Published in final edited form as:
Vaccine. 2007 March 1; 25(11): 2085–2094.

Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination

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Abstract

The development of safe, novel adjuvants is necessary to maximize the efficacy of new and/or available vaccines. Chitosan is a non-toxic, biocompatible, biodegradable, natural polysaccharide derived from the exoskeletons of crustaceans and insects. Chitosan's biodegradability, immunological activity and high viscosity make it an excellent candidate as a depot/adjuvant for parenteral vaccination. To this end, we explored chitosan solution as an adjuvant for subcutaneous vaccination of mice with a model protein antigen. We found that chitosan enhanced antigen-specific antibody titers over 5-fold and antigen-specific splenic $CD4^+$ proliferation over 6-fold. Strong increases in antibody titers together with robust delayed-type hypersensitivity (DTH) responses revealed that chitosan induced both humoral and cell-mediated immune responses. When compared with traditional vaccine adjuvants, chitosan was equipotent to incomplete Freund's adjuvant (IFA) and superior to aluminum hydroxide. Mechanistic studies revealed that chitosan exhibited at least two characteristics that may allow it to function as an immune adjuvant. First, the viscous chitosan solution created an antigen depot. More specifically, less than 9% of a protein antigen, when delivered in saline, remained at the injection site after 8 hours. However, more than 60% of a protein antigen delivered in chitosan remained at the injection site for 7 days. Second, chitosan induced a transient 67% cellular expansion in draining lymph nodes. The expansion peaked between 14 and 21 days after chitosan injection and diminished as the polysaccharide was degraded. These mechanistic studies, taken together with the enhancement of a vaccine response, demonstrate that chitosan is a promising and safe platform for parenteral vaccine delivery.

Keywords

adjuvant; depot; vaccination

1. Introduction

Since the discovery of the adjuvant activity of aluminum compounds 80 years ago [1], over 100 empirically-derived adjuvants and adjuvant variations have been tested both preclinically and clinically [2]. Nearly all of these adjuvants failed to win approval for use in routine vaccines due to toxicity concerns. Even aluminum hydroxide, the only adjuvant widely licensed for human use, has come under recent scrutiny due to concerns about aluminum-related macrophagic myofasciitis [3] and the potential for cumulative aluminum toxicity which has

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been associated with amyotrophic lateral sclerosis, Alzheimer's disease and dialysis-associated dementia [4].

According to Gupta and Siber, an "ideal" adjuvant would elicit a persistent, high quality immune response to an antigen while being non-toxic, biodegradable, non-immunogenic and chemically defined for reproducible manufacture [5]. Chitosan is an abundant, natural linear polysaccharide derived by the deacetylation of chitin from crustaceans, insects and fungi [6, 7]. Chitosan is non-toxic (LD_{50} > 16 g/kg [8]), biodegradable [9], non-immunogenic [6] and can be manufactured reproducibly in accordance with GMP guidelines. Chitosan is a widely used biomaterial with an established safety profile in humans. It is used as a pharmaceutical excipient [7], a controversial weight loss supplement [10,11], an experimental mucosal adjuvant [12-14] and in an FDA-approved hemostatic dressing [15]. When administered intranasally, chitosan is well-tolerated with only transient mild-to-moderate symptoms mostly of rhinorrhea [12-14]. When ingested, differences in nonserious adverse events, which included bloating, constipation and diarrhea, were not significant in participants given chitosan versus placebo [10].

Over 20 years ago, chitin derivatives, including chitosan, were found to be potent activators of macrophages and NK cells [16,17]. This immunostimulating activity along with the structural similarities between chitin derivatives and glucans, an immunoadjuvant class of natural polysaccharides, led several scientists to study the adjuvant capabilities of chitosan. Nishimura et al. formulated various chitin derivatives with antigen and incomplete Freund's adjuvant (IFA) to measure adaptive immune responses [18]. Both 70% and 30% deacetylated chitosan, when formulated with IFA, increased antigen-specific serum antibody titers in mice by over 3-fold versus IFA alone. Similarly, in guinea pigs, chitosan plus IFA induced greater DTH responses than IFA alone [18]. Marcinkiewicz et al. found that intraperitoneal (i.p.) administration of a water insoluble chitosan suspension enhanced humoral responses but not cell-mediated immune responses in mice [19]. Subcutaneous administrations of chitosan suspensions were found to be ineffective [19]. In other studies, Seferian and Martinez found that chitosan particles, formulated in an emulsion with antigen, squalene and Pluronic® L121, gave a prolonged, high antigen-specific antibody titer and sensitized animals for antigen-specific DTH responses following an i.p. injection [20]. Chitosan particles alone offered no enhancement of an adaptive immune response [20]. In all of the aforementioned studies, chitosan was regarded as an immune stimulant, and therefore, never considered as a subcutaneous or intraperitoneal vaccine delivery system.

However, because of its mucoadhesive properties, chitosan has also been explored as an adjuvant for mucosal vaccination. Intranasal administration of chitosan solutions have enhanced adaptive immune responses to several antigens [21,22]. The mechanisms of vaccine enhancement by chitosan are believed to be due to both retention of vaccine in the nasal passages via mucoadhesion and opening of endothelial cell junctions for paracellular transport of vaccine [21]. Recent clinical studies have confirmed that chitosan is a promising adjuvant platform for intranasal vaccination [12-14].

Nonetheless, chitosan solution alone has never been tested as a vaccine delivery system or depot for subcutaneous administration. This is most likely due to two reasons. First, the mucoadhesive advantage of chitosan is lost during a non-mucosal administration. Second, the high viscosities of chitosan solutions have been overlooked as a way to control the release of antigens. Chitosan, by virtue of its long polymer backbones, forms a highly viscous solution in mild aqueous solvents; a 1% (w/v) chitosan solution is two orders of magnitude more viscous than water. Viscous solutions are widely used for the controlled release of drugs and macromolecules [23-25].

Thus, in this study, we hypothesized that a viscous chitosan solution, when administered subcutaneously, would not only provide immune stimulation as previously described [16,18] but also act as an antigen depot. We quantified vaccine responses to a model antigen protein, β-galactosidase, delivered subcutaneously with or without chitosan solution. We also compared chitosan with the traditional adjuvants, aluminum hydroxide and IFA. Finally, we conducted non-invasive imaging of subcutaneous injections of antigen and phenotypic analysis of draining lymph nodes in an effort to uncover the mechanisms by which chitosan enhanced a subcutaneous vaccination. This study is the first to demonstrate and describe the adjuvant characteristics of chitosan solution formulated with a model protein antigen for subcutaneous vaccination.

2. Materials and Methods

2.1 Animals, antigens and adjuvants

Female C57BL/6 mice (8-12 weeks old) were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). Mice were housed and maintained under pathogen-free conditions in microisolator cages. Animal care was in compliance with recommendations of *The Guide for Care and Use of Laboratory Animals* (National Research Council). β-galactosidase was purchased from Prozyme (San Leandro, CA). Ovalbumin (Grade VI) and concanavalin A were purchased from Sigma-Aldrich (St. Louis, MO). Chitosan (Protosan G 213) was purchased from NovaMatrix (Drammen, Norway). Incomplete Freund's adjuvant (IFA) was purchased from Rockland (Gilbertsville, PA). Aluminum hydroxide (Imject Alum) was purchased from Pierce Biotechnology, Inc. (Rockford, IL).

2.2 Vaccinations

Vaccinations consisted of a prime and one boost, separated by 1 week, with 100 μg β-galactosidase. Vaccinations were given as two 50 μl s.c. injections administered bilaterally in the lumbar region. β-galactosidase was formulated via simple addition with either PBS or 1.5% chitosan dissolved in PBS. β-galactosidase was formulated with aluminum hydroxide or IFA according to the manufacturer's instructions.

2.3 Splenic $CD4^+$ proliferation assay

All proliferation assays were initiated 1 week following the booster vaccination and performed as described previously with minor modifications [26]. Briefly, harvested spleens were mechanically disrupted with a syringe plunger and passed through a 70 μm nylon mesh strainer (BD Biosciences; Bedford, MA). Erythrocytes were lysed with ACK lysing buffer (Cambrex Bio Science; Walkersville, MD). $CD4^+$ splenocytes were isolated via Dynal® CD4 negative isolation kits (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. Splenic $CD4^+$ cells from immunized mice were co-incubated with 5×10^5 irradiated (20 Gy) naïve syngeneic splenocytes in individual wells of a 96-well plate. Cells were stimulated with 6.25-100 μg/ml β-galactosidase for 5 days. For positive controls, cells were stimulated with 0.0625-1 μg/ml concanavalin A, a T cell mitogen, for 3 days. For non-specific antigen controls, cells were stimulated with 100 μg/ml ovalbumin for 5 days. In all cases, cells were labeled with 1 μCi/well [^{3}H]-thymidine (Amersham Biosciences; Piscataway, NJ) for the final 18 h of culture. Following incubation, cultures were harvested onto glass fiber filtermats via a Tomtec Harvester 96 (Hamden, CT). Incorporated radioactivity was measured by liquid scintillation counting on a 1450 Betaplate (Perkin-Elmer; Shelton, CT). Results from individual mice in triplicate wells were combined to yield a mean ± SEM for each immunization group.

2.4 Serum antibody responses

Antigen-specific serum antibody responses were measured 1 week following the booster vaccination via ELISA. Briefly, microtiter plates were sensitized overnight at 4°C with 100 ng/well β-galactosidase or ovalbumin (as a negative control). Wells were blocked with 5% BSA in PBS for 1h at 37°C. Wells were then incubated with serum serially diluted (1:20-1:1,526,500). Anti-β-gal was used as positive control (Promega; Madison, WI). Following a 1 h incubation, wells were washed thrice with 1% BSA in PBS and incubated with Horseradish peroxidase-conjugated goat-anti-mouse IgG (Pierce; Rockford, IL), IgG_1 or IgG_{2a} (Southern Biotech;Birmingham, AL). Following a 1 h incubation, wells were washed thrice with 1% BSA in PBS and incubated with o-phenylenediamine (Sigma-Aldrich; St. Louis, MO) according to the manufacturer's instructions. The reaction was stopped with 3N HCl and the absorbance of each well was read at 490 nm using a Bio-Tek Synergy HT multi-detection microplate reader (Winooski, VT).

2.5 Delayed-type hypersensitivity

Seven days after the booster vaccination, the baseline thickness of both ears was measured with a spring-loaded dial gauge (Mitutoyo Corp., Tokyo, Japan). Ten minutes prior to antigen challenge, mice were anesthetized with 15 mg/kg xylazine + 75 mg/kg ketamine. Ten microliters of PBS or β-galactosidase (5 mg/ml) were injected into opposite pinnae. Ear thickness was measured in triplicate 24 h after challenge. The thickness of the ear challenged with antigen was divided by the thickness of the ear challenged with PBS to obtain percent increase in ear thickness.

2.6 Flow cytometry

Inguinal lymph nodes were harvested, via gross dissection, mechanically disrupted with a syringe plunger and passed through a 70 μm nylon mesh strainer (BD Biosciences; Bedford, MA). Cells were washed twice with cold PBS. FcγII and FcγIII receptors on lymphocytes were blocked via incubation with 1 μg purified anti-mouse CD16/CD32 (clone: 2.4G2) (BD Biosciences; San Jose, CA) per 1×10^6 cells for 15 min on ice. Cells were stained with fluorescence-labeled antibodies (1 μg/1×10^6 cells) to the following markers (BD Biosciences; San Jose, CA): CD3e (clone: 145-2C11), CD19 (clone: 1D3), CD4 (clone: RM4-5), CD8a (clone: 53-6.7), NK1.1 (clone: PK136), CD25 (clone: PC61), CD11b (clone: M1/70), CD11c (clone: HL3), and Gr-1 (clone: RB6-8C5). Antibody isotype controls (BD Biosciences; San Jose, CA) included: mouse IgG_1 (clone: MOPC-31C), mouse IgG_{2a} (clone: G155-178), rat IgG_1(clone: A110-1), rat IgG_{2a} (clone: R35-95), rat IgG_{2b} (clone: A95-1) and Hamster IgG_1 (clone: A19-3). Following a 45 min incubation on ice, cells were washed twice with cold PBS and read in six colors on a LSR II (BD Biosciences; San Jose, CA). Data analyses were performed using BD FACSDiva Software (BD Biosciences; San Jose, CA).

2.7 Non-invasive fluorescence imaging of antigen depots

Non-invasive animal imaging was carried out in the Mouse Imaging Facility (MIF), a division of the NIH MRI Research Facility (NMRF). Fluorescence and photographic images of anesthetized mice that were given a single s.c. injection of Alexa Fluor 660-labeled β-galactosidase formulated in either PBS or 1.5% chitosan were acquired over a 2-week period with an IVIS 100 Imaging System (Xenogen; Alameda, CA). Anesthesia was induced in a chamber with 4-5% isoflurane delivered by a gas mixture of oxygen, nitrogen and medical air. Once mice were unconscious and unresponsive to toe pinch, anesthesia was maintained with 1-2% isoflurane administered via nosecone. Following each imaging session, mice were allowed to recover in the MIF/NMRF on a circulating warm water pad until they could breathe unassisted and walk. Prior to the initial imaging session, the lumbar regions of mice were shaved with electric shears. Residual hair was removed with a depilatory cream. Approximately

60 μg of β-galactosidase, labeled with an Alexa Fluor 660 protein labeling kit (Invitrogen; Carlsbad, CA), were injected s.c. in a total volume of 50 μl. The fluorescence intensity of the injection site was used as a surrogate for β-galactosidase concentration. The fluorescence intensity of a region of interest drawn around the injection site was calculated at each time point with Living Image® software (Xenogen; Alameda, CA). Background/autofluorescence from non-injected control mice was subtracted. Fluorescence data for each mouse were normalized by the initial measurement, which was taken immediately after injection, for that mouse.

2.8 Histopathology

Similar to the vaccinations described above, mice (n=9) were given bilateral s.c. injections of 50 μl of 1.5% chitosan in the lumbar region. Mice were sacrificed 2, 7 or 14 days after the injection. The skin/subcutis containing the injection site was removed, embedded in paraffin, sectioned and stained with hematoxylin and eosin to document inflammation and chitosan regression. Slides were blinded and read by a board certified pathologist.

2.9 Statistical analysis

Statistical analyses of differences between means of antigen-specific splenic $CD4^+$ proliferation, antibody titer and lymphocyte percentages from flow cytometry experiments were performed using Student's two-tailed *t* test assuming unequal variances (JMP Software; Cary, NC). Differences in means were accepted as significant if P was less than 0.05.

3. Results

3.1 Chitosan enhances both humoral and cell-mediated vaccine responses

C57BL/6 mice were vaccinated subcutaneously with a model antigen, β-galactosidase, in either PBS or chitosan solution. Proliferation of $CD4^+$ splenocytes from mice receiving the vaccine in chitosan is significantly greater ($P<0.05$) than that of $CD4^+$ splenocytes from mice receiving the vaccine in PBS when re-exposed to the vaccine antigen (Fig. 1). Chitosan also increased serum IgG titers to β-galactosidase (Fig. 2a). Antibody titers in mice administered β-galactosidase in chitosan were increased 5.3 fold, as linearly approximated at an optical density of 1.0. Similarly, chitosan enhanced antigen-specific IgG_1 and IgG_{2a} titers 5.9- and 8.0-fold respectively, implying a mixed T_H1/T_H2 response (Figs. 2b-c). All increases in antibody titers were statistically significant ($P<0.001$).

Delayed-type hypersensitivity responses were measured as an in vivo assay of cell-mediated immune function. One week after the booster vaccination, mice were challenged with 50 μg of β-galactosidase in the pinnae. Opposite pinnae were injected with PBS to control for non-specific inflammation. Twenty-four hours after challenge, mice originally vaccinated with β-galactosidase in PBS had, on average, less than a 10% increase in ear thickness. However, mice originally vaccinated with β-galactosidase formulated with chitosan had a substantial 116% increase in ear thickness indicating a robust cell-mediated immune response (Fig. 3).

3.2 Chitosan is equipotent to IFA and superior to aluminum hydroxide

After it was shown that chitosan had vaccine enhancing properties, the next objective was to compare chitosan with commonly used adjuvants, IFA and aluminum hydroxide. Antigen-specific $CD4^+$ proliferative and serum antibody responses were similar in mice vaccinated with β-galactosidase in either chitosan solution or IFA (Fig. 4). Antigen-specific $CD4^+$ proliferative responses were significantly greater ($P<0.05$) in mice vaccinated with β-galactosidase in a chitosan solution rather than aluminum hydroxide (Fig. 5a). Chitosan also enhanced antigen-specific antibody titers 6.6-fold over aluminum hydroxide at optical density of 1.0 (Fig. 5b).

3.3 Chitosan expands local lymph nodes

During the aforementioned studies, mice were dissected to note any gross pathological changes that may have resulted from the s.c. injection of chitosan. We noted a significant increase in the size of the lymph nodes draining the s.c. chitosan injections. Mice were otherwise healthy at the time of sacrifice. To characterize the leukocyte expansion, inguinal lymph nodes were resected, disrupted, counted and stained for phenotypic analysis via six-color flow cytometry. The number of leukocytes in inguinal lymph nodes from mice injected with chitosan increased by more than 67% from 4.9×10^6 leukocytes per node at day 0 to 8.2×10^6 leukocytes per node at day 14 (Table 1). Phenotypic analysis revealed chitosan modestly increased the number of $NK1.1^+$ cells in the lymph node and spleen as well as the number of $CD11b^+$ cells in the lymph nodes. All other compartments were not significantly altered by chitosan.

3.4 Chitosan retains antigen at the injection site

Another possible mechanism of vaccine enhancement is the maintenance of an antigen depot at the injection site. Dissemination of macromolecules from an injection site can be hindered greatly by highly viscous solutions [25]. The use of 1.5% chitosan solution, which, according to the manufacturer, is approximately more than two orders of magnitude more viscous than water, was expected to result in a depot of antigen at the injection site. To verify this hypothesis, β-galactosidase was labeled with Alexa Fluor 660 prior to injection in order to track the spatiotemporal distribution of antigen when administered in PBS versus chitosan solution. Mice receiving a single s.c. injection with Alexa Fluor 660-labeled β-galactosidase were imaged over the course of 2 weeks (Fig. 6).

Fluorescence intensity was used as a surrogate for β-galactosidase concentration. Analysis of the injection site revealed that within 24 h, less than 3% of the antigen delivered in PBS remained at the injection site (Fig. 7). This is contrasted with greater than 60% of antigen delivered in chitosan remaining 7 days after injection.

3.5 Chitosan is highly biodegradable

In order to document pathological changes in the subcutis, chitosan solution alone was injected as in the vaccination studies. Tissues surrounding the subcutaneous injection site of chitosan were removed 2, 7 and 14 days after injection and stained with hematoxylin and eosin. Histopathological analysis revealed that chitosan was infiltrated and degraded, mainly by macrophages and neutrophils, in 2-3 weeks (Fig. 8). This rate of degradation coincided with the dissipation of antigen from the injection site (Fig. 7).

4. Discussion

This study is the first to demonstrate that chitosan solution improved humoral and cell-mediated immune responses to a subcutaneous vaccination with a model protein antigen in the absence of additional adjuvants. More specifically, chitosan significantly enhanced antigen-specific serum IgG titers over 5-fold and antigen-specific $CD4^+$ proliferation over 6-fold. Antibody isotype analysis revealed that the nature of the immune responses facilitated by chitosan was a mixed T_H1/T_H2 response. DTH responses were used to confirm that chitosan also induced a robust cell-mediated immune response. Further characterization of the cell-mediated immune response and the role of chitosan in eliciting a $CD8^+$ response will be the foci of future studies.

Chitosan exhibited two adjuvant characteristics that were responsible for the enhanced immune response. First, subcutaneous injections of chitosan solution led to a 67% cellular expansion in local lymph nodes. The proportions of all leukocyte subsets did not change significantly with the exception of small increases in the percentages of $NK1.1^+$ and $CD11b^+$ cells. These increases agreed with previous findings that chitosan can stimulate NK cells and macrophages

[16-18,27]. The increase in NK cells, together with the recent discovery of their regulatory role [28,29], indicate that this population may be important in chitosan's vaccine enhancement. Future studies will examine the role of NK cells during vaccination in the presence of chitosan.

The second adjuvant characteristic observed was chitosan's ability to form an antigen depot. The chitosan solution used in this study was over two orders of magnitude more viscous than water. The large difference in viscosities led to large differences in antigen dissemination from the injection site (Figs. 6-7). Within 8 h, less than 9% of antigen injected with a saline vehicle remained at the injection site. This is contrasted with a chitosan vehicle that maintained a depot of over 60% injected antigen for 1 week and over 10% injected antigen for 11 days. The retention of antigen in chitosan creates an ideal scenario for vaccination - a depot of antigen at a site of inflammation that introduces the critical danger signals and costimulation to generate an adaptive immune response.

When compared to known parenteral vaccine adjuvants, chitosan was found to be equipotent to IFA (Fig. 4) and superior to aluminum hydroxide (Fig. 5). Although a great many preclinical studies have employed IFA due to its robust vaccine enhancing properties, toxicity has limited its clinical use. In general, water-in-oil emulsions, such as IFA, are not readily biodegradable. In fact, studies have shown that a water-in-oil emulsion similar to IFA persisted up to 22 weeks [30] and only 70% of an injected mineral oil is metabolized in 1 year [31,32]. The documented side effects of IFA include painful granulomas [4], sterile abscesses [33], degenerative arthritis [34,35] and lupus-like autoimmune responses [36,37].

Aluminum hydroxide remains the only vaccine adjuvant widely licensed in the U.S.; however, aluminum-related toxicities have become a recent concern [4,38,39]. Aluminum hydroxide, like IFA, is not readily biodegradable. Previous studies have shown that aluminum hydroxide persisted for at least 6 months after intramuscular administration [40]. The documented side effects following subcutaneous administration of aluminum hydroxide include pruritic subcutaneous nodules [41,42], delayed hypersensitivity [41] and severe granulomatous inflammation [5].

Chitosan, on the other hand is a natural polysaccharide that is cleared by enzymatic digestion [43,44]. As mentioned previously, chitosan has been used safely in humans for topical, intranasal and oral applications [10-15]. Figure 8 clearly depicts the infiltration and degradation of a subcutaneous chitosan injection over a 2-week period. No overt side effects were observed in any mouse receiving a subcutaneous injection of chitosan in any of our experiments. Nevertheless, we are planning to follow up with full toxicology screens addressing short and long-term uses of subcutaneous, intraperitoneal and intramuscular chitosan.

At this point it should be noted that not all chitosan will degrade at the rate depicted in Figure 8. Chitosan degradation, which is mediated largely by lysozyme digestion [45], is dependent on degree of deacetylation [18] and most likely molecular weight and concentration as well. Chitosan variability will no doubt lead to confusion when trying to interpret results from different labs using different chitosans at different concentrations. However, this variability provides tremendous opportunity to engineer chitosan for specific vaccine delivery. For example, manipulating the viscosity of chitosan via molecular weight and concentration will change the density of the chitosan matrix and would be expected to control vaccine release and lymphocyte infiltration. Also, by manipulating the degree of deacetylation of chitosan, one can control the rate of lysozomal digestion and therefore vaccine release. This adaptable platform may be engineered to degrade in 1 week to several months. It is also probable that simple chemical modifications of chitosan will affect its immunological activity [16] and, thus, the nature of a vaccine response. Audibert's commentary that "it is not possible to describe a

universal and appropriate adjuvant" [46] is well-taken; however, the potential of chitosan to control the context and duration of antigen exposure is unprecedented.

Altogether, chitosan has shown promise as a parenteral vaccine adjuvant platform. Chitosan, via lymph node expansion and antigen depot, is a robust adjuvant that enhances both humoral and cell-mediated immune responses. The biocompatibility of this natural polysaccharide offers a potentially safer alternative to IFA and aluminum hydroxide and perhaps even bacterial cell wall-based adjuvants. The versatility of chitosan through engineering polymer characteristics offers an additional advantage. To our knowledge, no other adjuvant platform has this combination of vaccine enhancement, biocompatibility and versatility.

Acknowledgements

We are grateful for the outstanding technical support of Garland Davis and Eileen Thompson. We also thank Debra Weingarten for editorial assistance and Dr. Miriam Anver for histopathological interpretation. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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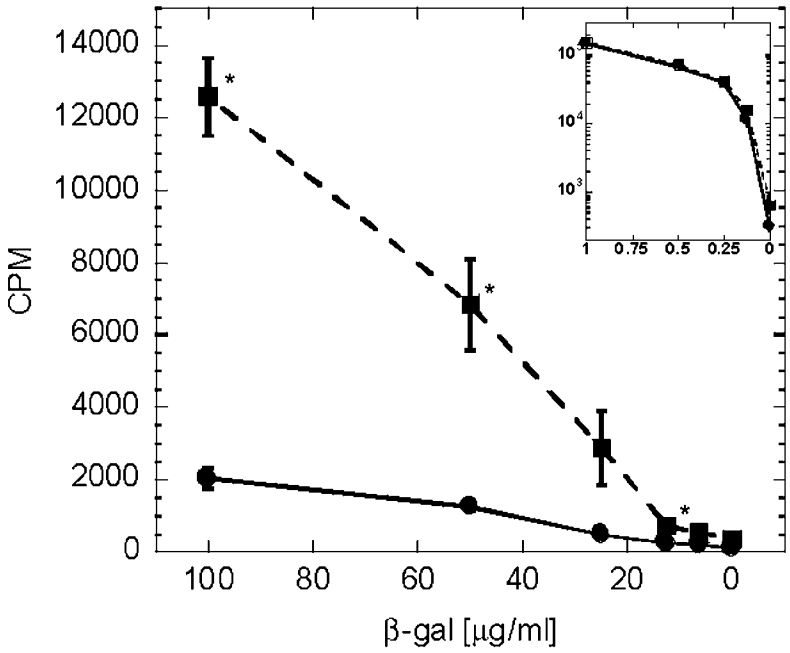

FIGURE 1.

Chitosan enhanced antigen-specific $CD4^+$ proliferation. Splenic $CD4^+$ proliferative responses from C57BL/6 mice (n=3) vaccinated with 100 μg β-galactosidase in PBS (●) or 1.5% chitosan (■) were assessed 1 week after the booster vaccination. 150,000 $CD4^+$ cells from experimental animals were cultured with 500,000 irradiated antigen presenting cells from unvaccinated control mice in the presence of increasing concentrations of β-galactosidase (β-gal). Mice vaccinated with β-gal in chitosan demonstrated a robust enhancement in antigen-specific $CD4^+$ proliferation. Chitosan did not affect the quality of $CD4^+$ splenocytes as judged by the $CD4^+$ response to a non-specific T cell mitogen, concanavalin A (Insert). Data are represented as mean ± SEM. Data are representative of two independent experiments. *, $P < 0.05$.

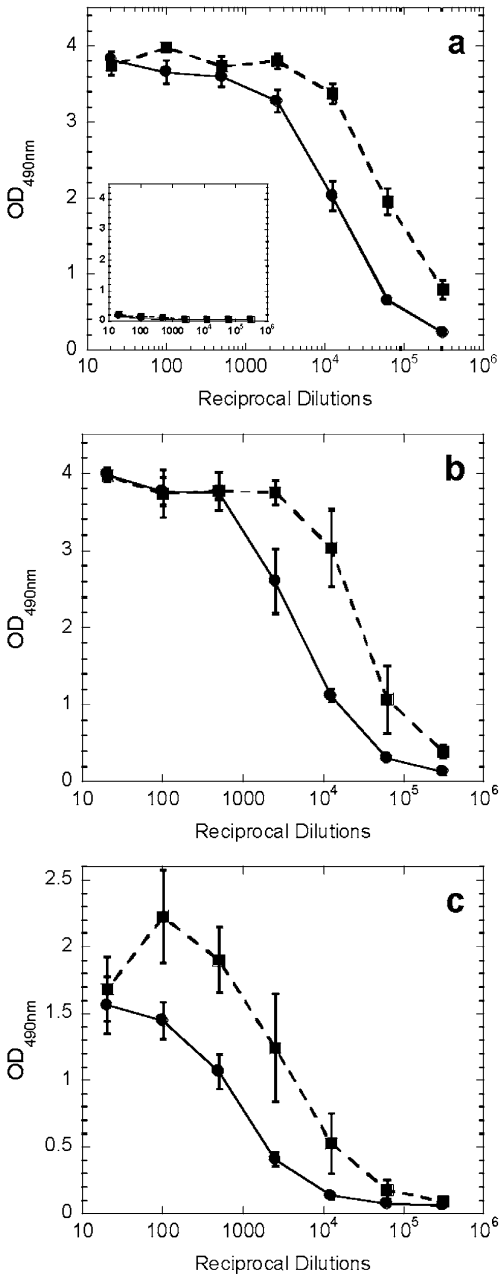

FIGURE 2.

Chitosan enhanced antigen-specific serum IgG. *a*, β-galactosidase-specific serum IgG from C57BL/6 mice (n=3) vaccinated with 100 μg β-galactosidase in PBS (●) or 1.5% chitosan (■) were measured 1 week after the booster vaccination via ELISA. Chitosan enhanced the antigen-specific antibody titer in the linear portion of the titration (O.D. = 1.0) approximately 5.3-fold. There were no differences in serum IgG against control antigen (ovalbumin) (Insert). *b*, β-galactosidase-specific serum IgG_1 and *c*, IgG_{2a} from vaccinated mice were measured 1 week after the booster vaccination via ELISA. Chitosan enhanced antigen-specific IgG_1 and IgG_{2a} titers 5.9- and 8.0-fold, respectively, at O.D. = 1.0, implying a mixed T_H1/T_H2 response. All data are represented as mean ± S.E.M. Data are representative of two independent experiments

(n=3). All increases in antibody titer approximated at an optical density of 1.0 were statistically significant ($P<0.001$).

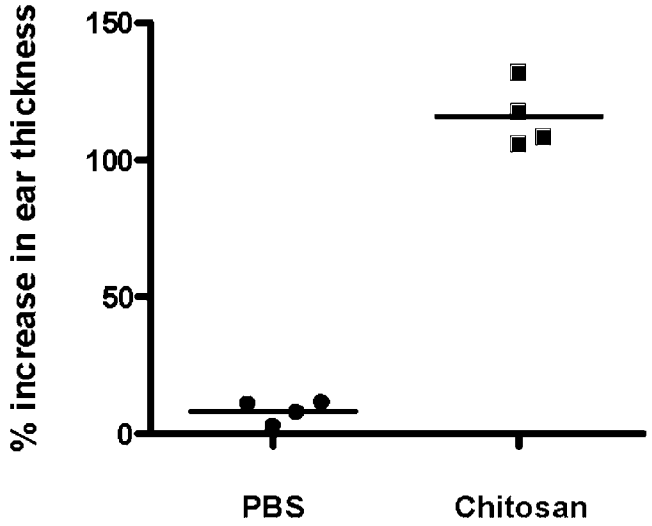

FIGURE 3.

Chitosan elicited a robust DTH response. Delayed-type hypersensitivity responses in C57BL/6 mice (n=4) primed and boosted with 100 μg β-galactosidase in PBS (●) or 1.5% chitosan (■) were measured 1 week after the booster vaccination. 50μg β-galactosidase in 10 μl PBS were injected into the pinnae of vaccinated mice. Opposite pinnae were injected with 10 μl PBS. Ear thickness was measured 24 h after ear injections. The thickness of the ear challenged with antigen was divided by the thickness of the ear challenged with PBS to obtain percent increase in ear thickness. Ear swelling was significantly greater ($P < 0.01$) in mice vaccinated with β-galactosidase in chitosan.

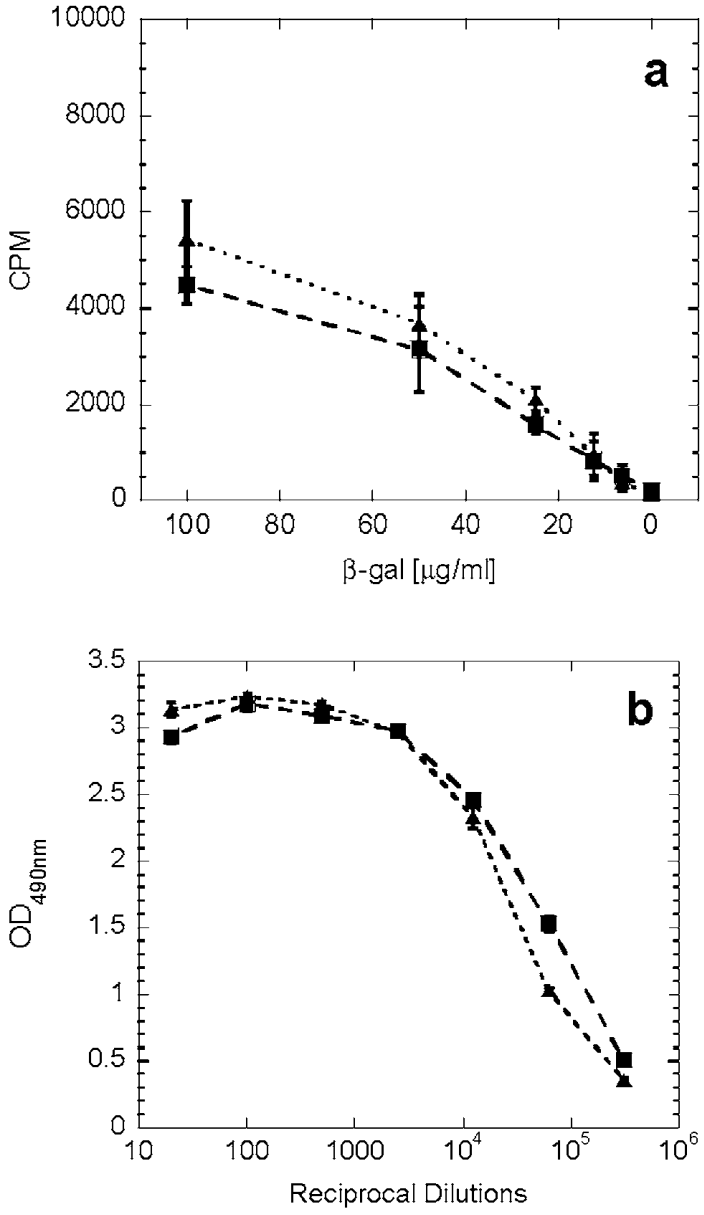

FIGURE 4.

Chitosan was equipotent to IFA as a subcutaneous vaccine adjuvant. *a*, Splenic $CD4^+$ proliferative responses and *b*, β-galactosidase-(β-gal)-specific serum IgG from C57BL/6 mice (n=3) vaccinated with 100 μg β-gal in 1.5% chitosan (■) or IFA (▲) were assessed 1 week after the booster vaccination. Approximately 100,000 $CD4^+$ cells from experimental animals were cultured with 500,000 irradiated antigen presenting cells from unvaccinated control mice in the presence of increasing concentrations of β-gal. Proliferative responses were indistinguishable ($P > 0.1$). Data are represented as mean ± SEM.

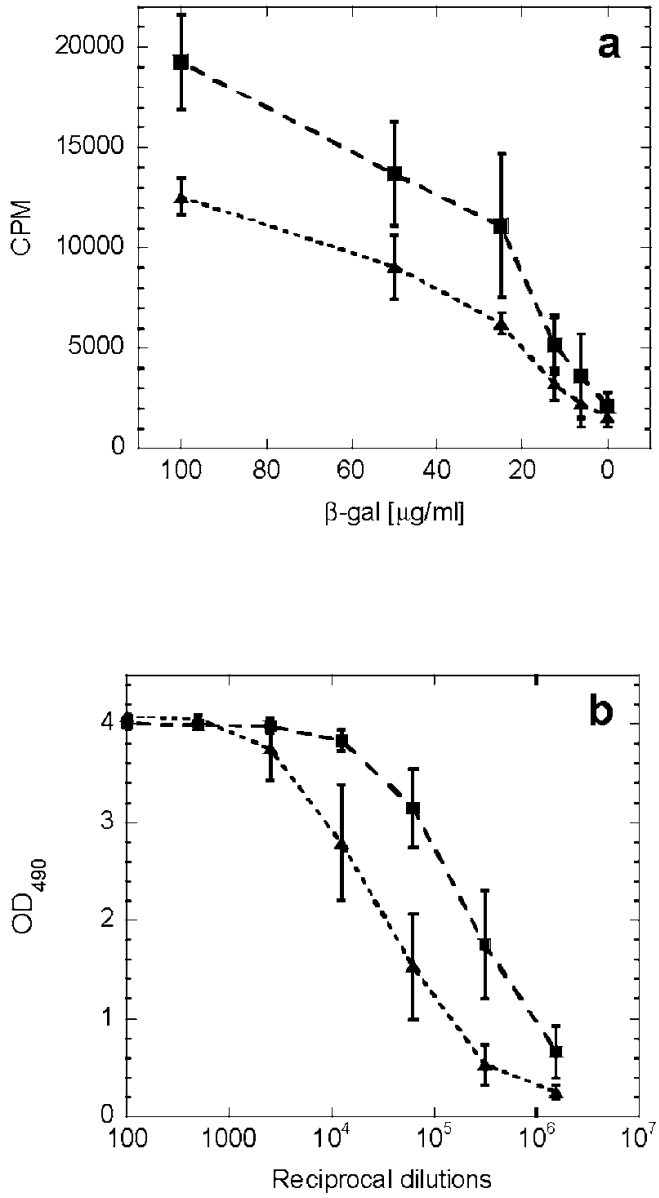

FIGURE 5.

Chitosan was superior to aluminum hydroxide as a subcutaneous vaccine adjuvant. *a*, Splenic $CD4^+$ proliferative responses and *b*, β-galactosidase-(β-gal)-specific serum IgG from C57BL/6 mice (n=3) vaccinated with 100 μg β-gal in 1.5% chitosan (■) or aluminum hydroxide (▲) were assessed 1 week after the booster vaccination. Approximately 200,000 $CD4^+$ cells from experimental animals were cultured with 500,000 irradiated antigen presenting cells from unvaccinated control mice in the presence of increasing concentrations of β-gal. Chitosan outperformed aluminum hydroxide in enhancing antigen-specific $CD4^+$ proliferation and serum IgG titers. Chitosan increased antigen-specific antibody titer 6.6-fold over aluminum

hydroxide at an optical density of 1.0. Data are represented as mean ± SEM. * $P < 0.05$ versus aluminum hydroxide.

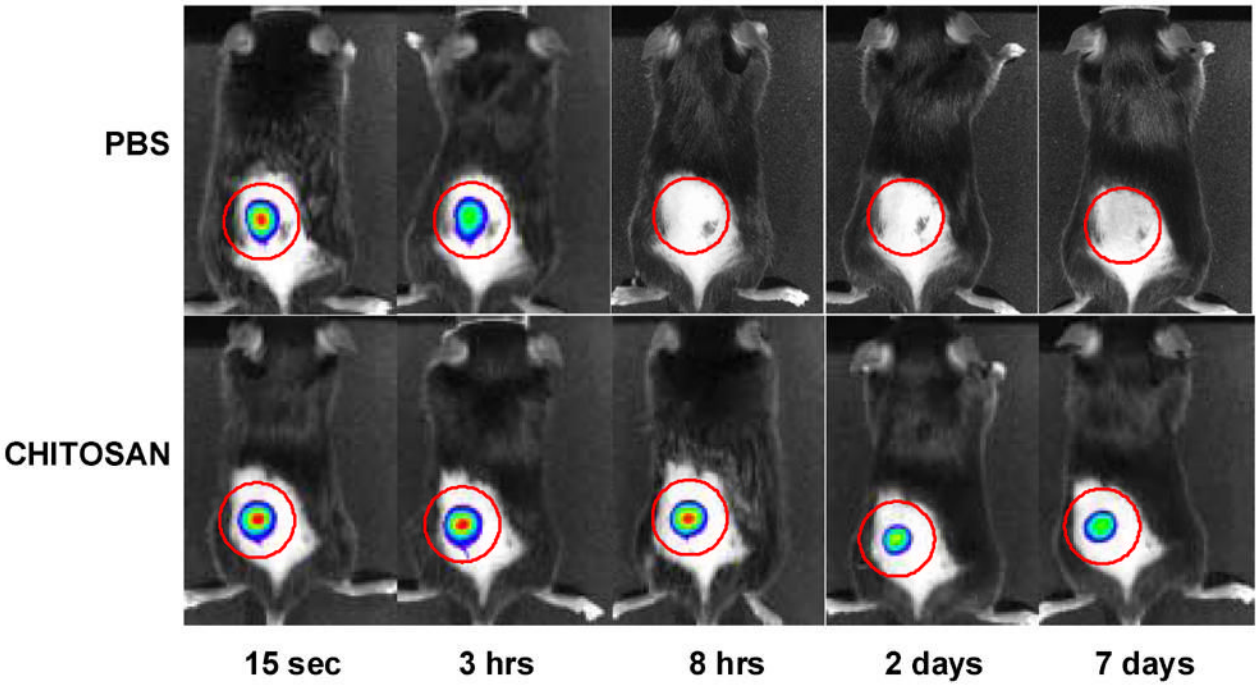

FIGURE 6.

Chitosan maintained a depot of β-galactosidase. Spatiotemporal distributions of a single subcutaneous administration of a fluorescently-labeled model antigen (Alexa Fluor 660-labeled β-galactosidase) were acquired via non-invasive fluorescence imaging. Fluorescence intensity was used as surrogate for β-galactosidase concentration. The region of interest used to quantify fluorescence intensity is denoted by the red circle.

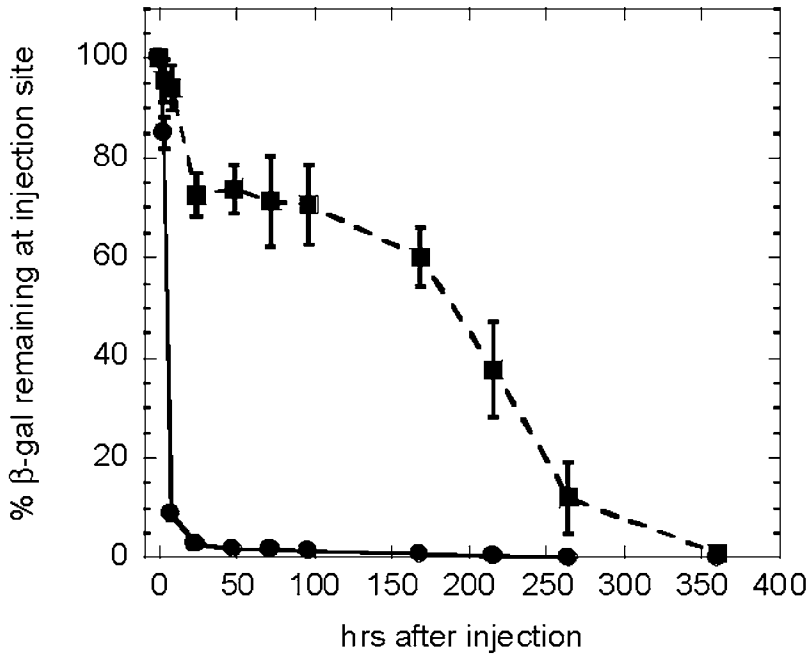

FIGURE 7.

Dissipation of a model antigen (β-galactosidase(β-gal)) from a subcutaneous injection site. C57BL/6 mice were shaved and treated with a depilatory cream prior to injection of Alexa Fluor 660-labeled β-gal in PBS (●) or 1.5% chitosan (■). The fluorescence intensity of Alexa Fluor 660-labeled β-gal in a region of interest around the injection site was used as a surrogate of β-gal concentration. Within 24 h, less than 3% of the antigen delivered in PBS remained at the injection site. Greater than 60% of antigen delivered in chitosan remained 7 days after injection. Data are represented as mean ± SEM from four mice per group.

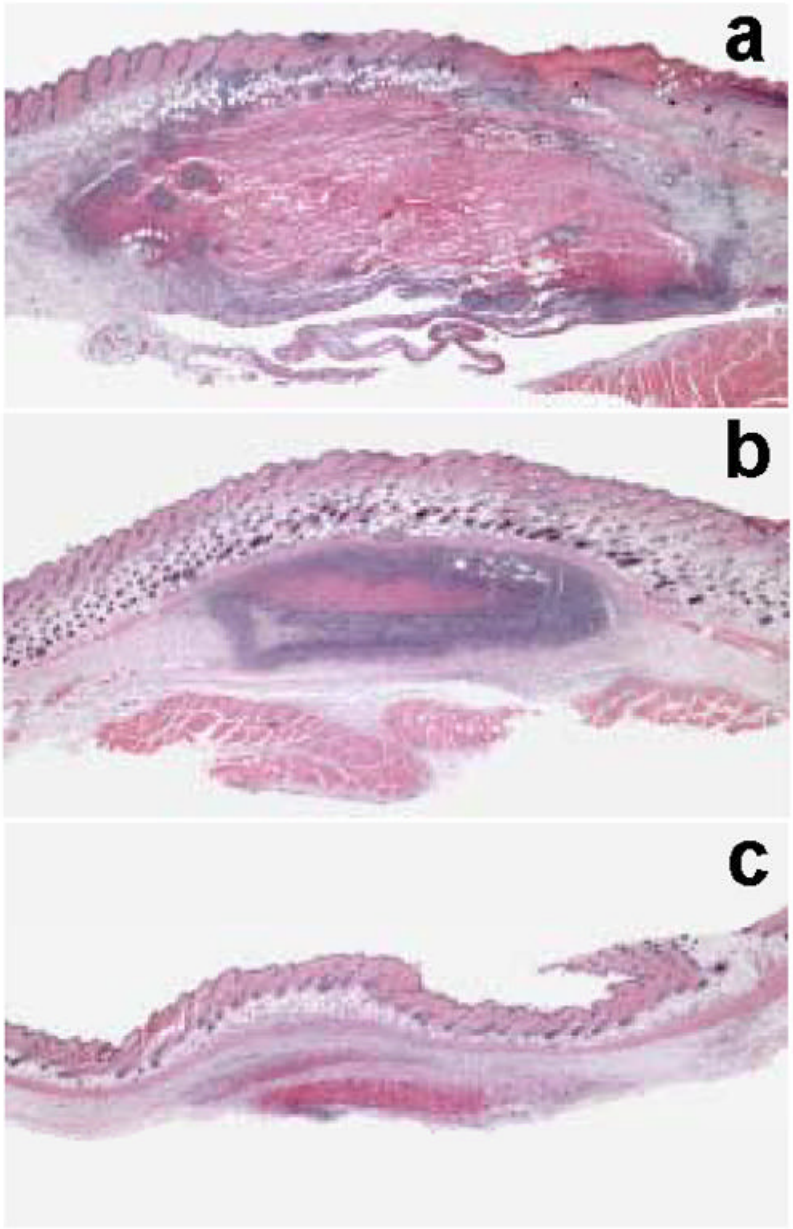

FIGURE 8.
A subcutaneous chitosan depot is infiltrated and degraded in 2-3 weeks. H&E staining of the subcutis (*a*) 2 days, (*b*) 7 days and (*c*) 14 days after a subcutaneous injection of 1.5% chitosan.

Table 1

The effect of chitosan on the total number and percent of lymphocyte subsets in the spleen and inguinal lymph nodes (ILN). Chitosan, without antigen, was injected subcutaneously at Day 0. Spleens and lymph nodes were harvested at Days 0, 2, 7, 14 and 21. Chitosan increased the number of lymphocytes per ILN by 67% (Day 14). Chitosan modestly increased the number of NK1.1$^+$ cells in the lymph node and spleen as well as the number of CD11b$^+$ cells in the lymph nodes. All other compartments were not significantly altered by chitosan. Data are represented as the mean (*SD*) of 5 mice.

	Spleen							
	lymphocyte number	**CD3$^+$**	**CD19$^+$**	**CD8$^+$**	**NK 1.1$^+$**	**Gr-1$^+$**	**CD11c$^+$**	**CD11b$^+$**
Day 0	114.8 (*14.4*)	31.6 (*2.4*)	60.4 (*2.5*)	12.2 (*1.4*)	4.6 (*0.3*)	5.7 (*0.3*)	3.4 (*0.3*)	4.4 (*0.6*)
Day 2	118.4 (*13.4*)	28.5 (*1.9*)	62.2 (*2.4*)	12.6 (*1.0*)	5.6 (*0.6*)*	5.5 (*0.2*)	3.7 (*0.2*)	5.0 (*0.4*)
Day 7	117.6 (*22.6*)	31.3 (*2.7*)	59.5 (*2.5*)	12.3 (*0.8*)	5.7 (*0.6*)*	5.7 (*0.3*)	3.5 (*0.2*)	4.9 (0.3)
Day 14	116.9 (*8.2*)	28.7 (*1.4*)	63.4 (*2.0*)	10.6 (*0.6*)	4.6 (*0.3*)	5.5 (*0.5*)	3.2 (*0.4*)	4.0 (0.4)
Day 21	112.5 (*19.3*)	31.5 (*2.6*)	56.2 (*3.6*)	12.5 (*1.1*)	5.1 (*0.5*)*	5.9 (*0.5*)	3.2 (*0.2*)	4.3 (0.5)

	ILN							
	lymphocyte number	**CD3$^+$**	**CD19$^+$**	**CD8$^+$**	**NK 1.1$^+$**	**Gr-1$^+$**	**CD11c$^+$**	**CD11b$^+$**
Day 0	4.9 (*0.4*)	67.6 (*6.0*)	24.8 (*5.2*)	28.0 (*2.8*)	1.5 (*0.1*)	7.4 (*1.0*)	1.2 (*0.4*)	1.5 (*0.2*)
Day 2	6.2 (*0.9*)*	62.9 (*3.2*)	30.7 (*5.0*)	25.8 (*2.0*)	2.8 (*0.4*)*	7.8 (*1.4*)	1.8 (*0.6*)	2.7 (*0.8*)*
Day 7	7.4 (*1.1*)*	65.3 (*1.3*)	30.0 (*1.5*)	27.9 (*0.9*)	3.6 (*0.4*)*	7.8 (*1.2*)	1.9 (*0.7*)	2.8 (*0.7*)*
Day 14	8.2 (*0.8*)*	66.6 (*2.2*)	30.2 (*2.3*)	26.6 (*1.1*)	1.8 (*0.2*)	8.6 (*0.8*)	1.1 (*0.2*)	1.8 (*0.3*)
Day 21	6.6 (*0.6*)*	66.1 (*3.1*)	30.9 (*3.0*)	26.0 (*1.2*)	1.8 (*0.2*)	8.2 (*0.5*)	1.0 (*0.2*)	1.5 (*0.2*)

* indicates $P < 0.05$ compared to Day 0.