Vaccine 37 (2019) 4031–4039

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Chitosan alters inactivated respiratory syncytial virus vaccine elicited immune responses without affecting lung histopathology in mice

Abenaya Muralidharan [a,e], Marsha S. Russell [a], Louise Larocque [a], Caroline Gravel [a], Simon Sauvé [a], Ze Chen [b], Changgui Li [c], Wangxue Chen [d], Terry Cyr [a], Michael Rosu-Myles [a,e], Lisheng Wang [e], Xuguang Li [a,e,*]

[a] Centre for Biologics Evaluation, Biologics and Genetic Therapies Directorate, Health Canada, Sir Frederick G. Banting Research Centre, AL: 2201E, 251 Sir Frederick Banting Driveway, Ottawa, Ontario K1A 0K9, Canada
[b] Shanghai Institute of Biological Products, 1262 Yan'an W Rd, Changning Qu, Shanghai Shi 200052, China
[c] National Institutes for Food and Drug Control and WHO Collaborating Center for Standardization and Evaluation of Biologicals, No. 2; Tiantan Xili, Beijing, China
[d] Human Therapeutics Portfolio, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario K1N 5A2, Canada
[e] Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, Roger Guindon Campus, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

ARTICLE INFO

Article history:
Received 4 January 2019
Received in revised form 30 May 2019
Accepted 3 June 2019
Available online 8 June 2019

Keywords:
Respiratory syncytial virus
Vaccine
Immunopathology
Vaccine safety

ABSTRACT

Chitosan is a polysaccharide capable of augmenting immune responses with a proven safety record in animals and humans. These properties make it a potentially attractive agent for the prevention and treatment of infectious disease. Infection by respiratory syncytial virus (RSV) is the leading cause of serious lower respiratory disease in young children throughout the world. There is no licensed vaccine available against RSV whereas inactivated vaccine is known to cause enhanced respiratory disease instead of protection. Here, we investigated whether chitosan administered one or three days post-infection could protect animals against RSV infection and whether it could alter immune responses or immunopathology induced by inactivated RSV vaccine when administered twice before RSV infection. We found chitosan could modestly protect animals against RSV infection when given post-infection, while, in conjunction with inactivated RSV vaccine when given pre-infection, it could significantly reduce RSV infection in mice. Further mechanistic investigation revealed that chitosan enhanced antigen-specific immune responses through augmenting the induction of regulatory T cells, lung resident T cells and neutralizing antibodies while reversing Th2-skewed immune responses induced by inactivated RSV vaccine but, surprisingly, failing to reverse lung histopathology. Overall, this study sheds more light on the molecular mechanisms underlying inactivated RSV vaccine-induced disease.

1. Introduction

Chitosan is a generic term for a family of linear polysaccharides that is commercially available as a partially deacetylated α-chitin produced from the exoskeletons of crustacean or the cell walls of fungi [1–3]. It is a biocompatible, non-toxic and non-allergenic material that has been tested for safety and toxicity in a variety of animal species for various applications through various routes of administration [4–6]. In solution, the positive charge of chitosan confers it with mucoadhesive properties, which are crucial for its use in intranasal applications [7,8]. When nasally administered with an antigen, it has been shown to augment antigen-specific immune responses in many animal models [9–12] and in human subjects [9,13–16]. Interestingly, chitosan alone is also able to enhance the immune responses against some viral infections [10–12]. While its activities of immune enhancement have been well documented, the molecular mechanisms remain to be fully understood. It would be of interest to investigate whether chitosan could alter immune responses induced by a vaccine known to be generally less effective or even induce severe adverse reactions.

Formaldehyde-inactivated respiratory syncytial virus (FIRSV) vaccine was initially developed to protect humans against RSV infection known to cause severe disease in young children, elderly and immunocompromised patients [17–20]. However, instead of protection, the vaccine was found to be associated with severe vaccine-induced enhanced respiratory disease (ERD), with 80% of the participants hospitalized and 2 deaths following subsequent

* Corresponding author at: Centre for Biologics Evaluation, Biologics and Genetic Therapies Directorate, Health Canada, Sir Frederick G. Banting Research Centre, A/L 2201E, 251 Sir Frederick Banting Driveway, Ottawa, ON K1A 0K9, Canada.
E-mail address: Sean.Li@canada.ca (X. Li).

https://doi.org/10.1016/j.vaccine.2019.06.003
0264-410X/

RSV infection [21–24]. It has been shown that Th2-skewed immune responses and poorly neutralizing antibodies lead to pulmonary inflammation, airway obstruction, and mucus hypersecretion [25–29]. Moreover, cell-mediated immune responses could also be involved in the development of ERD [30–32]. Here, we employed chitosan to gain better insight into FIRSV-induced immune responses, specifically its role in inducing regulatory and tissue resident T cells which are known to be critically important in inducing well-balanced and robust immune responses against microbial infections.

2. Materials and methods

2.1. Cells, virus and vaccines

Hep-2 (ATCC: CCL-23) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1.5 g/l sodium bicarbonate, 2 mM Glutamax, 1 mM HEPES, 20 U/ml Penicillin, 0.02 mg/ml Streptomycin, and 10% FBS.

RSV-A2 (ATCC: VR-1540) was grown in Hep-2 cells according to supplier's instructions and sucrose-purified for animal studies. FIRSV was prepared with the RSV-A2 strain in Hep-2 cells as described elsewhere [25,27]. Briefly, Hep-2 cells were infected at a MOI of 0.02 in Hep-2 growth media. Following 2 h of adsorption, the inoculum was removed, monolayer was washed with serum-free growth media, and cultured in serum-free growth media for 4 days. After 4 days, more than 80% cytopathic effect was observed and the cells along with the supernatant were harvested. The collected harvest was clarified by low speed centrifugation. The supernatant was collected and filtered while the pellet was resuspended in serum-free media for freeze-thawing twice. The freeze-thawed pellets were centrifuged at low speed to pellet the cell debris. The supernatant was filtered and combined with the previously collected supernatant. 100 μl of the combined filtered supernatant was removed for plaque assay conducted the same day. The rest of the filtrate was inactivated with formalin at a final dilution of 1:4000 and incubated at 37 °C for 3 days. Next, the formalin-inactivated media was ultracentrifuged at 26,000 rpm for 1 h at 4 °C. The resulting pellets were resuspended in 1/25th of the original volume in serum-free Hep-2 growth media. This was then adsorbed overnight to aluminium hydroxide added at a final concentration of 4 mg/ml. This formalin-inactivated alum-adjuvanted material was pelleted by low speed centrifugation and resuspended to 1/4th the volume in serum-free Hep-2 media. Formaldehyde-inactivated cell control (FI-Mock) was prepared alongside FIRSV using the same procedure starting with the same number of cells and media but without the RSV infection; Hep-2 growth media without RSV was added during the 2-hour adsorption step.

2.2. Animal studies

Six-week old female Balb/c mice (Charles River, Saint Constant, QC) were used for all animal studies. For a primary infection, mice were infected intranasally with 5×10^5 PFU of RSV-A2 in 25 μl per mouse. Chitosan (Sigma), also known as deacetylated chitin or poly β-1,4-D-glucosamine, derived from shrimp shells (≥75% deacetylated) is insoluble in water and organic solvents. Thus, chitosan was dissolved in 25 mM sodium acetate solution (pH 5.0) to a concentration of 4 mg/ml [11,12] and 25 μl was administered intranasally per mouse either one day or three days post RSV infection. Mice were euthanized four days post-infection for lung collection.

Twenty mice were given FIRSV or FI-Mock at 10^6 PFU intramuscularly in 50 μl volume per mouse. Each immunization was administered twice at the same dose and route 28 days apart (Day 0 and 28). On days 39 and 41, 10 mice given FIRSV and 10 mice give FI-Mock were administered 25 μl chitosan at 4 mg/ml intranasally. On day 42, 10 mice vaccinated with FIRSV (5 mice treated with chitosan) and 10 mice vaccinated with FI-Mock (5 mice treated with chitosan) were euthanized while the rest were challenged intranasally with 5×10^5 PFU of RSV-A2 in 25 μl per mouse. Four days post-challenge, mice were euthanized for blood and tissue collection.

In all animal studies, mice were intravenously injected with 1.5 μg of BV510-conjugated anti-mouse CD3 (clone 145-2C11; BD Biosciences) in 200 μl PBS 5 min before being euthanized. During flow cytometry analysis of the lungs, the cells that appear negative for the injected CD3 antibody will be considered tissue-resident [33,34].

All animal experiments were reviewed and approved by Institutional Animal Care and Use Committee of Health Canada and were conducted in accordance with Institutional Animal Care and Use Committee of Health Canada guidelines and regulations.

2.3. Lung viral titer

Lungs were removed four days post RSV challenge and the RSV titer was determined as described elsewhere [27]. Briefly, lungs were collected in serum-free RPMI media and weighed prior to mechanical homogenization using Ultra-Turrax® T25 homogenizer at 24,000 min^{-1}. The homogenates were clarified by centrifugation at 2000 rpm for 10 min at 4 °C and the supernatants were frozen at −80 °C until further use. Serial dilutions of the supernatant were done and incubated on Hep-2 cells for 2 h at 37 °C. A 1:1 overlay of 2x DMEM media and 0.8% agarose was added. Following 6 days of incubation, the overlay was removed and the cell monolayer was stained with crystal violet before counting plaques. Results are expressed as PFU/g lung tissue.

2.4. Histopathology

Four days post RSV challenge, whole lungs were collected from the Balb/c mice and fixed in 10% neutral buffered formalin. They were then trimmed, processed and embedded into paraffin blocks. Four-micron Hematoxylin-Phloxine-Eosin (HPE) and Periodic Acid Schiff (PAS) stained slides were made for evaluation. Scoring was done by a veterinary pathologist who was blinded to the experimental design. The lesion assessment protocol outlined by Weiss et al. was adopted [35]. Perivascular leukocytic infiltration was evaluated where 1 means within normal parameters; 2 means small numbers of solitary cells with uncommon aggregates; 3 means multifocal small to moderate aggregates; 4 means moderate to high cellularity with multifocal large cellular aggregates that may be expansive into adjacent tissues. Mucus was visualized with PAS stain and graded as follows: 1 means none; 2 means epithelial mucinous hyperplasia with none to rare luminal mucus accumulation in airways; 3 means epithelial mucinous hyperplasia with luminal mucus accumulation in airways; 4 means there is severe mucinous hyperplasia with airway obstruction by mucus. Total pathological score was calculated as the average of the individual scores.

2.5. ELISA

Serum from immunized mice was collected for determination of IgG1 and IgG2a titer. Ninety six-well plates were coated with 0.5 μg/ml recombinant RSV fusion (F) protein (Sino Biological) overnight at 4 °C. Next day, the plates were washed and blocked with BSA in PBS containing 0.05% Tween 20 for 2 h at 37 °C. Serial dilutions of the mouse serum in blocking buffer were then added for 1 h at 37 °C. After washing, HRP-conjugated anti-mouse IgG1

or IgG2a (Jackson Immunoresearch Laboratories) were added for 1 h at 37 °C. The plates were again washed and Tetramethylbenzidine substrate (Cell Signaling Technology) was added for 20 min at room temperature. The reaction was then stopped with 0.16 M sulfuric acid. The plates were read spectrophotometrically at 450 nm wavelength. The resulting OD values for IgG1 and IgG2a for each mouse at each serum dilution were divided to calculate the IgG1:IgG2a ratio.

2.6. Microneutralization

RSV-neutralizing ability of the serum from immunized mice was determined. Serial dilutions of the serum were incubated with 800 PFU of purified RSV-A2 for 1 h at 37 °C, 5% CO_2. The virus-antibody mixture was added to HEp-2 cells seeded the previous day and incubated at 37 °C. After 3 days, the cells were fixed with ice-cold methanol for 10 min at room temperature, air-dried, and blocked with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 2 h at 37 °C. Then, the plates were washed and a HRP-conjugated anti-RSV (Meridian Life Science) was added for 1 h at 37 °C. The plates were again washed and Tetramethylbenzidine substrate (Cell Signaling Technology) was added for 20 min at room temperature. The reaction was then stopped with 0.16 M sulfuric acid. The plates were read spectrophotometrically at 450 nm.

2.7. Flow cytometry

Cells were isolated from lungs of immunized mice using a lung dissociation kit (Miltenyi Biotec) and a mechanical tissue homogenizer. Single-cell suspensions were washed with PBS and first, stained with Fixable Viability Stain 700 (BD Biosciences) for 30 min at 4 °C, then, with purified anti-mouse CD16/CD32 (eBioscience) as a Fc block for 5 min. Next, cells were washed with FACS wash buffer (PBS with 1% BSA and 0.05% sodium azide) and stained for 30 min at 4 °C with a panel containing BV711-conjugated anti-mouse CD127 (clone SB/199), BV650-conjugated anti-mouse CD25 (clone PC61), BV421-conjugated anti-mouse CD44 (clone IM7), PE-Cy7-conjugated anti-mouse CD62L (clone MEL-14), PE-CF594-conjugated anti-mouse CD103 (clone M290), FITC-conjugated anti-mouse CD8a (clone 53–6.7), APC-H7-conjugated anti-mouse CD4 (clone GK1.5), and AF647-conjugated CCR7 (CD197; clone 4B12). After washing the stained cells, BD Pharmingen™ Transcription Factor Buffer Set (BD Biosciences) was used as per manufacturer's instructions to stain with PE-conjugated anti-mouse Foxp3 (clone MF23). All antibodies were purchased from BD Biosciences. Using a BD LSRFortessa flow cytometer, 50,000 singlet events were recorded. FMO controls and compensation beads were used where appropriate to correct for spectral overlap. Data analysis was completed using FlowJo X 10.0. Gating strategy is outlined in Fig. S1.

2.8. Secreted cytokines

Cells were isolated from lungs using a lung dissociation kit (Miltenyi Biotec) and a mechanical tissue homogenizer. The cells were then stimulated with an immunodominant H-2K^d-restricted RSV F_{85-93} peptide (KYKNAVTEL; ProImmune) at 5 μg/ml for 48 h at 37 °C, 5% CO_2. Supernatants were collected following centrifugation and stored at −80 °C for later analysis. A ProcartaPlex Mouse Cytokine Panel (eBiosciences) was used to determine the levels of IL-5 and IL-13 in the supernatants. The samples were read on a Luminex 200 System (xMAP Technology). Data analysis was performed using MILLIPLEX Analyst version 5.1 for determining the pg/ml of each cytokine.

2.9. Statistical analysis

Analysis was conducted using one-way or two-way ANOVA where appropriate. Bonferroni posttest was used to adjust for multiple comparisons between different test groups. Tests were done at a 5% significance level. All statistical analyses were performed using GraphPad Prism 7 software.

3. Results

3.1. Chitosan enhances induction of resident effector T cells and regulatory T cells following RSV infection

We first investigated whether chitosan administered therapeutically could afford protection against RSV and the underlying mechanism. To that end, Balb/c mice were infected with RSV-A2 and intranasally inoculated with chitosan either one day or three days post-infection [10]. A 'no chitosan' control was included where mice were infected with RSV but did not receive any chitosan. Four days post-infection, all mice were injected with a fluorophore-conjugated anti-mouse CD3 antibody [33,34] and euthanized five minutes later for lung collection (Fig. 1A). Minimally lower RSV titer, albeit statistically significant, was observed in mice given chitosan one day post-infection (Fig. 1B), while chitosan treatment on day 3 failed to significantly inhibit viral replication. Notably, we found that the decreased levels of viral replication in chitosan treated mice was accompanied by significantly increased T cell populations known to be crucial for protection against RSV [30,36–39]. Specifically, treating with chitosan one-day post RSV infection significantly increased the number of lung-resident CD8+ and CD4+ effector T cells compared to day 3 and no treatment groups (Fig. 1C and D). In addition, the population of CD4+ regulatory T cells (Tregs) expressing transcription factor box P3 (Foxp3) also increased considerably in mice treated with chitosan one day post-infection compared to day 3 and no chitosan treatment groups (Fig. 1E). As expected, due to the use of a low-dose RSV primary infection, lung histopathological presentations associated with severe RSV disease [40] were not observed in the infected mice. As such, we did not observe the effects of chitosan on RSV-induced histopathology. Collectively, these results indicate that chitosan augments the induction of tissue-resident effector T cells and Tregs, and the timing of chitosan administration following infection has a profound effect on T cell activation.

3.2. Chitosan augments viral clearance elicited by inactivated RSV vaccine

Previous studies have demonstrated the use of chitosan in increasing neutralizing antibody levels and promoting a balance between Th1 and Th2 responses during viral infections when used as a vaccine adjuvant or a therapeutic treatment [9,41–44]. FIRSV, however, has been shown to induce skewed immune responses with high levels of Th2 antibodies with poor RSV neutralizing ability [28]. Therefore, we investigated if chitosan treatment of FIRSV-immunized mice prior to RSV challenge may enhance FIRSV-induced protection. To that end, Balb/c mice were twice immunized with FIRSV or formaldehyde-inactivated cell control (FI-Mock) and given chitosan intranasally twice before RSV challenge (Fig. 2A). As previously reported, the time points for chitosan treatment that provided the most effective protection against influenza [10] were chosen. Four days post-challenge, lung viral titer was significantly lower in FIRSV-immunized mice treated with chitosan compared to all other treatment groups (Fig. 2B). Similar to other studies, mice vaccinated with 10^5–10^6 PFU of FIRSV and challenged with 10^5–10^6 PFU RSV had lung viral titer of 10^4 PFU/g

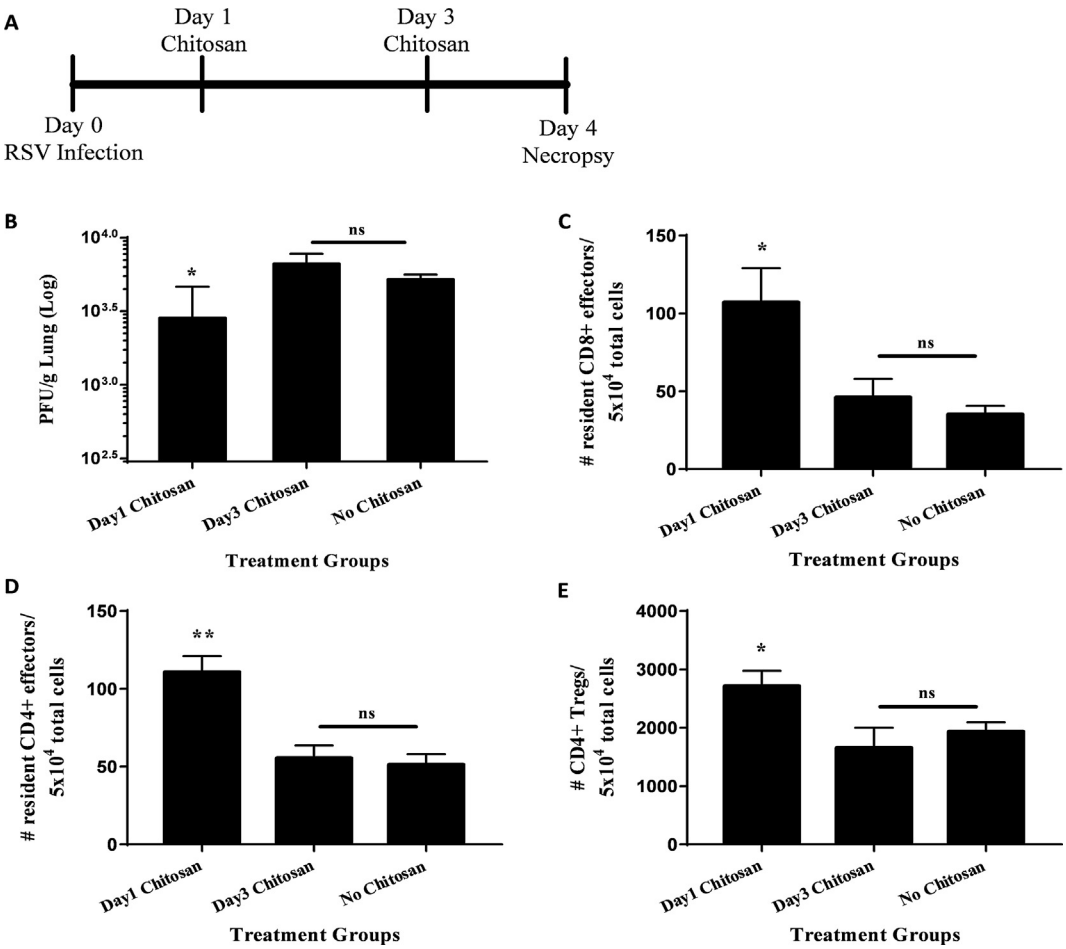

Fig. 1. Chitosan treatment following RSV infection leads to enhanced induction of resident T cells and Tregs. (A) Schematic diagram of the RSV infection and chitosan treatment timeline. (B) Lung viral titer determined using plaque assay 4 days post infection (n = 5). Flow cytometry was used to determine the number of lung resident T effector cells and Tregs. (C) Resident CD8+ T effector cells are CD3− CD8+ CD44+ CD62L− CCR7− CD103+, (D) resident CD4+ T effector cells are CD3− CD4+ CD44+ CD62L− CCR7− CD103+, and (E) Tregs are CD3− CD4+ CD127− CD25+ Foxp3+ in mice that were intravenously injected with BV510-conjugated anti-mouse CD3 prior to necropsy. Data shown is mean ± SEM; n = 7 per group; $^{*}p < 0.05$, $^{**}p < 0.01$ (one-way ANOVA with Bonferroni posttest). The Day 1 chitosan group was compared to Day 3 chitosan and No chitosan groups in panels B, C, D and E. PFU: Plaque forming units.

4 days post-challenge [28,31]. Although some studies have reported better clearance of RSV in FIRSV-immunized mice, these studies used different immunization schedules and doses along with higher challenge doses resulting in either undetectable lung viral titer [45,46] or low lung viral titer of 10^2–10^3 PFU/g lung [47,48]. It is interesting to note that chitosan treatment prior to RSV challenge did not reduce virus replication (FI-Mock+Chitosan; Fig. 2B), an observation different from that with treatment one day after RSV challenge (Fig. 1B). These results collectively suggest that chitosan, when used alone or in the absence of antigen administration, has a small time window to effectively inhibit RSV replication, whereas FIRSV itself shows minimal inhibition of virus but FIRSV in conjunction with chitosan can significantly suppress virus replication.

As FIRSV can induce exacerbated lung inflammatory reaction, we investigated whether chitosan treatment could reduce lung pathology. To this end, lung tissues from the animals were HPE and PAS stained for histopathological analysis of interstitial disease, edema, perivascular aggregates of leukocytes and mucus, respectively. All groups except the naïve group showed some histopathological changes, which was also reported by others [40]. Specifically, other studies showed RSV challenge of wild-type RSV primed animals resulted in mild perivasculitits and peribronchiolitis in the animals, which peaked 2 days post-challenge and diminished to undetectable levels by day 9; there was no sign of interstitial inflammation or alveolitis in these animals. Importantly, all FIRSV-immunized mice regardless of chitosan treatment exhibited severe ERD as demonstrated by significantly higher perivascular cell infiltration and mucus cell hyperplasia with mucus in the bronchiolar lumen (Fig. 2C and D), while vaccination with FI-Mock resulted in minimal cellularity and mucus irrespective of the chitosan treatment. These findings suggest that the exacerbated pulmonary inflammation is unrelated to virus replication but is greatly influenced by other immunopathological mechanisms (see below for more discussion).

3.3. The effect of chitosan on specific anti-RSV antibody responses

Having observed chitosan in conjunction with FIRSV significantly reduced viral loads in the lung, we set out to determine the antibody responses in these animals. As shown in Fig. 3A, a high RSV F-specific IgG1 to IgG2a ratio indicative of a Th2 skew, and a hallmark of FIRSV [28], was observed exclusively in the FIRSV group without chitosan treatment (Fig. 3B), whereas chitosan was able to restore the balance between IgG1 and IgG2a in FIRSV-immunized mice. As expected, a 1:1 ratio between IgG1 and IgG2a was observed in the other two control groups. Importantly, neutralizing antibody levels were significantly higher in the FIRSV group with chitosan treatment compared to other groups

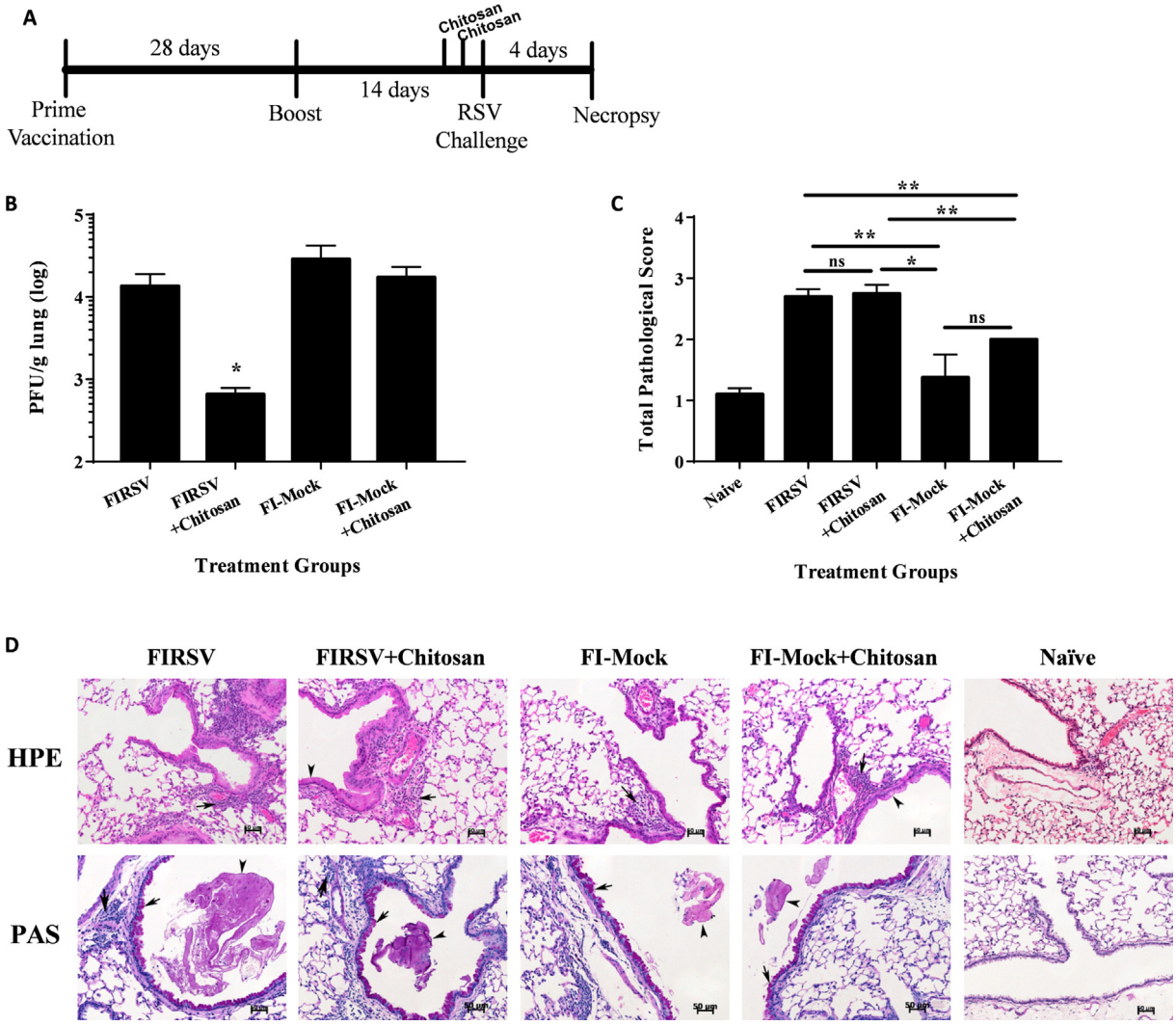

Fig. 2. Chitosan enhances FIRSV-induced viral clearance without reversal of ERD. (A) Schematic diagram of the immunization, chitosan treatment, RSV challenge and necropsy timeline. (B) Lung viral titer determined using plaque assay 4 days post infection. (C) Pathological scoring of lung tissue. Perivascular leukocyte infiltration and mucus were scored using HPE and PAS stained slides, respectively, 4 days post-challenge. An average of the two scores is shown. (D) Representative images of HPE and PAS stained mouse lungs post-challenge at 20X magnification. In the HPE slides, the arrows point to the extensive cell infiltration and in the PAS stained slides, the arrows point to the mucus-positive cells and mucus in the bronchiolar lumen. Data shown is mean ± SEM; n = 5 per group; $^{*}p < 0.05$, $^{**}p < 0.01$ (one-way ANOVA with Bonferroni posttest). FIRSV: Formaldehyde-inactivated RSV, FI-Mock: Formaldehyde-inactivated cell control, PFU: Plaque forming units.

(Fig. 3C, Fig. S2). It is understood that chitosan itself would not change the structure of the viral antigens in the FIRSV preparation; it would be worthy studying its adjuvant effects on native RSV antigens, which have not been inactivated (more in discussion). Overall, interestingly, chitosan was able to have an effect on FIRSV-induced serum antibody isotype and neutralizing titer in only 3 days post treatment. The exact mechanism remains to be fully understood.

3.4. Chitosan increases FIRSV-elicited Tregs

Next, we examined the cell influx in the lungs of immunized/chitosan-treated mice (Fig. 4A). The number of total lung-resident CD4+ T cells was drastically increased in FIRSV-vaccinated mice following chitosan treatment compared to other groups (Fig. 4B), while the highest levels of CD4+ Tregs were observed following FIRSV vaccination in conjunction with chitosan treatment (Fig. 4C). It is of note that FIRSV immunization alone significantly decreased total tissue-resident CD4+ T cells and CD4+ Tregs compared to FI-Mock immunization (Fig. 4B and C), accompanied by a significant increase in RSV F protein specific Th2 cytokines, IL-5 (Fig. 4D) and IL-13 (Fig. 4E). These FIRSV-induced pulmonary T cells responses were altered with chitosan treatment as demonstrated by the significant increase in the number of tissue-resident CD4+ T cells and Tregs compared to the other treatment groups as well as decreased levels of IL-5 and IL-13, similar to that of the FI-Mock control group. Taken together, chitosan exerts its adjuvant activity by increasing pulmonary resident CD4+ T cells and Tregs (Fig. 4) and inducing higher levels of neutralizing antibody (Fig. 3C, Fig. S2).

4. Discussion

Because of its proven safety record and a variety of immunomodulatory properties, chitosan has been extensively studied for its potential as a vaccine adjuvant. Not only does it have a profound effect on humoral immunity [9,44], it also affects cell-mediated immunity [49]. Several lines of evidence prompted us to conduct the current study in which FIRSV vaccine was used as a model vaccine to study the mechanisms of chitosan as a therapeu-

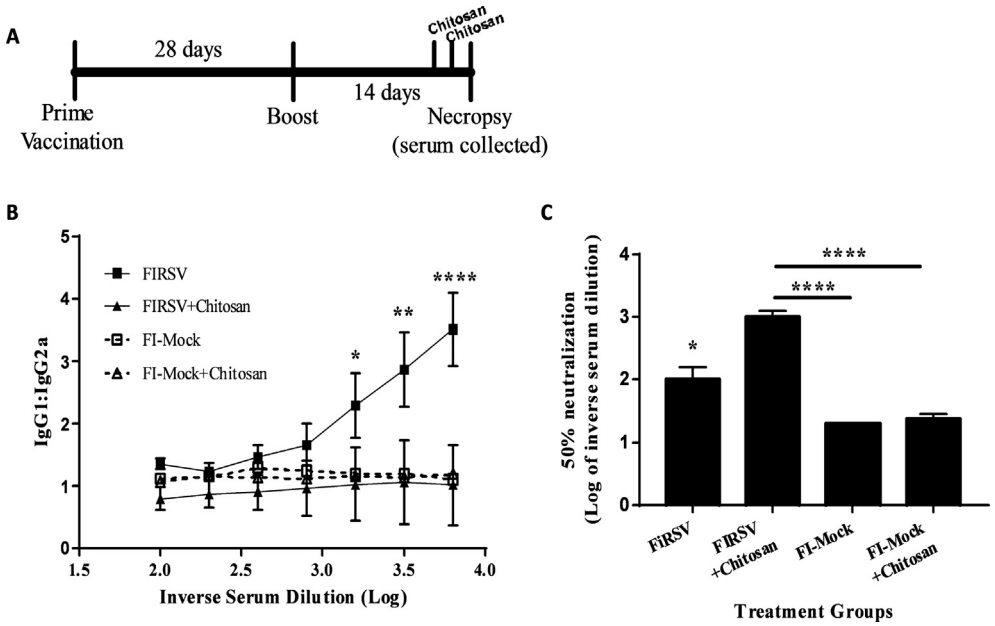

Fig. 3. FIRSV-induced antibodies are significantly enhanced with chitosan treatment. (A) Schematic diagram of the immunization, chitosan treatment and necropsy timeline. (B) RSV F-specific IgG1/IgG2a ratio in the serum before challenge was determined using ELISA. (C) RSV neutralizing ability of the mice serum pre-challenge. The serum dilution at which 50% neutralization is achieved is shown. Data shown is mean ± SEM; n = 5 per group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ (two-way ANOVA (B) or one-way ANOVA (C) with Bonferroni posttest). The FIRSV group was significantly different from all the other treatment groups.

tic. First, although the role of chitosan in stimulating NK cells and macrophages is well established [10,49], its effect on tissue-resident T cells still remains to be understood. This is an important issue as tissue resident effector/memory T cells play a vital role in tissue-specific protection. By secreting cytokines and chemokines, these cells recruit innate and adaptive immune cells into the infected tissue [34,50]. Indeed, these cells are believed to be the first line of defense against infection mainly due to their ability to proliferate rapidly and kill infected cells directly as well as through immune cell recruitment [51–54]. Although macrophages, dendritic cells, B cells and other immune cells can contribute to viral clearance by secreting antibodies, presenting antigens, inducing phagocytosis and other antibody or cell-mediated responses, tissue-resident T cells are crucial for effective protection against infection. Secondly, the effect of chitosan on regulatory T cells (Tregs) remains unknown. These cells play important roles in regulating the balance between effective antimicrobial immune responses and excessive effector T cell activation or antigen-presenting cell maturation and functionality [55–59] during immune response against infections. Third, by employing FIRSV vaccine as an model antigen to study the adjuvant activity of chitosan, we could gain better insight into chitosan-mediated immune enhancement, given FIRSV is known to induce skewed immune responses and poorly neutralizing antibodies, and that lung resident T cells are critical for robust RSV virus clearance and early establishment of cell-mediated responses [30]. Moreover, following RSV infection, Tregs have been shown to accumulate in the lungs and mediastinal lymph nodes in mice and recruit RSV-specific CD8+ cytotoxic T cells to the lungs thereby facilitating viral clearance [36,38,39]. Given these considerations, we conducted animal studies to investigate how chitosan could alter immune responses in the RSV vaccine model.

We report for the first time the effect of chitosan on Tregs and tissue-resident T cells following a RSV infection and FIRSV immunization. Specifically, we found that chitosan given post RSV infection resulted in a significant increase in pulmonary resident CD8+ and CD4+ T effector cells, and Tregs. Furthermore, the reversal of FIRSV-induced Th2-skewed immune responses by chitosan could be mediated by elevated levels of resident CD4+ T cells and CD4+ Tregs, leading to a decrease in Th2 cytokines (Fig. 4). These functional activities of chitosan, along with the capacity of substantially facilitating antigen-specific neutralizing antibodies (Fig. 3C), could have contributed to the significant inhibition of RSV replication (Fig. 2B).

While we are able to gain some insight into the mechanisms relating to the adjuvant activity of chitosan, it remains to be understood as to why chitosan failed to reduce exacerbated pulmonary inflammatory reactions or ERD in animals following FIRSV vaccination even if diminished Treg responses and increased Th2 responses have been implicated in ERD development [28,31,60]. However, it is unlikely that the development of ERD was correlated to the level of viral replication, as the viral loads were the lowest in FIRSV/chitosan group.

Our findings provide some mechanistic insight into chitosan-induced balanced host responses between Th1 and Th2. In agreement with studies that have shown that Tregs could downregulate Th2 responses [61,62], we show that chitosan upregulates Tregs, resulting in decreased levels of pulmonary Th2 cytokine production. The acute effects of chitosan on modulating Tregs, IgG isotypes and neutralizing antibody titers were observed under our experimental conditions where the host was primed with FIRSV or live RSV. Clearly, such speculation would need more data to be substantiated.

In conclusion, through this study, we have gained some mechanistic insight into the functional activities of chitosan as a potential treatment/adjuvant in the context of FIRSV-immunization and RSV infection. In addition to some well-characterized functional pathways reported by others, we show that chitosan can substantially augment antigen-specific immune responses through upregulating critical tissue-specific resident T cells and Tregs while significantly stimulating generation of neutralizing antibodies. Nonetheless, the exact molecular mechanisms remains to be

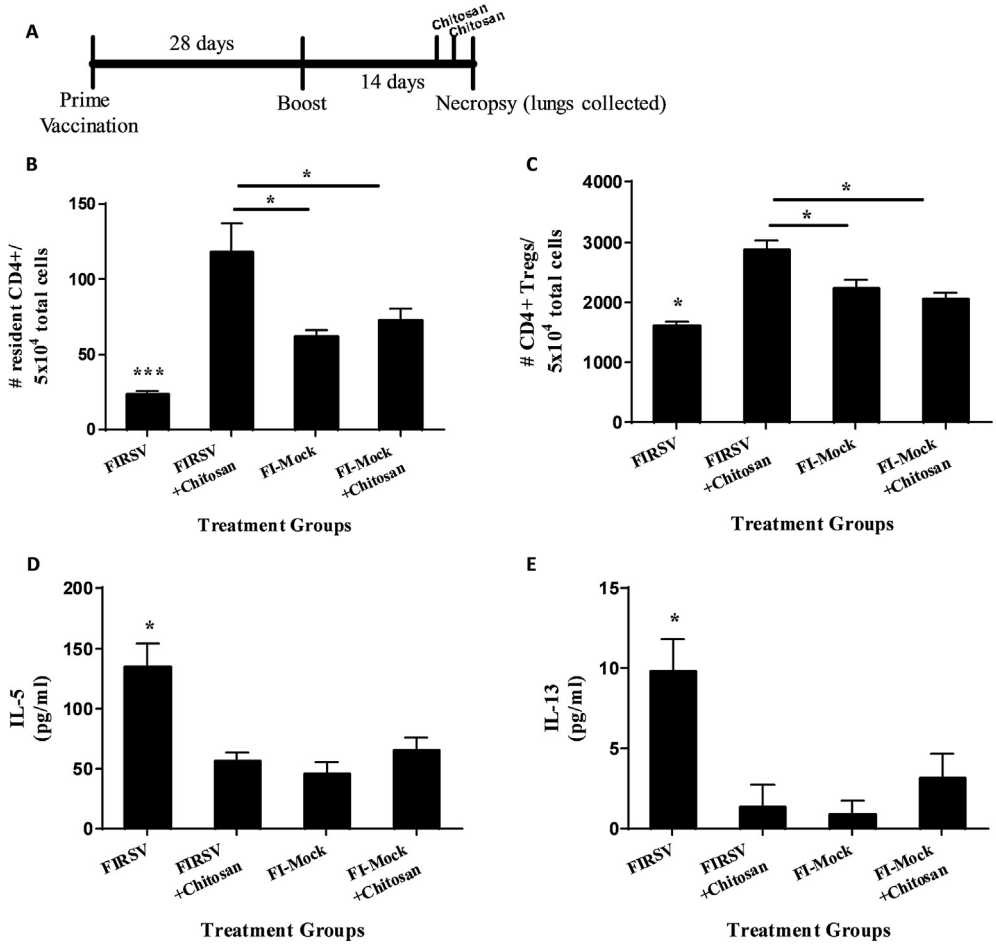

Fig. 4. FIRSV-elicited Tregs are improved with chitosan treatment leading to a decrease in Th2 cytokines. (A) Schematic diagram of the immunization, chitosan treatment and necropsy timeline. Flow cytometry was used to determine the number of lung resident CD4+ T cells and Tregs before RSV challenge. (B) Resident CD4+ T cells are CD3− CD4+ CD103+, and (C) Tregs are CD3− CD4+ CD127− CD25+ Foxp3+ in mice that were intravenously injected with BV510-conjugated anti-mouse CD3 prior to necropsy. Secreted levels of IL-5 (D) and IL-13 (E) were determined using the Luminex system in the lungs of mice collected before challenge following 48-hour ex-vivo stimulation with RSV F85-93 peptide. Data shown is mean ± SEM; n = 5 per group; $^{*}p < 0.05$ (one-way ANOVA with Bonferroni posttest). The FIRSV group was significantly different from all the other treatment groups.

elucidated; further studies are necessary to delineate the definitive roles of Tregs and resident T cells induced by chitosan with respect to protection against RSV infection.

Funding statement

This work was funded by Genomics Research and Development Initiative Grant, Government of Canada.

Contributors

AM, XL, SS, ZC, CL, WC, TC, MRM and LW conceived the overall study. AM, MSR, LL and CG performed the experiments and analyzed the data. AM and XL wrote the manuscript. All authors edited and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

Acknowledgements

We thank Bozena Jaentschke, Sathya Raman and Emily Dupuis for technical assistance. We are so grateful to Dr. Martha Navarro and all technicians in the animal facility for help. Thank you to Dr. Don Caldwell, veterinary pathologist at Health Canada, for his expert analysis of the lung tissues. Drs Roger Tam and Michael Johnston are acknowledged for their critical review of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.003.

References

[1] Brück WM, Slater JWCB. Chitin and chitosan from marine organisms. In: Kim S-KE, editor. Chitin, chitosan, oligosaccharides their deriv. New York: CRC Press; 2011. p. 11–23.
[2] Knezevic-Jugovic Z, Petronijevic ZSA. Chitin and chitosan from microorganisms. In: Kim S-KE, editor. Chitin, chitosan, oligosaccharides their deriv. New York: CRC Press; 2011. p. 25–36.
[3] Nwe N, Furuike TTH. Chitin and chitosan from terrestrial organisms. In: Kim S-KE, editor. Chitin, chitosan, oligosaccharides their deriv. New York: CRC Press; 2011. p. 3–10.
[4] Dornish M, Hagen A, Hansson E, Pecheur C, Verdier F, Skaugrud Ø. Safety of Protasan™: ultrapure chitosan salts for biomedical and pharmaceutical use. In: Adv chitin sci. Lyon, France: Andre Publisher; 1997. p. 664–70.
[5] Arca HÇ, Günbeyaz M, Şenel S. Chitosan-based systems for the delivery of vaccine antigens. Expert Rev Vaccines 2009;8:937–53. https://doi.org/10.1586/erv.09.47.

[6] Kean T, Thanou M. Biodegradation, biodistribution and toxicity of chitosan. Adv Drug Deliv Rev 2010;62:3–11. https://doi.org/10.1016/j.addr.2009.09.004.
[7] Harding S. Characterisation of chitosan-mucin complexes by sedimentation velocity analytical ultracentrifugation. In: Muzzarelli RAAPM, editor. Chitin handbook Eur chitin soc. p. 457–66.
[8] Morris G, Kök S, Harding S, Adams G. Polysaccharide drug delivery systems based on pectin and chitosan. Biotechnol Genet Eng Rev 2010;27:257–84.
[9] Smith A, Perelman M, Hinchcliffe M. Chitosan: a promising safe and immune-enhancing adjuvant for intranasal vaccines. Hum Vaccin Immunother 2014;10:797–807.
[10] Zheng M, Qu D, Wang H, Sun Z, Liu X, Chen J, et al. Intranasal administration of chitosan against influenza A (H7N9) virus infection in a mouse model. Sci Rep 2016;6:28729. https://doi.org/10.1038/srep28729.
[11] Sui Z, Chen Q, Fang F, Zheng M, Chen Z. Cross-protection against influenza virus infection by intranasal administration of M1-based vaccine with chitosan as an adjuvant. Vaccine 2010;28:7690–8. https://doi.org/10.1016/j.vaccine.2010.09.019.
[12] Sui Z, Chen Q, Wu R, Zhang H, Zheng M, Wang H, et al. Cross-protection against influenza virus infection by intranasal administration of M2-based vaccine with chitosan as an adjuvant. Arch Virol 2010;155:535–44. https://doi.org/10.1007/s00705-010-0621-4.
[13] Roon KI, Soons PA, Uitendaal MP, de Beukelaar F, Ferrari MD. Pharmacokinetic profile of alniditan nasal spray during and outside migraine attacks. Br J Clin Pharmacol 1999;47:285–90.
[14] Stoker DG, Reber KR, Waltzman LS, Ernst C, Hamilton D, Gawarecki D, et al. Analgesic efficacy and safety of morphine-chitosan nasal solution in patients with moderate to severe pain following orthopedic surgery. Pain Med 2008;9:3–12. https://doi.org/10.1111/j.1526-4637.2007.00300.x.
[15] Newman SP, Simpson M, Fisher T IK. Quantification of lung deposition and nasal mucociliary clearance for a nasally administered drug formulation containing chitosan. In: Dalby RN, Byron PR, Peart J SJ, FS, editor. Respir. Drug Deliv. IX. Virginia: Virginia Commonw. Univ.; 2004, p. 617–9.
[16] Aspden TJ, Mason JDT, Jones NS, Lowe J, Skaugrud Ø, Illum L. Chitosan as a nasal delivery system: the effect of chitosan solutions on in vitro and in vivo mucociliary transport rates in human turbinates and volunteers. J Pharm Sci 1997;86:509–13. https://doi.org/10.1021/js960182o.
[17] Calvo C, García-García ML, Blanco C, Pozo F, Flecha IC, Pérez-Breña P. Role of rhinovirus in hospitalized infants with respiratory tract infections in Spain. Pediatr Infect Dis J 2007;26:904–8. https://doi.org/10.1097/INF.0b013e31812e52e6.
[18] Hall CB, Weinberg GA, Blumkin AK, Edwards KM, Staat MA, Schultz AF, et al. Respiratory syncytial virus-associated hospitalizations among children less than 24 months of age. Pediatrics 2013;132:e341–8. https://doi.org/10.1542/peds.2013-0303.
[19] Falsey AR. Respiratory syncytial virus infection in elderly and high-risk adults. Exp Lung Res 2005;31(Suppl 1):77.
[20] Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet 2010;375:1545–55. https://doi.org/10.1016/S0140-6736(10)60206-1.
[21] Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969;89:422–34.
[22] Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am J Epidemiol 1969;89:449–63.
[23] Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am J Epidemiol 1969;89:435–48.
[24] Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. Epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated Rs virus vaccine. Am J Epidemiol 1969;88:405–21.
[25] Prince GA, Curtis SJ, Yim KC, Porter DD. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. J Gen Virol 2001;82:2881–8. https://doi.org/10.1099/0022-1317-82-12-2881.
[26] Muralidharan A, Li C, Wang L, Li X. Immunopathogenesis associated with formaldehyde-inactivated RSV vaccine in preclinical and clinical studies. Expert Rev Vaccines 2017. https://doi.org/10.1080/14760584.2017.1260452.
[27] Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, et al. A role for immune complexes in enhanced respiratory syncytial virus disease. J Exp Med 2002;196:859–65.
[28] Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 2009;15:34–41. https://doi.org/10.1038/nm.1894.
[29] Jaberolansar N, Chappell KJ, Watterson D, Bermingham IM, Toth I, Young PR, et al. Induction of high titred, non-neutralising antibodies by self-adjuvanting peptide epitopes derived from the respiratory syncytial virus fusion protein. Sci Rep 2017;7:11130. https://doi.org/10.1038/s41598-017-10415-w.
[30] Morabito KM, Ruckwardt TR, Redwood AJ, Moin SM, Price DA, Graham BS. Intranasal administration of RSV antigen-expressing MCMV elicits robust tissue-resident effector and effector memory CD8+ T cells in the lung. Mucosal Immunol 2017;10:545–54. https://doi.org/10.1038/mi.2016.48.
[31] Knudson CJ, Hartwig SM, Meyerholz DK, Varga SM. RSV vaccine-enhanced disease is orchestrated by the combined actions of distinct CD4 T cell subsets. PLoS Pathog 2015. https://doi.org/10.1371/journal.ppat.1004757.
[32] Schmidt ME, Knudson CJ, Hartwig SM, Pewe LL, Meyerholz DK, Langlois RA, et al. Memory CD8 T cells mediate severe immunopathology following respiratory syncytial virus infection. PLOS Pathog 2018;14:. https://doi.org/10.1371/journal.ppat.1006810e1006810.
[33] Anderson KG, Mayer-Barber K, Sung H, Beura L, James BR, Taylor JJ, et al. Intravascular staining for discrimination of vascular and tissue leukocytes. Nat Protoc 2014;9:209–22. https://doi.org/10.1038/nprot.2014.005.
[34] McMaster SR, Wilson JJ, Wang H, Kohlmeier JE. Airway-resident memory CD8 T cells provide antigen-specific protection against respiratory virus challenge through rapid IFN-γ production. J Immunol 2015;195:203–9. https://doi.org/10.4049/jimmunol.1402975.
[35] Weiss KA, Christiaansen AF, Fulton RB, Meyerholz DK, Varga SM. Multiple CD4+ T cell subsets produce immunomodulatory IL-10 during respiratory syncytial virus infection. J Immunol 2011;187:3145–54. https://doi.org/10.4049/jimmunol.1100764.
[36] Fulton RB, Meyerholz DK, Varga SM. Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. J Immunol 2010;185:2382–92. https://doi.org/10.4049/jimmunol.1000423.
[37] Raiden S, Pandolfi J, Payasliàn F, Anderson M, Rivarola N, Ferrero F, et al. Depletion of circulating regulatory T cells during severe respiratory syncytial virus infection in young children. Am J Respir Crit Care Med 2014;189:865–8. https://doi.org/10.1164/rccm.201311-1977LE.
[38] Lee DCP, Harker JAE, Tregoning JS, Atabani SF, Johansson C, Schwarze J, et al. CD25+ natural regulatory T cells are critical in limiting innate and adaptive immunity and resolving disease following respiratory syncytial virus infection. J Virol 2010;84:8790–8. https://doi.org/10.1128/JVI.00796-10.
[39] Ruckwardt TJ, Bonaparte KL, Nason MC, Graham BS. Regulatory T cells promote early influx of CD8+ T cells in the lungs of respiratory syncytial virus-infected mice and diminish immunodominance disparities. J Virol 2009;83:3019–28. https://doi.org/10.1128/JVI.00036-09.
[40] Power UF, Huss T, Michaud V, Plotnicky-Gilquin H, Bonnefoy J-Y, Nguyen TN. Differential histopathology and chemokine gene expression in lung tissues following respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV- or BBG2Na-immunized mice. J Virol 2001;75:12421–30. https://doi.org/10.1128/JVI.75.24.12421-12430.2001.
[41] Atmar RL, Bernstein DI, Harro CD, Al-Ibrahim MS, Chen WH, Ferreira J, et al. Norovirus vaccine against experimental human norwalk virus illness. N Engl J Med 2011;365:2178–87. https://doi.org/10.1056/NEJMoa1101245.
[42] El-Kamary SS, Pasetti MF, Mendelman PM, Frey SE, Bernstein DI, Treanor JJ, et al. Adjuvanted intranasal norwalk virus-like particle vaccine elicits antibodies and antibody-secreting cells that express homing receptors for mucosal and peripheral lymphoid tissues. J Infect Dis 2010;202:1649–58. https://doi.org/10.1086/657087.
[43] Mann AJ, Noulin N, Catchpole A, Stittelaar KJ, de Waal L, Veldhuis Kroeze EJB, et al. Intranasal H5N1 vaccines, adjuvanted with chitosan derivatives, protect ferrets against highly pathogenic influenza intranasal and intratracheal challenge. PLoS ONE 2014;9:. https://doi.org/10.1371/journal.pone.0093761e93761.
[44] Wen Z-S, Xu Y-L, Zou X-T, Xu Z-R. Chitosan nanoparticles act as an adjuvant to promote both Th1 and Th2 immune responses induced by ovalbumin in mice. Mar Drugs 2011;9:1038–55. https://doi.org/10.3390/md9061038.
[45] Boelen A, Andeweg A, Kwakkel J, Lokhorst W, Bestebroer T, Dormans J, et al. Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice. Vaccine 2000;19:982–91.
[46] Kamphuis T, Meijerhof T, Stegmann T, Lederhofer J, Wilschut J, de Haan A. Immunogenicity and protective capacity of a virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid A in mice. PLoS ONE 2012;7:. https://doi.org/10.1371/journal.pone.0036812e36812.
[47] Johnson TR, Teng MN, Collins PL, Graham BS. Respiratory syncytial virus (RSV) G glycoprotein is not necessary for vaccine-enhanced disease induced by immunization with formalin-inactivated RSV. J Virol 2004;78:6024–32. https://doi.org/10.1128/JVI.78.11.6024-6032.2004.
[48] Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J Virol 1996;70:2852–60.
[49] Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine 2007;25:2085–94. https://doi.org/10.1016/j.vaccine.2006.11.034.
[50] Schenkel JM, Fraser KA, Beura LK, Pauken KE, Vezys V, Masopust D. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. Science (80-) 2014;346:98–101. https://doi.org/10.1126/science.1254536.
[51] Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, Carbone FR. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. Nat Immunol 2009;10:524–30. https://doi.org/10.1038/ni.1718.
[52] Ariotti S, Hogenbirk MA, Dijkgraaf FE, Visser LL, Hoekstra ME, Song J-Y, et al. Skin-resident memory CD8+ T cells trigger a state of tissue-wide pathogen

alert. Science (80-) 2014(346):101–5. https://doi.org/10.1126/science.1254803.
[53] Schenkel JM, Masopust D. Tissue-resident memory T cells. Immunity 2014;41:886–97. https://doi.org/10.1016/j.immuni.2014.12.007.
[54] Jiang X, Clark RA, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS. Skin infection generates non-migratory memory CD8+ TRM cells providing global skin immunity. Nature 2012;483:227–31. https://doi.org/10.1038/nature10851.
[55] Shalev I, Schmelzle M, Robson SC, Levy G. Making sense of regulatory T cell suppressive function. Semin Immunol 2011;23:282–92. https://doi.org/10.1016/j.smim.2011.04.003.
[56] Afzali B, Mitchell P, Lechler RI, John S, Lombardi G. Translational Mini-Review Series on Th17 Cells: induction of interleukin-17 production by regulatory T cells. Clin Exp Immunol 2010;159:120–30. https://doi.org/10.1111/j.1365-2249.2009.04038.x.
[57] O'Connor RA, Taams LS, Anderton SM. Translational mini-review series on Th17 Cells: CD4 $^{+}$ T helper cells: functional plasticity and differential sensitivity to regulatory T cell-mediated regulation. Clin Exp Immunol 2010;159:137–47. https://doi.org/10.1111/j.1365-2249.2009.04040.x.
[58] Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. Trends Mol Med 2007;13:108–16. https://doi.org/10.1016/j.molmed.2007.01.003.
[59] Huang C-T, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. Immunity 2004;21:503–13. https://doi.org/10.1016/j.immuni.2004.08.010.
[60] Liu J, Cao S, Peppers G, Kim S-H, Graham BS. Clonotype-specific avidity influences the dynamics and hierarchy of virus-specific regulatory and effector CD4 $^{+}$ T-cell responses. Eur J Immunol 2014;44:1058–68. https://doi.org/10.1002/eji.201343766.
[61] Durant LR, Makris S, Voorburg CM, Loebbermann J, Johansson C, Openshaw PJM. Regulatory T cells prevent Th2 immune responses and pulmonary eosinophilia during respiratory syncytial virus infection in mice. J Virol 2013;87:10946–54. https://doi.org/10.1128/JVI.01295-13.
[62] Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+ CD25+ regulatory T cells is interleukin 10 dependent. J Exp Med 2005;202:1539–47. https://doi.org/10.1084/jem.20051166.