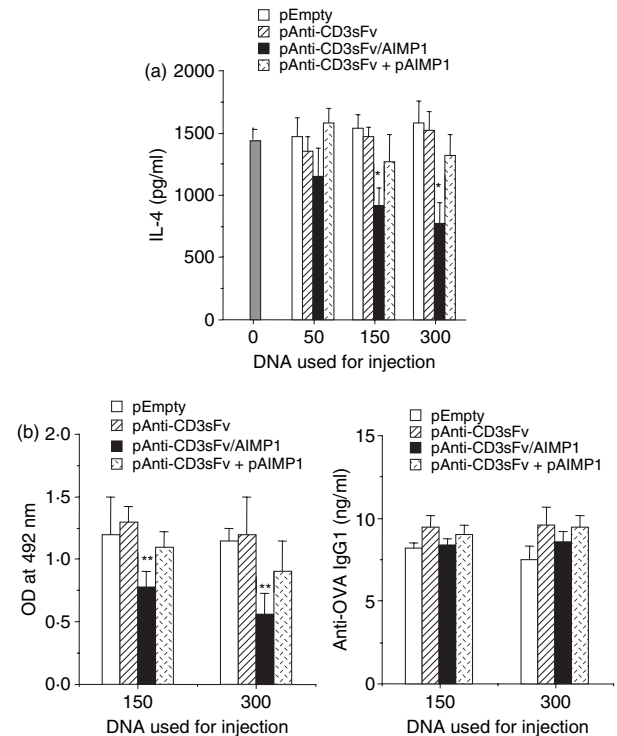


Figure 6. Injection with pAnti-CD3sFv/AIMP1 DNA inhibited ovalbumin (OVA)-specific T helper type 2 (Th2) responses in OVA-sensitized mice. (a) BALB/c mice (five per group) were serially injected intraperitoneally with OVA/alum and intramuscularly with the indicated amounts of pAnti-CD3sFv/AIMP1 or pAnti-CD3sFv DNA, or with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA. Two weeks after injection, production of interleukin-4 (IL-4) from splenic CD4⁺ T cells was determined after *in vitro* stimulation with 100 µg/ml OVA. The values represent the mean ± SE ($n = 5$). * $P < 0.01$, relative to the group injected with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA. (b) BALB/c mice were serially injected intraperitoneally with OVA/alum and intramuscularly with pAnti-CD3sFv/AIMP1 or pAnti-CD3sFv DNA, or with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA. The mice were individually bled at 2 weeks after injection and the levels of anti-OVA immunoglobulin E (IgE) and anti-OVA IgG1 were determined by enzyme-linked immunosorbent assay. The values represent the mean ± SE of five mice. ** $P < 0.05$, relative to the group injected with a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA.

pAnti-CD3sFv/AIMP1 DNA, although the levels of anti-OVA IgG1 did not significantly differ among mice injected with pAnti-CD3sFv/AIMP1, pAnti-CD3sFv, or a mixture of pAnti-CD3sFv and pAIMP1 (Fig. 6).

DNA immunization is an effective means of eliciting protective immune responses to a variety of pathogens, such as viruses and parasites. The protection appears to be highly influenced by the capacity of the DNA vaccination to modulate Th1/Th2 responses.²⁶ Efficient uptake of plasmid vectors by myofibres without a carrier molecule is most likely the result of intrinsic characteristics of striated muscles. Proteins expressed by DNA vaccines can

induce both local and systemic immune responses.^{27–29} Several factors, including the individual properties of the antigen and chemical adjuvants, as well as the route of DNA administration, can influence the types of immune responses following DNA immunization; these properties could also be manipulated to enhance the immunogenicity of plasmid DNA vaccines in animal models.³⁰ In the present study, we found that directly linking the anti-CD3sFv to AIMP1 with a Th1-polarizing effect could efficiently shift immune responses toward a Th1 profile in antigen-sensitized mice.

Moreover, the pAnti-CD3sFv/AIMP1 DNA induced a stronger OVA-specific Th1 response than a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA, although both experimental groups exhibited a Th1-like response. One explanation for this effect is that the anti-CD3sFv/AIMP1 fusion protein may have a longer *in vivo* half-life than the AIMP1 protein, prolonging the stimulatory effect of AIMP1 and inducing a more potent immune response. Gillies *et al.* reported that fusing IL-2 or granulocyte-macrophage colony-stimulating factor to antibodies prolonged the half-life of the cytokines in serum.³¹ It is also possible that the linkage between anti-CD3sFv and AIMP1 localized the Th1-polarizing effect of AIMP1 to sites within immune organs where CD3⁺ T cells were being activated. In a previous study, an anti-CD3 mAb combined with IL-18 induced IFN- γ production by Th1 cells, whereas neither anti-CD3 mAb nor IL-18 induced IFN- γ production.³² Activation of APC is likely to be a mechanism of AIMP1-mediated up-regulation of Th1 responses, because we previously demonstrated that AIMP1 enhanced Th1 responses by increasing IL-12 production in activated APCs, such as macrophages and dendritic cells.^{13,14} However, as the AIMP1 was covalently linked to the anti-CD3sFv in the anti-CD3sFv/AIMP1 fusion construct, an involvement of fusion DNA-mediated APC activation on Th1 response might be indirect.

In addition, injection with pAntiCD3sFv/AIMP1 may have induced or increased other T-cell type-mediated immune responses in antigen-sensitized mice, including cytotoxic T-cell type 1 (Tc1) responses. Th1 cytokines can generate potent CD4⁺ and CD8⁺ anti-tumour effector cells by polarizing activated T cells towards Th1 and Tc1 phenotypes.³³ Although the present study focused on the Th cell-polarizing effect of anti-CD3sFv/AIMP1 fusion DNA in OVA/alum-injected BALB/c mice, we are currently investigating whether injection with this fusion DNA construct induces or enhances a cytotoxic T-lymphocyte response in tumour-bearing mice and OVA/CFA-injected C57BL/6 mice. In another study, direct injection of vectors encoding therapeutic proteins (such as cytokines) into tumour cells, or injection of cells that secrete specific cytokines, resulted in tumour regression and acquisition of systemic anti-tumour immunity.³⁴

These results emphasize that the presence of immune-modulating molecules in the microenvironment of antigen-specific immune responses provides advantages in T-cell immunobiology and cancer therapy.

Importantly, the mice injected with DNA adjuvants exhibited no adverse effects. They showed no evidence of autoimmune or other diseases upon extensive microscopic examination of multiple organs and tissues, and upon analysis of the serum for anti-nuclear antibody. We noted no lymphoid hyperplasia or tissue necrosis in livers and spleens of mice that were injected with the fusion DNA adjuvant (data not shown).

The pAnti-CD3sFv/AIMP1 fusion DNA was therefore more efficient than a mixture of pAnti-CD3sFv DNA and pAIMP1 DNA in inducing OVA-specific Th1-mediated immune responses and in inhibiting OVA-specific IL-4 production. These studies indicate that directly linking AIMP1 to anti-CD3sFv can confine the Th1-polarizing effect of AIMP1 to CD3⁺ T cells in antigen-sensitized mice. The pAnti-CD3sFv/AIMP1 plasmid may therefore be beneficial not only in cancer immunotherapy, but also in treating certain parasitic infections and allergic diseases in which down-regulation of an aberrant Th2 immune response inhibits the immunopathological state.³⁵

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