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Enhancement of *In Vivo* and *In Vitro* Immune Functions by a Conformationally-Biased, Response-Selective Agonist of Human C5a: Implications for a Novel Adjuvant in Vaccine Design

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Abstract

A conformationally-biased, agonist of human $C5a_{65–74}$ (EP67) was assessed for its adjuvant activities *in vitro* and *in vivo*. EP67 induced the release of the inflammatory (Th1) type cytokines from C5a receptor (CD88)-bearing antigen presenting cells (APC). Serum from mice immunized with EP67-ovalbumin (OVA) contained high OVA-specific antibody (Ab) titers [IgG1, IgG2a (IGg2c), IgG2b]. Mice receiving OVA alone produced only IgG1 Abs, indicating the ability of EP67 to induce a Th1-like antibody (A)b class switch. Spleen cell cultures from wild type mice but not $CD88^{-/-}$ mice showed an enhanced OVA-specific proliferative response *in vitro*. These results indicate the ability of EP67 to drive a Th1-mediated immune response and its potential use as a unique adjuvant

1. Introduction

A fundamental objective sought in the design of a vaccine is the invocation of a robust and sustained immune response. Important factors to be considered in vaccine designs include, vaccine formulation, route of administration, ease of production, harmful side effects, and the use of an adjuvant capable of augmenting the desired immune outcome. Different adjuvants can bias the observed immune response to the pathogen of interest and the capacity of the adjuvant to bias an immune response either towards a humoral or a cell-mediated phenotype has been shown to be important in controlling certain diseases [1–9]. For human vaccines, the only licensed adjuvant is aluminum hydroxide (alum), which induces a strong humoral immune response but only a weak T cell-mediated immune response [1–4]. The design of new vaccines to problematic pathogens may require a bias towards a Th1 type (humoral and cell mediated) immune response to be effective. Thus, an important design feature for the next generation of vaccines will be the incorporation of an adjuvant that can safely invoke T cell-mediated responses that are difficult to achieve with alum [5–9].

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Toward this end, we have generated conformationally restricted analogues of the biologically active C-terminal region of the human complement-derived component C5a ($C5a_{65–74}$, ISHKDMQLGR). Backbone flexibility in $C5a_{65–74}$ was restricted by specific residue substitutions with the objective of biasing or "locking in" certain topochemical features that would allow a distinction between C5a-like immune stimulatory activities versus C5a-like inflammatory activities. One such analogue, YSFKPMPLaR or EP54, retained 3–5% of full C5a activity in the induction of spasmogen release from tissue-resident macrophages, but only 0.1% of C5a activity in the induction of proteolytic enzyme release from PMNs (15–16) - the first C5a agonist to exhibit this differential C5a activity. Detailed NMR structural analysis of EP54 revealed unique conformational features that were shown to be well accommodated by C5a receptors (C5aR) expressed on APCs (macrophages), but not by C5aRs expressed on PMNs [16]. Of particular note, was a region of extended backbone conformation to the N-terminal side of the Leu residue, which appeared to play an important role in differentiating PMN and APC activities. Thus, EP67 was designed to enhance this extended backbone conformation with a methyl group on the nitrogen atom that forms the amide bond between the Pro and Leu residues of EP54. The presence of this methyl group forces extended backbone conformation to its N-terminal side in a manner similar to a Pro substitution (17), but without the loss of the biological contribution made by the Leu residue side chain. The resulting EP67 analogue (YSFKDMP(MeL)aR), unlike EP54, is devoid of PMN activity and binding of C5aRs on PMNs, yet retains C5a-like engagement of C5aR-bearing APCs [17].

EP54 has been used as a "built in" adjuvant in simple peptide-based vaccines in which a well-characterized B-cell or T-cell epitope is covalently attached to the N-terminus of EP54. Such EP54-containing vaccines have been shown to induce robust antigen (Ag)-specific humoral (30) and cell-mediated [xx] immune responses, which have the characteristics of Th1-driven immune outcomes. The EP54 moiety of the vaccine targets the epitope to and simultaneously activates C5aR-bearing cells (30). While EP67 has received only limited attention as a possible adjuvant, it does appear to have the ability to engage C5aR-bearing APCs in a manner similar to EP54 to induce Ag-specific cell mediated immune responses [18]. However, since EP67 is devoid of inflammatory properties via engagement of PMNs, it represents a more attractive adjuvant for human use for vaccine designs aimed at inducing Th1 immune outcomes.

Consequently, the objective of this study was to evaluate and characterize the adjuvant activities of EP67 particularly within the context of its ability to drive a Th1-mediated immune response. The results are discussed against the backdrop of the need for improved vaccination approaches for the treatment of bacterial, fungal, and viral infections, particularly the need to invoke robust antibody (Ab) and T cell-mediated immune responses.

2. Materials and Methods

2.1 Reagents

The reagents were purchased from the sources indicated. RPMI 1640 (Mediatech Cellgro, Manassas, VA), fetal bovine serum (Gibco/Invitrogen, Carlsbad CA), newborn calf serum (Gibco), biotin-donkey anti-mouse IgG (Biosource, CA), AP-goat anti-mouse IgG1, anti-mouse IgG2a and IgG2b (Bethyl Laboratories), anti-mouse IgG2c (Beckman Coulter, Fullerton, CA), ExtrAvidin-AP (Sigma, St. Louis, MO), Sigma Fast p-nitrophenly phosphate Tablet sets (Sigma), unconjugated and biotin-conjugated anti-mouse IL-6, TNFα, and INFγ (BD Biosciences Pharmingen, San Diego CA), recombinant mouse IL-6, TNFα, and INFγ (eBioSciences, San Diego CA). Ovalbumin (OVA) was obtained from Sigma. The Bio-Plex Cytokine Assay reagents for IL-6 and IL-2 were purchased from Bio-Rad Laboratories (Richmond, CA).

2.2 EP67-OVA Conjugate Preparation

Peptides—EP67 [YSFKDMP(MeL)aR] and scrambled EP67 [(MeL)RMYKPaFDS] were synthesized by standard Fmoc (9-fluoreny-lmethoxy-carbonyl) solid-phase methods on a pre-loaded Arg Wang resin by sequential coupling of the HBTU (2-(1H-benzotriazole-1-yl-1,1,3,3-tetramethyluronium hexafluoro-phosphate) esters of each amino acid. Photoactive BB-EP67 was generated by coupling the HBTU ester of benzyolbenzoic acid (BB) to the N-terminal amino group of EP67 while attached to the resin. The course of this coupling reaction was monitored by the loss of the N-terminal amino group on EP67 via the ninhydrin reaction. EP67 and BB-EP67 were cleaved from the resin via standard acidolysis with a TFA (trifluoroacetic acid) cocktail containing phenol (5%), water (2%), and triisopropylsilane (2%) as scavangers. Peptides were purified by analytical and preparative reverse-phase HPLC on C18-bonded silica columns with 0.1% TFA as the running buffer (solvent A) and 60% acetonitrile in 0.1% TFA (solvent B) as the eluant. All peptides were characterized by molecular mass (MH^+) with MALDI mass spectrometry.

Photoconjugation—Conjugation of BB-EP67 to OVA was accomplished by stirring a solution of BB-EP67 and OVA (10:1 molar ratio) in PBS pH 7.0 in the presence of short wave UV light (ca. 300 nm) for 2 – 2.5 hours. The solution was dialyzed to remove unconjugated BB-EP67 and the final protein concentration was determined using standard Bronsted-Lowrey methods. The EP67-to-OVA ratio ranged from 5–7:1 as determined by amino acid compositional analysis. Amino acid analysis was performed by the Protein Structure Core Facility at the University of Nebraska Medical Center using a Hitachi L-8800A amino acid analyzer. Conjugate samples were hydrolyzed for 20 hours at 110° C in 6N HCl vapor under an argon atmosphere with 1% phenol and 0.5 % sodium sulfite in the hydrolysis mix. After hydrolysis, the samples were dissolved in 200 ml of 0.02N HCl and 50 ml was injected automatically onto the amino acid analyzer. Norleucine was added to a measured amount of sample as an internal standard. Chromatographic runs were monitored at 570 and 440 nm. In data analysis, a correction was made to the amount of the internal standard, thus minimizing dilution errors.

2.3. Animals

Female C57Bl/6 mice and Balb/C mice were obtained from Harlan Laboratories (Los Angeles, CA). Mice were utilized at 8–12 weeks of age. Breeding pairs of C5aR deficient ($CD88^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor, ME). C5L2 deficient mice were kindly provided by Dr. Lu Bio, Harvard University. The $CD88^{-/-}$ and $C5L2^{-/-}$ mice ($C5L2^{-/-}$) were bred and maintained at the Sidney Kimmel Cancer Center.

2.4. Preparation of Splenic Cells

Spleens were removed aseptically in complete media (CM) consisting of RPMI 1640, 10% FBS, 2mM L-glutamine, pen-strep, and 5×10^{-5} M 2-mercaptoethanol. Single cell preparations were prepared as previously described (19,20). Cells were maintained on ice in CM prior to use.

Cell Culture Procedures—For the generation of cytokines, spleen cells were cultured in CM at a concentration of 2.5×10^6/ml in 12 well or 96 well plates (Costar Corning, Cambridge, MA) in a humidified atmosphere containing 5% CO_2 for 24–48 hrs. Supernatants were collected and frozen at –20°C prior to analysis by ELISA (21).

Methods of Immunization—For the generation of Ab-specific responses and antigen Ag-specific spleen cell proliferation, mice were immunized IP on day 0 boosted on days 7, 14 and

21 with either 100 μg (delivered in 0.1 ml) of EP67-OVA construct or OVA alone. Blood and spleen cells were collected on day 28.

2.5. Measurement of Cytokines

A sandwich ELISA was performed according to established protocols (21). Samples were run in triplicate and the data presented as pg/ml ± SD. Each experiment was performed at least three times. The Bio-Plex Cytokine Assay was performed according to manufacturer's instructions. Triplicate samples were utilized.

2.6. Measurement of Cell Proliferation

Spleen and cells were cultured as described above. During the last 18–24 hr of culture, cells were pulsed with 1 μCi of [^{3}H]-TdR (ICN Biomedicals, Aurora, OH) and thymidine incorporation was subsequently measured on a beta plate scintillation counter (22). Results are reported as CPM ± SD from triplicate cultures. Each experiment was performed at least three times.

2.7. Measurement of Anti-OVA Ab Responses

A direct ELISA was utilized to measure OVA specific antibodies derived from mouse serum. 100 μl of a 10 μg/ml OVA solution in conjugation buffer (0.1M $NaCO_3$, pH 9) was added to high binding 96 well plates (Immulon II, Shantally, VA) and allowed to bind for 24 hrs at 4° C. The plates were washed with PBS (pH 7) containing 0.1% Tween 20. The plates were subsequently blocked for 90 min at room temperature (RT) with 200 μl of blocking buffer (PBS containing 10% new born calf serum). The plates were then washed three times with PBS-Tween. Samples, at various dilutions, and incubated for 24 hr at 4°C. Plates were washed three times with PBS-Tween and 100 μl of an optimal concentration of biotin labeled donkey anti-mouse IgG was added and allowed to incubate at RT for 60 min. The plates were then washed three times with PBS-Tween. StrepAv-AP was added and the plates incubated for 30 min. The plates were then washed three times with PBS-Tween. The presence of AP was assessed by the addition of substrate. Plates were allowed to develop and read on a BioRad Microplate Reader utilizing dual wave lengths OD 490 and 405. The results are recorded as ng or pg/ml ± SD. Each experiment was performed at least three times.

3. Results

3.1 Cytokine Production

Numerous laboratories have previously shown that intact human C5a is capable of inducing a variety of cytokines derived from C5aR-bearing cells (e.g. macrophages/monocytes) (23–25). To assess the ability of EP67 to induce cytokine production, whole spleen cell cultures were stimulated with various concentrations of EP67 and scrambled EP67 (negative control) for 48 hours, culture supernatants collected, and assessed for IL-6 by indirect ELISA. The results presented in Figure 1 (Panel A) indicate that EP67 induced the synthesis/release of IL-6, in a dose dependent fashion, with the optimal concentration of EP67 being approximately 50 μg/ml. The EP67-scrambled sequence was unable to induce cytokine synthesis/release. In addition, spleen cell cultures were stimulated with an optimal amount of EP67 (50 μg/ml), EP67-scrambled (50 μg/ml), or LPS (10 μg/ml) for 48 hrs, the culture supernatants collected, and assessed for cytokine production (IL-6, TNF-α, and INFγ) by ELISA. The results presented in Figure 1 (Panel B) indicate that EP67 was able to induce the production/release of IL-6, TNF-α, and IFNγ. In contrast, scrambled EP67 did not induce a cytokine response beyond that observed in un-stimulated control cultures. The positive control, LPS, induced the synthesis of all three cytokines. Taken together these results indicate that EP67 was a potent inducer of pro-inflammatory cytokines from murine spleen cells. The fact that EP67-scrambled was

unable to induce cytokine synthesis lends credence to the hypothesis that the biologic activity of EP67 is sequence specific.

Our working hypothesis is that the adjuvant activities of EP67 occurs via activation of C5aR-bearing APCs, as indicated by the cytokine profile it induces from these cells. To assess whether EP67 could also induce T cells to produce cytokines, spleen cell cultures were stimulated with EP67 or scrambled EP67, supernatants collected at 48 hrs, and assayed for the presence of IL-2, IL-4, and IL-5 utilizing a bead-based fluorescence assay. The results shown in Table 1 indicate that, under the culture conditions employed, EP67 was unable to induce the synthesis of these Th2 type cytokines. In contrast, the supernatants contained measurable amounts of IL-6 and TNFα. Supernatants derived from spleen cells stimulated with scrambled EP67 contained cytokine concentrations similar to those observed in un-stimulated culture supernatants. These results indicate that, under the *in vitro* conditions employed, EP67 induces a cytokine profile indicative of APC activation but not a direct T cell activation.

The cloning of CD88 (27) and the alternate C5aR (C5L2) (28) and subsequent generation of knockout mice has provided the opportunity to evaluate the interaction of EP67 with these C5aRs for biologic activity. Spleen cells derived from $CD88^{-/-}$ and $C5L2^{-/-}$ knockout mice were stimulated *in vitro* with EP67, the culture supernatants collected after 48 hrs, and assessed for the presence of IL-6. The results presented in Figure 2 indicate that EP67 was capable of inducing cytokine synthesis in cultures containing cells from $C5L2^{-/-}$ mice but not $CD88^{-/-}$ mice.

3.2 EP67-induced Enhancement of OVA-Specific Ab Responses

To be an effective candidate as an adjuvant for vaccine development, the candidate must enhance Ag-specific responses *in vivo*. To assess the effects of EP67 on B-cell mediated immunity, a vaccine was generated in which EP67 was photo-conjugatged to the surface of OVA as described in Materials and Methods. Blood samples were collected on D=28 and assayed for the presence of OVA specifc IgG by direct ELISA. The results shown in Table 2 represent pooled serum from three mice immunized with OVA-EP67 and two mice immunized with OVA alone. As can be seen, serum from animals immunized with the OVA-EP67 construct had a higher titer of anti-OVA Ab compared to animals receiving OVA alone. Dilutions of serum derived from OVA-EP67 mice (1:125,000 and 1:625,000) contained a high titer anti-OVA response. In contrast to these results, mice immunized with OVA started to decline at a dilution of 1:2,500. These results indicate that the conjugation of EP67 to an intact protein resulted in an enhanced Ab response compared to mice immunized with OVA alone.

To determine whether mice immunized with OVA-EP67 expressed an anti-OVA isotype profile similar to immunization with OVA alone, the serum samples utilized in Table 2 were individually analyzed for IgG1 and IgG2b anti-OVA specific Ab. The results shown in Figure 3 indicated that serum from mice immunized with OVA-EP67 contained a high titer of IgG1, and IgG2b compared to mice immunized with OVA alone. Two of the three mice contained high titers of IgG2b anti-OVA. The IgG2b titers obtained from mice immunized with OVA alone were close to background levels. It should be noted that the sera from OVA-EP67 immunized mice were analyzed at a dilution of 1:10,000 whereas sera from mice immunized with OVA alone were assayed at a dilution of 1:1,000. These dilutions were chosen because of the disparity of Ab titers between mice immunized with OVA-EP67 and OVA. In addition, a large amount of IgG1 was also produced in OVA-EP67 immunized mice. It is noteworthy that the anti-OVA response from mice receiving OVA alone was almost entirely IgG1. Taken together, these results indicate that the conjugation of EP67 to OVA, in the absence of any additional adjuvants, is capable of enhancing an Ag-specific humoral immune response. Moreover, immunization with OVA-EP67 is capable of shifting the Ab isotype profile compared to immunization with Ag alone. Based on the observation that mice immunized with

EP67 produced a higher titer of IgG2b, we compared the IgG2b and IgG2c (IgG2a) ratios from mice immunized with OVA compared to mice receiving OVA-EP67. IgG2c was chosen because it has been demonstrated that certain inbred mouse strains, including C57BL/6, with the Igh-1b allele do not have the gene for IgG2a and instead express the IgG2c isotype 1. Thus commercial anti-IgG2a Abs may cross react inadequately with IgG2c in ELISA (28–29). The results presented in Figure 4 indicate that mice immunized with OVA-EP67 produced a greater amount of IgG2b and IgG2c compared to OVA-immunized mice as indicated by the Ab ratio (OVA:OVA-EP67). Results were obtained from 6 OVA immunized mice and 5 OVA-EP67 immunized mice. Combined, the results presented in Figure 3 and Figure 4 demonstrated that mice immunized with OVA-EP67 resulted in higher IgG2b and IgG2c titers, which is indicative of a Th1 Ab profile (2). Immunization of mice with OVA + EP67 or OVA attached to the COOH terminus of EP67 did not enhance Ag-specific humoral immune responses in vivo (data not shown). These data are consistent with our previously reported results showing that the adjuvant peptide must be conjugated to the antigen of interest, via its amino terminus, in order to observe enhancement of humoral immune responses in vivo (30).

3.3 6EP67-Induced Enhancement of OVA Specific Cell Proliferation

Based on the earlier results showing that EP67 enhances OVA specific Ab responses, we assessed whether the *in vivo* administration of OVA-EP67 enhanced an Ag-specific proliferative response *in vitro*. Spleen cell cultures were prepared from the mice utilized in the OVA-specific Ab responses described above. Cultures were pulsed with various concentrations of OVA and cell proliferation measured on Day 4–5 of culture by [^{3}H-TdR] incorporation. The results presented in Figure 5 indicate that mice immunized with OVA alone or OVA-EP67 induced a dose dependent proliferative response to OVA. Mice receiving the OVA-EP67 construct produced an enhanced proliferative response compared to mice immunized with OVA alone.

Our observations indicate that EP67-induced cytokine production requires the presence CD88 on APCs. To confirm that the observed enhancement of cell proliferation required cells expressing CD88, similar proliferation experiments were performed comparing the responses of wild type, CD88$^{-/-}$, and C5L2$^{-/-}$ mice. When mice were immunized with OVA-EP67, an enhanced proliferative response was observed in the wild type and C5L2$^{-/-}$, but not the CD88$^{-/-}$ mice (Fig. 6). In contrast, all three groups of mice immunized with OVA alone showed similar proliferative responses (Fig. 6). These results indicate that CD88 is required to achieve the adjuvant activity associated with EP67.

4. Discussion

Ideally, an effective adjuvant should be able to increase Ag presentation and induce or support both T and B cell activation. Historically, the most potent adjuvants also induce undesirable inflammatory effects in the host animal and are therefore unsuitable for veterinary or human use. The major goal in adjuvant design is to promote its immuno-stimulatory properties while controlling the unwanted side effects. The objective of this study was to evaluate the adjuvant properties of EP67, both *in vitro* and *in vivo*. The results indicate that EP67 has potent adjuvant properties when conjugated to a protein Ag.

Numerous studies indicate that the Ag and/or Ag plus adjuvant can bias the type of observed immune response. The ability to bias an immune response has been shown to increase the efficacy of a vaccine (1–8). For example, vaccines capable of enhancing a Th1 type response have been shown to be more effective controlling both viral and fungal infections (1). Alum, which has been used for over 50 years as an adjuvant in vaccines in both human and veterinary medicine (1,2,4,5,8), produces a long lasting protective immunity, but drives mainly a Th2-type of immune response. (1,3). This type of a response is very effective in generating high

titer Ab response, but induces only limited T cell-mediated immune function. In contrast, a variety of adjuvants (bacterial products and oligonucleotides) have been shown to preferentially bias the immune response towards a Th1-like phenotype (high INF-γ, TNF-α, low IL-2, IL-4, IL-5, low IgG1:IgG2a/IgG2b ratio) (2,5–8).

The results presented in this report demonstrate that, under the culture conditions employed, EP67: 1) enhances the production of cytokines from spleen cells; 2) enhances Ag-specific humoral immunity with both Th1 and Th2 type response; and 3) enhances Ag-specific T cell proliferative responses. These findings suggest that EP67 may be an effective molecular adjuvant useful for vaccine development where both a Th1/Th2 response is warranted.

Previously, we showed that mice immunized with a vaccine made by the conjugation of a human Muc1 epitope to the N-terminus of EP54 generated high titers of IgG2a and IgG2b Abs with an absence of IgG1 (30). In contrast, mice immunized with a conventional vaccine in which the Muc1 epitope was conjugated to KLH generated almost exclusively IgG1, indicative of a Th1-driven Ab class switch in the EP54-containing vaccine. The results presented in this study are consistent with this observation using intact OVA-EP67 constructs. These results indicate that the OVA-EP67 construct is capable of biasing the immune response towards a Th1 like response. This observation is important in the context of vaccine development where protection to a particular pathogen requires the activation of a Th1 type immune response. In addition to enhancing primary Ag-specific Ab reponses, EP67 was able to augment a secondary Ag-specific T cell proliferative response *in vitro*. These results suggest that EP67 is capable of further activating primed APCs for the presentation of Ag to T cells.

C5a possesses a broad spectrum of biologic activities. The cloning and subsequent generation of $CD88^{-/-}$ mice has defined one receptor, CD88 (26). APCs (monocytes, macrophages, and DCs) are known to express CD88 on their cell surfaces (27). More recently, a second C5a-like receptor (C5L2/gpr77), which shares approximately 35% sequence homology with CD88 has been cloned. (27,28). The biologic activity of C5L2 alone or in combination with CD88 remains controversial (31–33). Originally defined as a decoy receptor, recent studies indicate that ligand binding to the C5L2 may result in a variety of biological responses (31–33). The results presented in this report indicate that the alternate C5aR, C5L2, does not play a role in the adjuvant induced effects associated with EP67.

Our hypothesis is that EP67 focuses the Ag on APCs, via binding to CD88, resulting in improved Ag presentation and in addition induces these cells to provide accessory stimulatory signals that further enhance immune function. This notion is underscored with published results showing that APCs (DCs) are able to bind and internalize the molecular adjuvant within 5–30 min (34). Further evidence to support our hypothesis that the adjuvant activity associated with EP67 is a result of receptor-ligand interaction resulted from studies performed with C5aR ($CD88^{-/-}$ and $C5L2^{-/-}$) knockout mice. Spleen cells derived from $C5L2^{-/-}$ were responsive to EP67. In contrast, $CD88^{-/-}$ mice did not respond to EP67 above background controls. Combined, these results are consistent with EP67 functioning via the interaction with the CD88 receptor.

The results presented in this report indicate that EP67 may represent a new class of adjuvants for vaccine development. Simple yet effective vaccines in which a peptide epitope is attached to the N-terminus of EP67 are readily generated by solid phase peptide synthesis. Such EP67-containing, peptide-based vaccines induce robust Ag-specific humoral and cell-mediated immune responses to the epitope with cross-reactivity to the protein and cell targets that express the epitope (35). Importantly, the results of this study also indicate that EP67 can induce similar immune outcomes when an intact protein is used at the target Ag, an observation that

significantly increases the repertoire of Ags that can now be used in EP67-containing vaccine designs.

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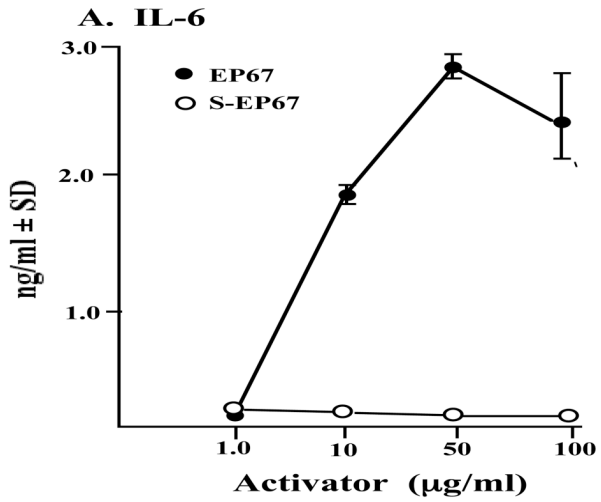

Panel B

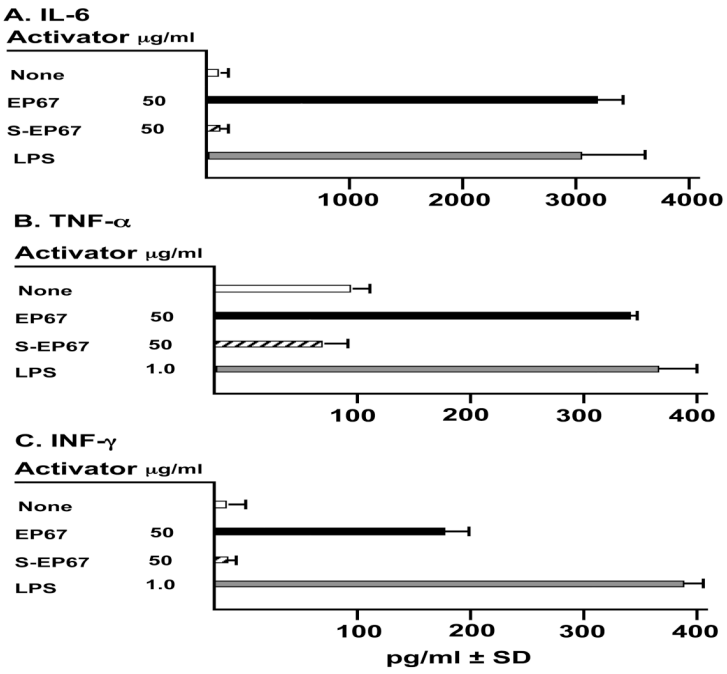

Figure 1.

Panel A: 5×10^5 spleen cells were stimulated with various concentrations of EP67 or scrambled EP67 for 24–48 hrs, the supernatants collected and assayed for the presence of cytokines by sandwich ELISA. Results are reported from triplicate cultures ± SD. Each experiment was performed at least three times. Panel B: 5×10^5 spleen cells were stimulated with 50 µg/ml of EP67 or scrambled EP67, or 1.0 µg/ml LPS for 24–48 hrs, the supernatants collected and assayed for the presence of cytokines by sandwich ELISA. Results are reported from triplicate cultures ± SD. Each experiment was performed at least three times.

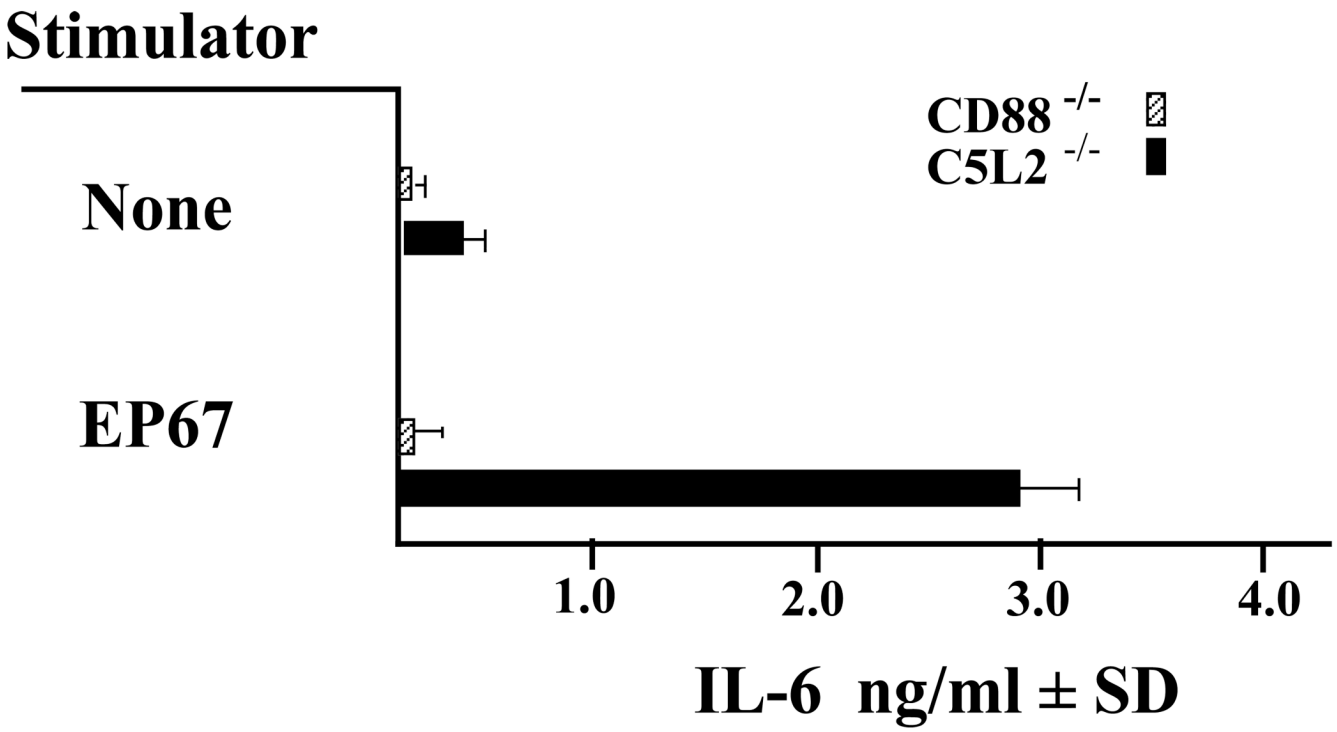

Figure 2.

5×10^5 spleen cells derived from wild type, $CD88^{-/-}$, or $C5L2^{-/-}$ mice were stimulated with 50 μg/ml of EP67 or scrambled EP67 for 24–48 hrs, the supernatants collected and assayed for the presence of cytokines by sandwich ELISA. Results are reported from triplicate cultures ± SD. Each experiment was performed at least three times.

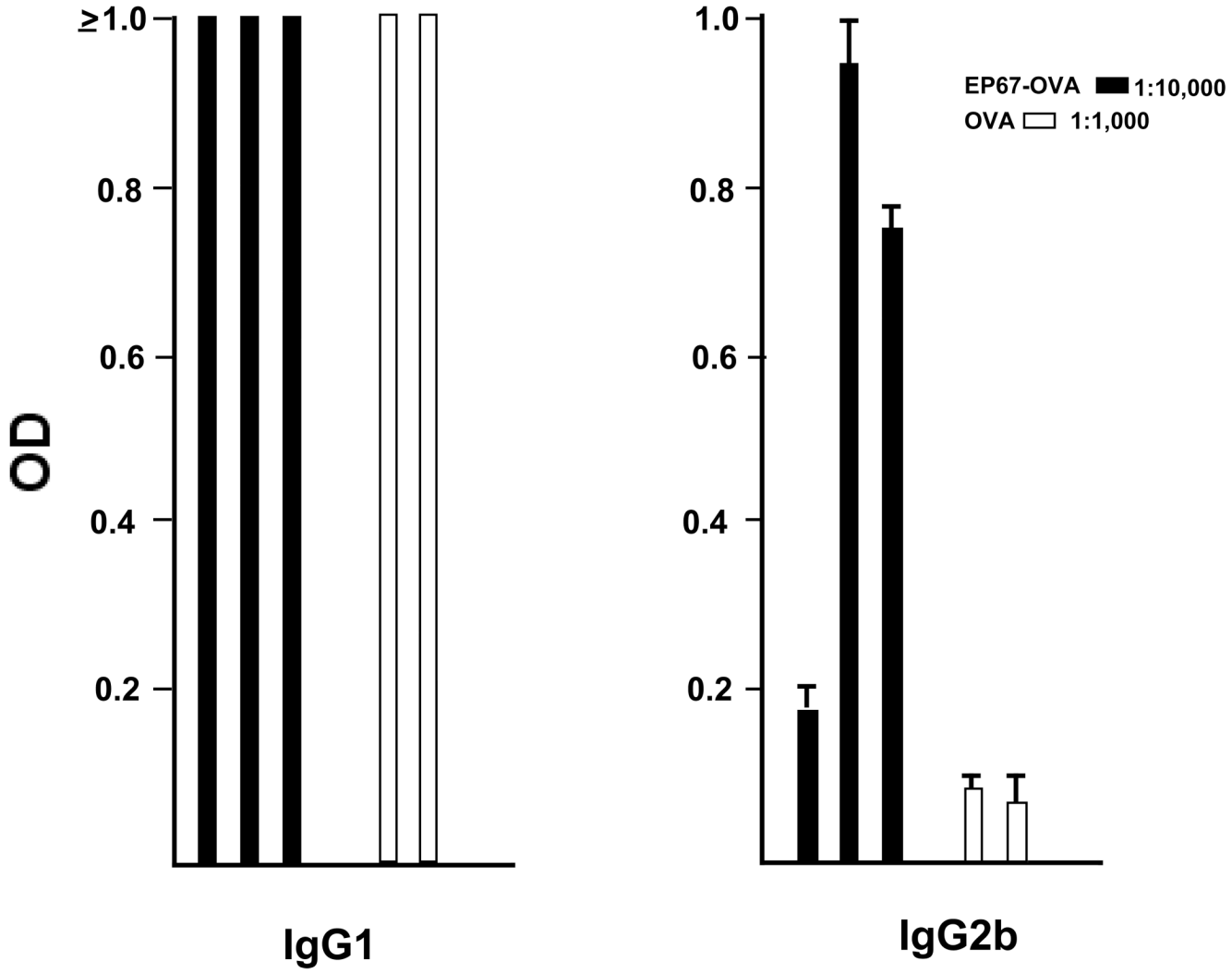

Figure 3.

Serum from mice immunized with OVA or EP67-OVA, as described in materials and methods, were assayed for the presence of anti-OVA Ab by a direct ELISA. Subclass analysis was preformed to determine the relative amounts (sera from OVA-EP67 immunized mice were analyzed at a dilution of 1:10,000 whereas sera from mice immunized with OVA alone were assayed at a dilution of 1:1,000 of IgG1- and IgG2b-anti-OVA). Results are reported from triplicate cultures ± SD. Each experiment was performed at least three times.

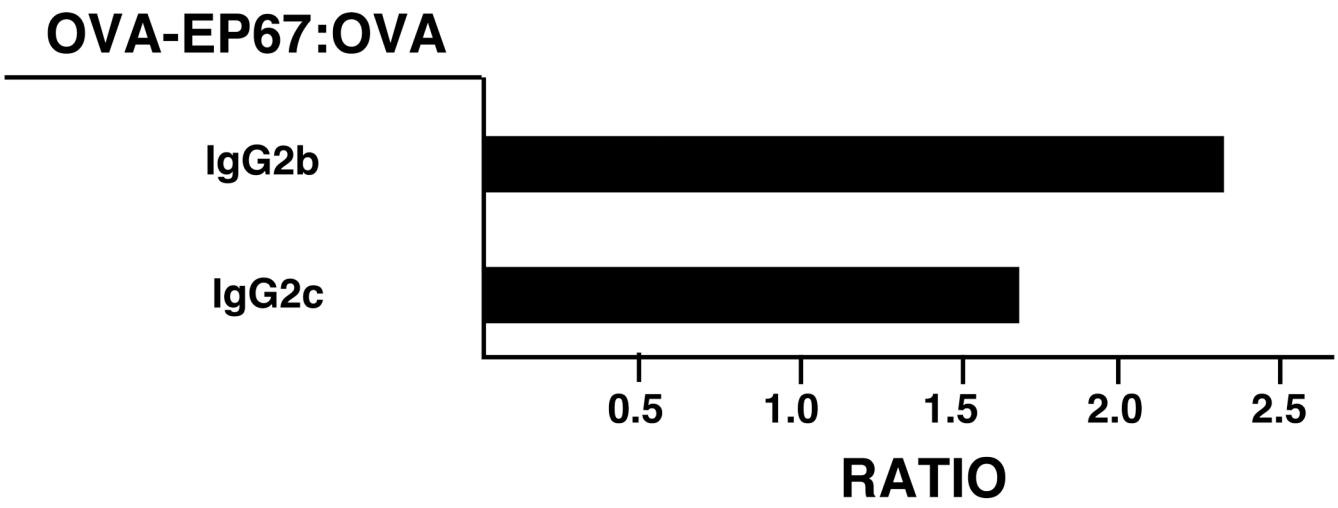

Figure 4.
Serum from mice immunized with OVA (n=6) or EP67-OVA (n=5), as described in materials and methods, were assayed for the presence of anti-OVA Ab by a direct ELISA. Subclass analyses of IgG2b and IgG2c, were preformed to determine the relative ratios of these subclasses (EP67-OVA:OVA).

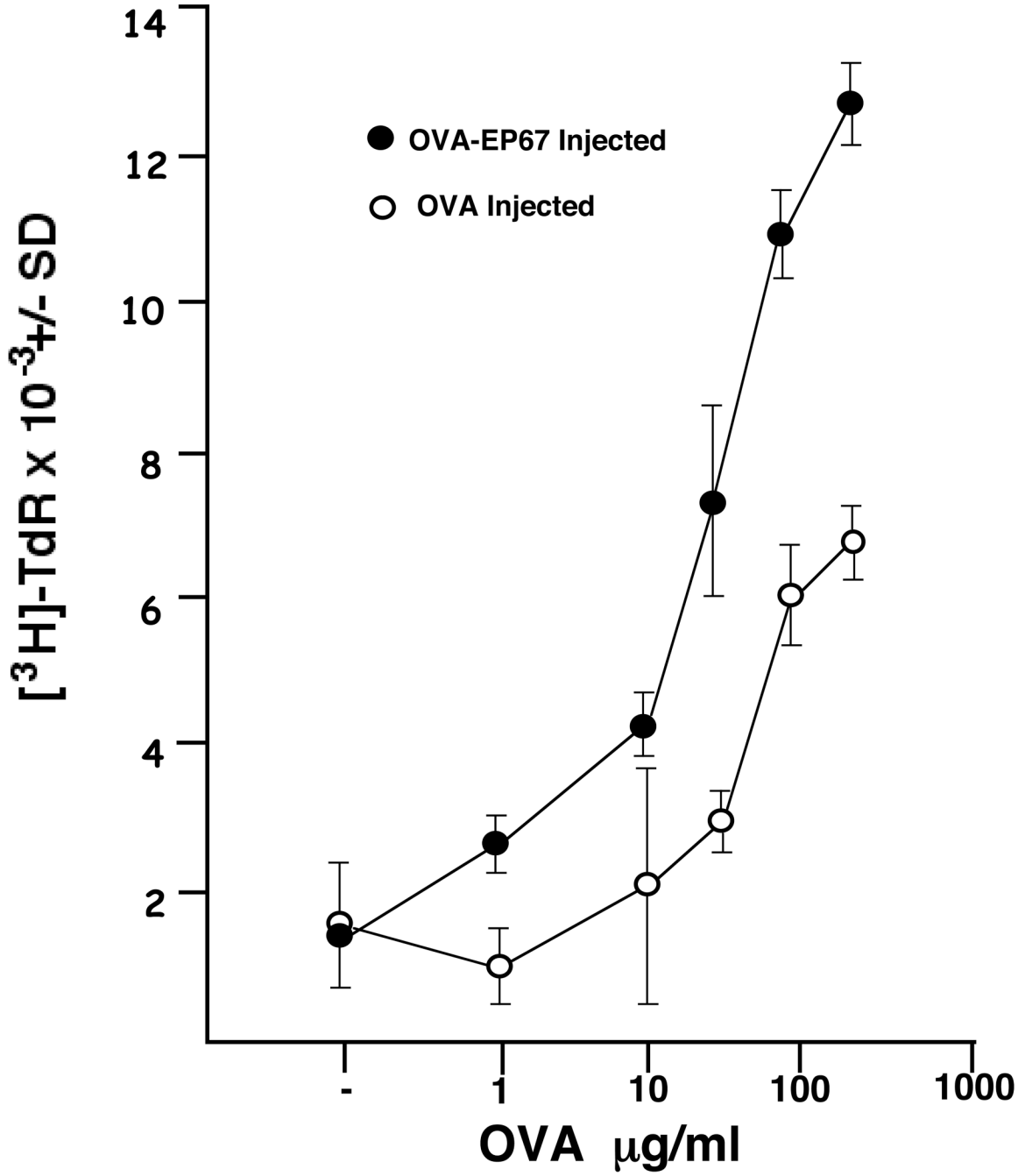

Figure 5.

Mice immunized with 100 μg/ml EP67 or scrambled-EP67, as described in materials and methods. Spleen cell cultures stimulated with various concentrations of OVA. Cultures were pulsed with 1.0 μCi [^{3}H]-TdR for the last 18–24 hrs of culture. Cells were harvested and processed for [^{3}H] incorporation by liquid scintillation analysis. Results are reported from triplicate cultures ± SD. Each experiment was performed at least three times.

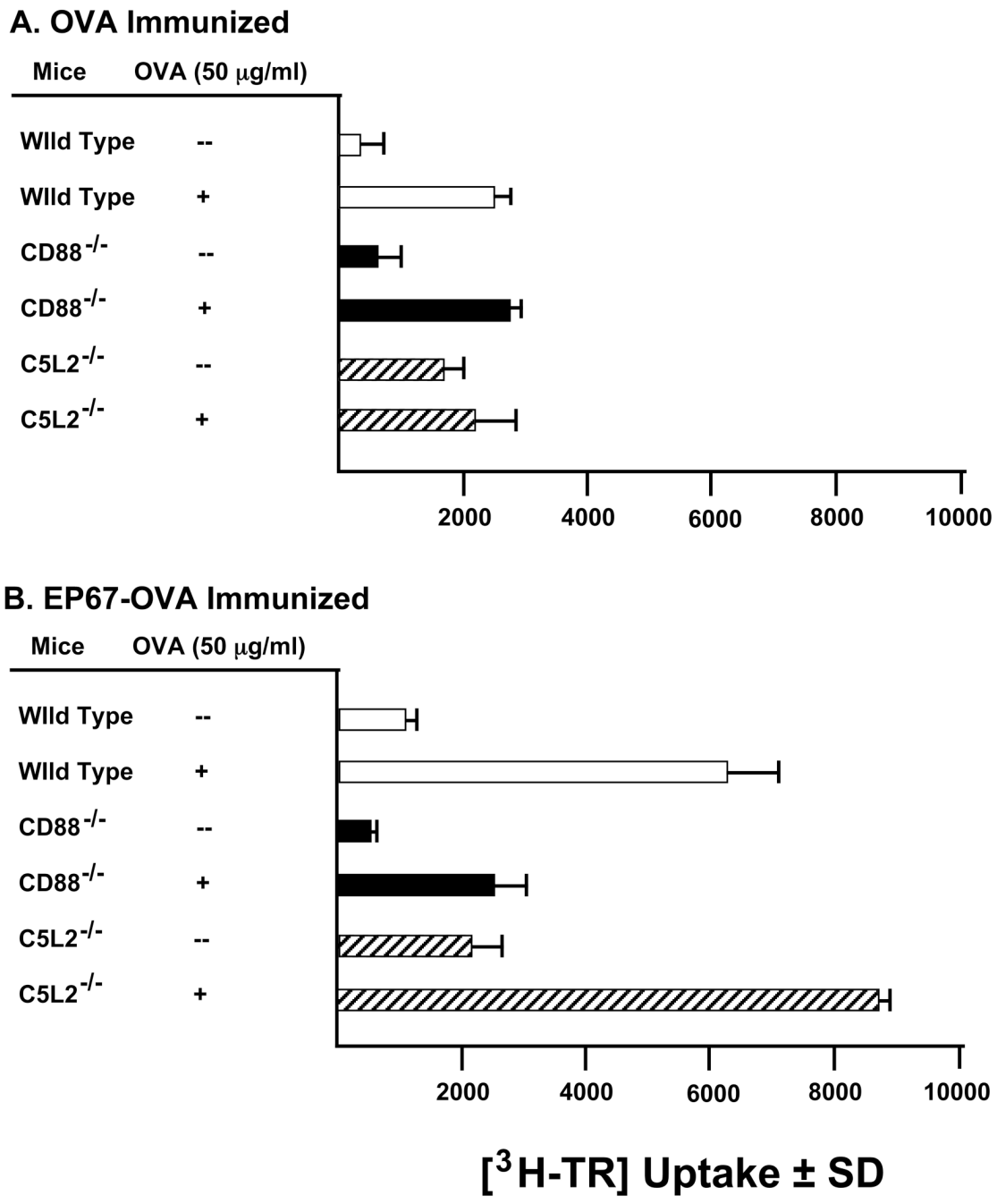

Figure 6.

Wild type, CD88$^{-/-}$, and C5L2$^{-/-}$ mice immunized with 100 μg/ml EP67 or scrambled-EP67, as described in materials and methods. Spleen cell cultures were stimulated with 50 μg/ml OVA. Cultures were pulsed with 1.0 μCi [^{3}H]-TdR for the last 18–24 hrs of culture. Cells were harvested and processed for [^{3}H] incorporation by liquid scintillation analysis. Results are reported from triplicate cultures ± SD. Each experiment was performed at least three times.

Table I

Induction of Cytokines By EP67

	pg/ml ± SD				
STIMULATOR	**IL-2**	**IL-4**	**IL-5**	**IL-6**	**TNFα**
EP67	BLT*	BLT*	BLT*	228 ± 15	16 ± 0.4
S-EP67	BLT*	BLT*	BLT*	BLT*	BLT*

Spleen cell cultures were stimulated with either 50μg/mlEP67 or S-EP67. The culture supernatants were collected at 48 hr and assayed for the presence of cytokines by a bead based fluorescence assay. Backgrounds were subtracted.

*Below the limit of detection.

Table II

OVA-EP67 vs. OVA for the Induction of an Antigen-specific Antibody Response

Immunizing Antigen	Serum Dilution (I/D)OD
OVA	5001.0
OVA	2,5000.62
OVA	12,5000.32
OVA	62,5000.13
EP67-OVA	1,0001.0
EP67-OVA	5,0001.0
EP67-OVA	25,0001.0
EP67-OVA	125,0000.85
EP67-OVA	625,0000.42

Mice were immunized IP on day 0, boosted on days 7, 14 and 21, serum was collected on day 28. Antibody concentrations were measured in a direct ELISA. OD_{405} was used as a measurement of antibody concentration following the subtraction of background values.