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Epithelial Immunomodulation by Aerosolized Toll-like Receptor Agonists Attenuates Allergic Responsiveness in Mice

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Abbreviations: TLRs, Toll-like receptors; ODN/PamCSK2, O/P; O/P, PUL-042; BAL, bronchoalveolar lavage; OVA, ovalbumin; PAMPS, pattern-associated molecular patterns; TSLP, thymic-stromal lymphopoietin

Abstract

Allergic asthma is a chronic inflammatory respiratory disease associated with eosinophilic infiltration, increased mucus production, airway hyperresponsiveness (AHR), and airway remodeling. Epidemiologic data has revealed that the prevalence of allergic sensitization and associated diseases has increased in the twentieth century. This has been hypothesized to be partly due to reduced contact with microbial organisms (the hygiene hypothesis) in industrialized society. Airway epithelial cells, once considered a static physical barrier between the body and the external world, are now widely recognized as immunologically active cells that can initiate, maintain, and restrain inflammatory responses, such as those that mediate allergic disease. Airway epithelial cells can sense allergens via myriad expression of Toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs). We sought to determine whether the innate immune response stimulated by a combination of Pam2CSK4 (“Pam2”, TLR2/6 ligand) and a class C oligodeoxynucleotide ODN362 (“ODN”, TLR9 ligand) when delivered together by aerosol (“Pam2ODN”), can modulate the allergic immune response to allergens. Treatment with Pam2ODN 7 days before sensitization to House Dust Mite (HDM) extract resulted in a strong reduction in eosinophilic and lymphocytic inflammation. This Pam2ODN immunomodulatory effect was also seen using Ovalbumin (OVA) and A. oryzae mouse models. The immunomodulatory effect was observed as much as 30 days before sensitization to HDM, but ineffective just 2 days after sensitization, suggesting that Pam2ODN mechanism of action involves immunomodulation of the response from airway epithelial cells to aeroallergens, possibly due to a repolarization of the immune response from type 2 to a type 3/type 17 direction. Furthermore, Pam2 and ODN cooperated synergistically to induce the immunomodulatory phenotype suggesting that this treatment is superior to all investigational TLR receptor agonists in the allergen immunotherapy setting which only utilize a single PRR agonist at one time. These data suggest that allergen immunotherapy using Pam2ODN might have a role in maximizing allergen immunotherapy effectiveness.

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Introduction

Allergic asthma is a chronic inflammatory respiratory disease associated with eosinophilic infiltration, increased mucus production, airway hyperresponsiveness (AHR), and airway remodeling.[1] Epidemiologic data has revealed that the prevalence of allergic sensitization and associated diseases has increased in the twentieth century. This has been hypothesized to be partly due to reduced contact with microbial organisms (the hygiene hypothesis) in industrialized society[2]. In one study comparing Amish and Hutterite children, higher levels of microbial elements in farm dust were found to be strongly protective against developing asthma.[3] In mechanistic studies, this effect was found to be mediated by downregulation of inflammatory pathways within airway epithelial cells.[4]

Airway epithelial cells, once considered a static physical barrier between the body and the external world, are now widely recognized as immunologically active cells that can initiate, maintain, and restrain inflammatory responses, such as those that mediate allergic disease.[5] Lung airway epithelial cells express myriad pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) that enable them to sense and respond to a variety of external triggers, usually referred to as pathogen-associated molecular patterns (PAMPs). The epithelial-derived cytokines IL-33, TSLP, and IL-25 are widely recognized as alarmins produced in response to allergens, such as those associated with house-dust mites (HDM). These cytokines activate leukocytes involved in allergic inflammation, such as eosinophils, T helper 2 (T_H2) cells, mast cells, basophils, type 2 innate lymphoid cells (ILC2), and dendritic cells (DC), which work together to polarize the immune response in a type 2 direction.

Our group has studied how to stimulate lung epithelial cells to defend against microbial pathogens.[6] We have shown that a combination of Pam2CSK4 (“Pam2”, TLR2/6 ligand) and a class C oligodeoxynucleotide ODN362 (“ODN”, TLR9 ligand), when delivered together by aerosol (“Pam2ODN”) synergistically activate an innate immune response in the lung mucosa (but not systemically) which results in host resistance to bacteria, fungi, and viruses.[7–10] Pam2ODN-mediated pathogen resistance is initiated very rapidly and has been shown to be mediated by lung epithelial cells.[11]

More recently, we showed that Pam2ODN can attenuate chronic asthma-like lung disease in mice infected with Sendai virus (SeV).[12] We reasoned that Pam2ODN would exert a protective effect against the chronic disease by reducing the viral burden in the acute infection, but we observed some efficacy when Pam2ODN treatment was given at remote time points when the virus burden was not reduced in the acute infection. This partial discordance between the reduction in viral burden and chronic disease severity led us to hypothesize that Pam2ODN also modulated the type 2 immune response that drives the chronic disease.

To disentangle antimicrobial and immunomodulatory effects of Pam2ODN, we now use allergic models where the antimicrobial effect is irrelevant. In this study, we show that Pam2ODN attenuates the type 2 allergic immune response to HDM, Ovalbumin (OVA), and *Aspergillus oryzae* (Ao) in the lung mucosa.

Results

Pam2ODN prevents allergic inflammation to HDM

Mice were sensitized by airway instillation of 100 μg HDM extract at day 0, followed by 6 daily challenges of 10 μg HDM extract from day 7 to 12, and evaluation of allergic inflammation on day 15 (Fig. 1A).[13] As previously reported, HDM-sensitized, HDM-challenged (HDM/HDM) mice exhibited robust allergic inflammation, though PBS-sensitized, HDM-challenged (PBS/HDM) did not (Fig. S1). Allergic inflammation to HDM was reflected by the substantial influx of eosinophils (Fig 1B, orange arrowhead) and lymphocytes (Fig. 1B, black arrowhead) with small numbers of neutrophils (Fig 1B, blue arrowhead). Macrophages were increased only slightly in number but were observed to be larger and more intensely stained than PBS/PBS, indicating activation (Fig. 1B, open arrowhead).

When mice were treated with a single Pam2ODN treatment 7 days before HDM sensitization, we observed strong reductions in leukocytes collected in lung lavage fluid, compared to HDM/HDM mice (Fig. 1B). Differential cell quantification with Wright-Giemsa stain revealed a >90% decrease in eosinophils (Fig. 1C), >85% decrease in lymphocytes (Fig. 1D), > 30% decrease in macrophages (Fig 1E), and > 95% decrease in neutrophils (Fig. 1F). Pam2ODN treatments similarly showed efficacy when administered 30- and 15-days before sensitization, with a trend toward less efficacy when the time between treatment and sensitization was increased.

On H&E staining of lung tissue, most of the infiltrating leukocytes were localized to the submucosal space between major airways and vasculature, though some alveolar inflammation was also observed (Fig. 1G-H, Fig. S2). Mucous metaplasia of lung epithelial cells was assessed by staining lung tissue with fluorescent PAS stain (PAFS) (Fig. 1I).[14] Quantitative image analysis of intracellular mucin content revealed that intracellular mucin accumulates moderately in PBS/HDM mice despite the near-complete absence of inflammatory leukocytes, though HDM sensitization was required for the full phenotype (Fig. 1J). A single Pam2ODN treatment 7 days before HDM sensitization reduced intracellular epithelial mucin content >35%.

Pam2ODN blocks sensitization to HDM in the airway

We next evaluated whether Pam2ODN prevented sensitization to HDM or attenuated allergic inflammation during the challenge phase. To do this, we varied the time-interval and the relative chronological order of Pam2ODN treatment and HDM sensitization. When Pam2ODN treatment was administered just 1 day before HDM sensitization, eosinophils were still significantly reduced, but this effect was completely absent when treatment was administered just 2 days after HDM sensitization, and at any later time point (Fig 2A). Since multiple treatments of Pam2ODN had higher efficacy than a single treatment (Fig. S3), we evaluated whether the lack of Pam2ODN efficacy after HDM sensitization could be due to insufficient treatment by administering 6 daily Pam2ODN treatments after HDM sensitization. As was observed using single treatments, multiple treatments after HDM sensitization did not reduce allergic inflammation (Fig. 2B).

We also evaluated whether Pam2ODN could prevent allergic inflammation when HDM sensitization occurred at a different location than the lungs. To do this, we used the same time

schedule for HDM sensitization and challenge, as previously shown, except that HDM sensitization was performed by intraperitoneal injection of 100 μg HDM (Fig S4A). Pam2ODN treatments were administered at day 0 and day 6 to cover the entire challenge period, however this resulted in no significant reduction in allergic inflammation (Fig. S4B-E).

Pam2 and ODN interact synergistically

The ligands Pam2 and ODN were selected for their remarkable synergy, however this characteristic has only been evaluated for antimicrobial resistance thus far.[7] To evaluate whether Pam2 and ODN also cooperate for allergic immunomodulation, the agonists were aerosolized individually or in combination 7 days before HDM sensitization. Neither Pam2 nor ODN exhibited any significant efficacy when administered alone, but the combination Pam2ODN treatment completely blocked allergic inflammation (Fig 3A-D).

Pam2ODN attenuates allergic inflammation to OVA

To evaluate the generalizability of Pam2ODN allergic immunomodulation, we also tested a conventional 3-week OVA model (Fig. 4A), consisting of 2 sensitizing intraperitoneal injections during the first week, and aerosol challenges beginning on day 8 administered every 2-3 days until day 21. From day 21 to day 24, OVA aerosol challenges were given daily, and allergic inflammation was assessed at day 24. Pam2ODN treatments were given immediately before OVA challenges. OVA-sensitized, OVA-challenged (OVA/OVA) mice had elevated cell counts in eosinophils, lymphocytes, and neutrophils, compared to PBS-sensitized, OVA-challenged (PBS/OVA) mice (Fig 4B-E). Pam2ODN treatment reduced eosinophils >75%, but macrophages and lymphocytes were unchanged. With this Pam2ODN treatment model, neutrophils were observed to be elevated, however this is likely a direct response to Pam2ODN, which elicits neutrophilic inflammation over a period of 72 hours, rather than a secondary effect on the allergic response.[15] Airway epithelial mucin content was also decreased >25% by Pam2ODN treatment (Fig. 4F).

Assessing changes in the systemic immune response showed that Pam2ODN treatment reduced serum immunoglobulin E (IgE) levels by >50% (Fig 4G), but total IgG (Fig. 4H) and IgG1 (Fig 4I) were unchanged. IgG2a levels, which increase with type 1 immune responses, decreased in OVA/OVA mice, compared to PBS/PBS, and Pam2ODN treatment reversed this change (Fig 4J).

Pam2ODN attenuates allergic inflammation to Aspergillus oryzae

Allergic sensitization can occur following exposure to fungal antigens[cite?]. To evaluate whether fungal allergic inflammation could also be attenuated by Pam2ODN, we tested a combined Ao-OVA model (Fig. 5A), with a similar time schedule as with OVA alone (Fig. 4A), except Ao-OVA or Pam2ODN were not administered from days 21 to 24. Pam2ODN treatment caused a >50% reduction in eosinophils (Fig 5B). Macrophages were decreased in Ao-OVA challenged mice, and this was reversed by Pam2ODN treatment (Fig. 5C). Lymphocytes were not significantly elevated in Ao-OVA challenged mice, compared to PBS/PBS (Fig. 5D). Neutrophil levels were not increased in Pam2ODN-treated groups (Fig 5E) due to the removal of Pam2ODN treatments within 3 days of evaluation (Fig. 5A). Airway epithelial mucin content was also observed to be decreased >30% with Pam2ODN treatment (Fig. 5F).

Discussion

Over the last two decades, scientific interest in utilizing PRR agonists, particularly TLRs, for the development of novel short-duration immunotherapy has grown considerably.[16] However, these novel agents have not achieved significant clinical success, such as a recent study using TLR9 agonists that failed to improve asthma control.[17] The inability to translate preclinical success into clinical results is likely due to the complexity of allergic sensitization and the interplay of different variables affecting the degree of type 2 polarization. Our study has attempted to isolate some of the variables that contribute to allergic sensitization in the airway. We have also identified a novel combination of TLR agonists which exhibit unique synergistic responses and thus are likely to be superior to any single compound such as those tested to date.

We have previously demonstrated that pharmacologic stimulation of innate immunity in the lungs of mice by aerosolized delivery of TLR agonists can induce host resistance to microbial pathogens.[18–22] In an earlier study, we found that Pam2ODN was effective in attenuating the severity of the chronic asthma-like disease that persists after viral clearance.[23] We hypothesized that reduced viral burden would be the primary mechanism of action by which Pam2ODN attenuated the severity of chronic disease, but upon investigation, we also observed direct immunomodulatory effects. To disentangle Pam2ODN's antiviral and immunomodulatory responses, we used allergic mouse models in which Pam2ODN's antimicrobial effects would not affect disease severity. In these studies, we observed Pam2ODN was effective at reducing allergic inflammation and mucous metaplasia from three different allergens – HDM, OVA, and Ao. While each model displays unique immunogenic characteristics, SeV, HDM, OVA, and Ao all lead to a chronic obstructive lung disease driven by polarization of the lung mucosa towards type 2 immunity. The effectiveness of this treatment against all of these different stimuli suggests that Pam2ODN is a generalizable immunomodulatory agent that can regulate the type 2 immune response.

In a healthy host, the antimicrobial response to Pam2ODN is robust but does not persist for very long.[15] Because of its short duration of action, we hypothesized that we'd be able to identify which part of the allergic sensitization process was blocked upon Pam2ODN exposure. Furthermore, one of the primary advantages of the acute HDM model is the ability to isolate host sensitization and recall phases.[13] By varying single treatments of Pam2ODN at different time points before and after sensitization, we were able to show that Pam2ODN treatment is vastly superior when administered before sensitization. We observed Pam2ODN efficacy in as little as 24 hours before sensitization, but a single treatment 2 days after sensitization was completely ineffective. These results suggest that once the host has already begun to polarize in a type 2 direction, it is very difficult to modulate in any other direction.

Independently, these results using the HDM model suggest that immunomodulation after sensitization may be impossible to achieve. However, HDM is an extremely strong pro-Th2 immunogen, and we wanted to evaluate weaker immunogenic stimuli could be modulated after sensitization. To evaluate this, we used an OVA allergic model, which involves systemic sensitization by intraperitoneal injection combined with an adjuvant, alum. Unlike what we observed with HDM, we were able to show significant Pam2ODN efficacy despite treatment

being delivered after systemic host sensitization. This suggests that Pam2ODN treatment, which is delivered directly into the lung mucosa and does not generate any detectable systemic immune response[15], acts locally in the lung mucosa by inducing an immunomodulatory stimulus that is capable of counteracting any incoming type 2 stimulus, regardless of whether that source of that stimulus is within the lungs or from systemically activated leukocytes.

Another important point to highlight is the speed of Pam2ODN efficacy, which was seen just 24 hours before an HDM sensitization. Leukocyte recruitment to the lung by Pam2ODN has been noted previously, but these responses occur later than 24 hours after exposure and are unlikely to be responsible for the immunomodulatory effect at this time point. On the other hand, Pam2ODN-inducible epithelial antimicrobial responses have consistently been observed within 8 hours.[15] Additionally, it has been well established that HDM sensing by lung epithelial cells, primarily via TLR4, is required for the development of the full allergic phenotype.[24] We further show that lung epithelial cells can respond to HDM directly by the observation that PBS/HDM mice accumulate intracellular mucins despite no detectable eosinophil recruitment, indicating that epithelial cells are capable of generating an allergic response directly, before the adaptive immune response has developed. Taken together, this suggests that integration between the type 2 immunogens and immunomodulatory responses of Pam2ODN occur within lung epithelial cells, which are optimally positioned to be first responders to either input.

We next wanted to evaluate whether Pam2ODN immunomodulation could always prevent airway allergic disease when hosts had been systemically sensitized to a strong immunogen to achieve a limit of effectiveness for Pam2ODN. To test this, we utilized a novel variation of the acute HDM model, and injected HDM extract intraperitoneally instead of intrapharyngeally. Unlike what was observed with OVA, Pam2ODN treatment had no effect on allergic inflammation in this setting. This potentially suggests that HDM is intrinsically a stronger type 2 immunogen than OVA, and that Pam2ODN cannot counteract it. This idea is further supported by the previous observations that HDM extract does not require any additional adjuvants to sensitize and is capable of sensitizing hosts directly in the lung mucosa, whereas OVA requires an adjuvant (i.e., alum) and cannot polarize airways independently of systemic sensitization.

Because different allergens might have different immunogenic strength, it is plausible that stronger immunogens require equivalently strong immunomodulatory stimulus. Any single PRR agonist will ultimately be restricted by receptor saturation, and thus cannot invoke a stronger stimulus past a certain concentration. However, Pam2ODN provides an important clue how to bypass this immunomodulatory strength limit by pairing a combination immunomodulatory agents that exhibit synergy. Previous work has shown that Pam2 and ODN cooperate synergistically to mediate protection against microbial pathogens, suggesting the importance of coincidence detection of multiple PRRs for innate immune sensing and generating antimicrobial responses,[11] and potentially highlighting a way to increase the strength of immunomodulatory responses. Our results confirm that synergistic interaction is required for Pam2ODN efficacy against allergic inflammation as neither Pam2 nor ODN showed any efficacy when delivered alone. We believe this highlights the importance of evaluating combinations of

immunomodulatory agents for future human studies. While Pam2 and ODN were the strongest combinations of PRR agonists for antimicrobial efficacy,[7] a systematic screen has never been performed that assessed immunomodulatory strength, and it is possible that different combinations of PRR agonists might show even more immunomodulatory strength.

To evaluate the precise cellular and molecular mechanism by which Pam2ODN modulates the type 2 immune response and reduces allergic responsiveness, further investigation is required. However, with the evidence generated from this study, we have shown that Pam2ODN is a generalizable immunomodulatory agent that counteracts allergic type 2 immune responses, and is likely superior to previously tested single agents, due to synergistic cooperation. The public need for immunomodulatory agents that can counteract the morbidity of type 2 is great, and clinical studies with Pam2ODN have already shown the drug to be well tolerated in humans (NCT02124278, NCT02566252), allowing Pam2ODN to be quickly evaluated in the setting of allergic immunotherapy.

Materials and Methods

Mice

Breeder BALB/cJ mice were obtained from the Jackson Laboratory (Sacramento, CA) and housed in specific pathogen-free conditions on a 12-hour light/dark cycle with free access to food and water. For euthanasia, mice were injected intraperitoneally with 2,2,2-tribromoethanol (250 mg/kg) and exsanguinated by transection of the abdominal aorta. All procedures were performed in accordance with the Institutional Animal Care and Use Committee of MD Anderson Cancer Center and the Texas A&M Institute for Biosciences and Technology. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Treatment with aerosolized Pam2ODN

This was performed as described.[15] Briefly, ODN 5’ TCG TCG TCG TTC GAA CGA CGT TGA T 3’ as the sodium salt on a phosphorothioate backbone (ODN M362) was purchased from TriLink BioTechnologies (San Diego, CA) and 2,3-bis (palmitoyloxy)-2-propyl-Cys-Ser-Lys-Lys-Lys-Lys-OH (Pam2CSK4) as the trifluoroacetic acid salt was purchased from Peptides International (Louisville, KY). A solution of ODN (1 μM) and Pam2CSK4 (4 μM) in endotoxin-free sterile water (8 ml) was placed in an Aerotech II nebulizer (Biodex Medical Systems, Shirley, NY) driven by 10 L/min of 5% CO2 in air to promote deep breathing. The nebulizer was connected by polyethylene tubing (30 cm × 22 mm) to a 10-L polyethylene chamber vented to a biosafety hood. Mice were exposed to the aerosol for 20 min, resulting in the nebulization of ~4 ml of O/P solution.

Sensitization and airway challenge with HDM

Lyophilized HDM extract (Stallergenes Greer, Lenoir, NC) was resuspended in PBS. Mice were sensitized to 100 μg of HDM (protein weight) by depositing 40 μl of reconstituted HDM into the oropharynx and allowing aspiration into the lungs, with mice suspended by the upper incisors on a board at 60 degrees from horizontal under isoflurane anesthesia. Mice were then challenged with 10 μg of HDM by depositing the same volume of reconstituted HDM into the nasal vestibule. This model and HDM dosages were selected from previously published data and confirmed by us during pilot studies (Fig. S1). In one case, mice were sensitized to 100 μg of

HDM by intraperitoneal injection of 40 ul of reconstituted HDM. For all experiments, mouse age at the time of sensitization to HDM was between 6 and 7 weeks old.

Sensitization and airway challenge with OVA

Mice were sensitized to 20 g ovalbumin (OVA) (Grade V, 2.25 mg alum in saline, pH 7.4; Sigma, St. Louis, MO) by intraperitoneal (i.p.) injection on day 0 and 7. Sensitized mice were exposed for 30 min to an aerosol of 2.5% (wt/vol) ovalbumin in PBS, using an Aerotech II nebulizer, as described above.

Airway challenge with Aspergillus oryzae and OVA

Aspergillus oryzae protease (Sigma-Aldrich, St. Louis, MO) was resuspended in PBS. Mice were challenged with 10 ug A.oryzae and 2.5% (wt/vol) OVA by aerosol 2.5 times per week for 3 weeks. A. oryzae dose was selected from previously published data.[25] Aerosol challenge was performed as described above for OVA.

Bronchoalveolar lavage (BAL) and differential leukocyte analysis

This was performed by instilling and collecting two 1 ml aliquots of ice-cold PBS through a 20 gauge cannula inserted through rings of the exposed trachea of euthanized animals, then combining the aliquots as described[15]. Total leukocyte count was determined using a hemocytometer, and differential counts by cytocentrifugation of 100 µl of lavage fluid at 300 g for 5 min followed by Wright-Giemsa staining.

Histochemistry

Lungs were fixed by intratracheal inflation with 10% formalin to 20 cm H2O pressure for 12 h, and then embedded in paraffin. Tissue blocks were cut into 5-µm sections, mounted on frosted glass slides (Medline, Northfield, IL), deparaffinized with xylene, washed with ethanol, then rehydrated and stained with hemoxtylin & eosin (H&E) (Sigma-Aldrich, St. Louis, MO).

Epithelial mucin content

Epithelial mucin content was measured as described.[26–28] Lungs were fixed and processed, as described above, but stained with periodic acid fluorescent Schiff reagent (PAFS). Left-axial bronchus sections were obtained using a custom precision cutting instrument (ASI Instruments, Warren, MI), as previously described.[23] Images were acquired by investigators blinded to mouse treatment, and morphometric analysis of the images for quantitation of intracellular mucin was performed using MATLAB Software (Mathworks Software, Natick, MD). Data are presented as the area of intracellular mucus, normalized to the length of the basement membrane.

Statistical analysis

All data sets were first analyzed with the Shapiro-Wilk test to determine normality. For analyses where there was only a single comparison between two groups, data was analyzed by Student's t-test or Mann-Whitney U test for normally and non-normally distributed data, respectively. For analyses where multiple experimental groups were compared against a single control, data were first analyzed by one-way ANOVA or ANOVA on ranks to determine if a significant difference between any groups was present for normally and non-normally distributed data, respectively. If a significant difference was found by ANOVA, data were further analyzed

using Dunnett's test. For analyses where each experimental group was compared to every other group, data were further analyzed using Tukey's test for one-way ANOVA or Dunn's test for all pairwise combinations for ANOVA on ranks. Significance was determined from adjusted P values. For studies comparing Pam2 and ODN individually and together, an interaction score from linear regression analysis was used to determine whether there was a synergistic effect. All data were analyzed using Prism (version 9, GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

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Main Figures

Figure 1.

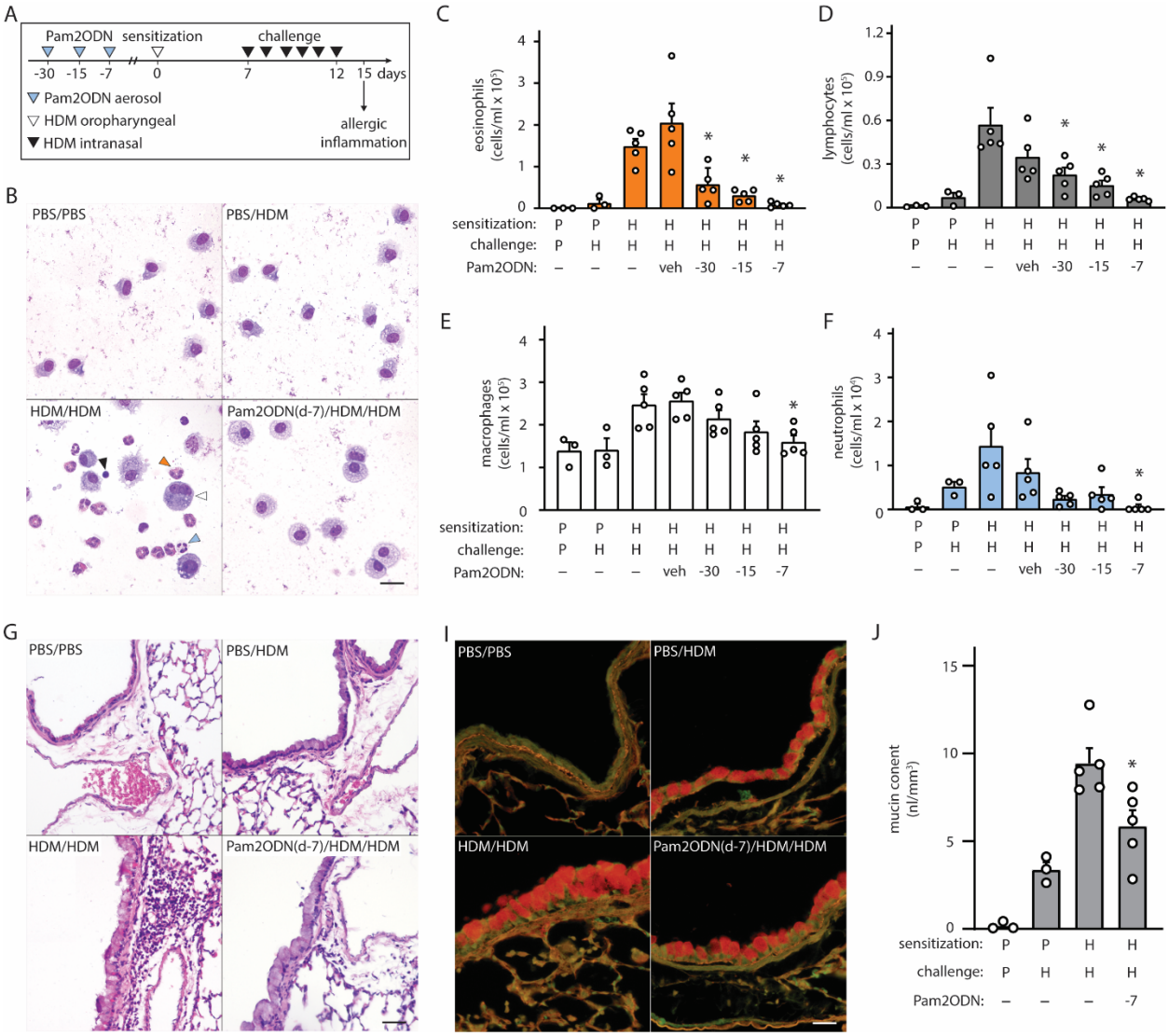

Figure 1. Exposure to Pam2-ODN attenuates allergic inflammation to HDM. (A) HDM experimental paradigm with a sensitization of 100 µg HDM and challenges of 10 µg HDM. **(B)** Leukocytes obtained by lung lavage were pelleted onto glass slides by centrifugation and stained with Wright-Giemsa. Scale bar = 20 µm. **(C-F)** Quantification of leukocytes in lung lavage for **(C)** eosinophils, **(D)** lymphocytes, **(E)** macrophages, and **(F)** neutrophils (N = 3-5 mice). **(G-H)** Airways stained with H&E to demonstrate submucosal inflammation. Scale bar = 20 µm **(I)** Airway epithelium stained with PAFS to demonstrate intracellular mucin in red. Scale bar = 20 µm. **(J)** Quantification of intracellular mucin content by image analysis of airway (N = 3-5 mice). Bars show mean +/- SEM. P* < 0.05 by one-way ANOVA with Dunnett's test for multiple comparisons against [HDM/HDM] control. P = PBS. H = HDM.

Figure 2.

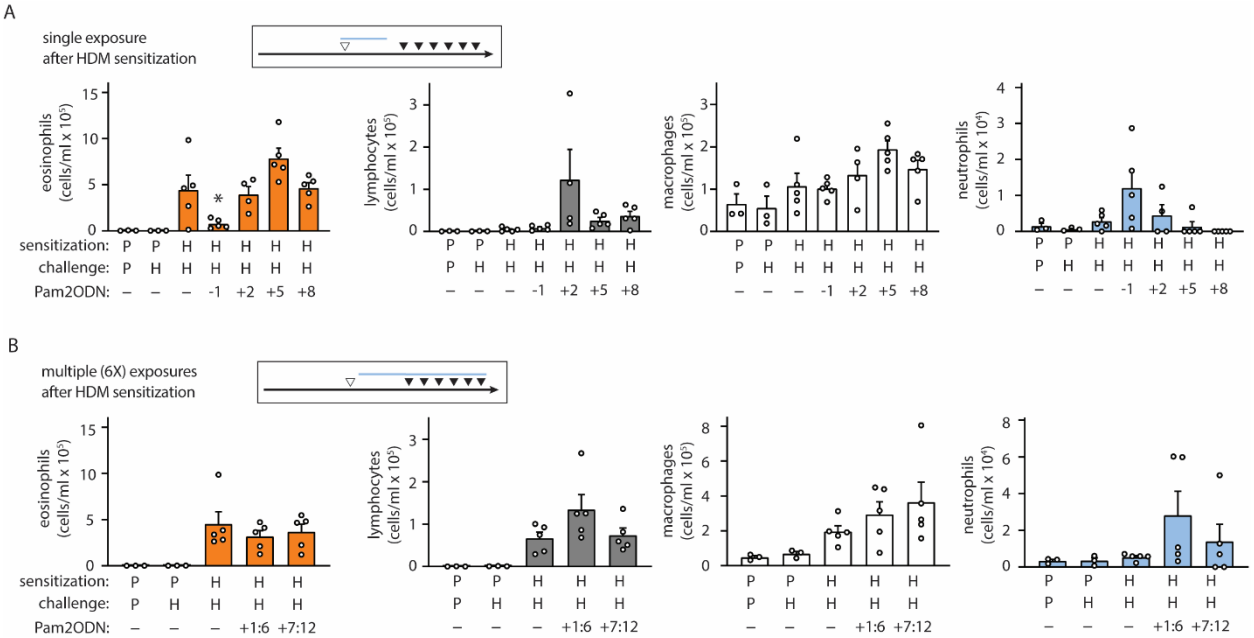

Figure 2. Pam2-ODN prevents sensitization to HDM. (A-B) Quantification of leukocytes in lung lavage when a single Pam2-ODN treatment was administered before or after sensitization to HDM (N = 3-5 mice). **(B)** Quantification of lung leukocytes when 6 consecutive daily Pam2-ODN treatments were administered after sensitization to HDM during the first week (days +1:6) or concurrently with challenge (days +7:12). Pictograms show blue bar at the approximate range of Pam2-ODN exposure in relationship to sensitization and challenge. Bars show mean +/- SEM. P* < 0.05 by one-way ANOVA with Dunnett's test for multiple comparisons against [HDM/HDM] control. P = PBS. H = HDM.

Figure 3.

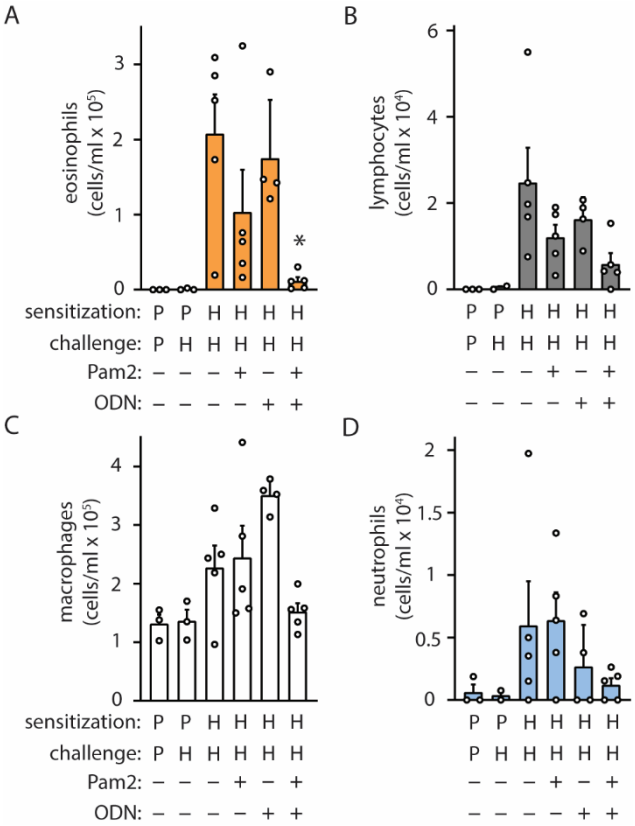

Figure 3. Pam2 and ODN interact synergistically to block sensitization to HDM. Pam2, ODN, or Pam2ODN was administered 7 days before sensitization to HDM. Quantification of leukocytes in lung lavage for **(A)** eosinophils, **(B)** lymphocytes, **(C)** macrophages, and **(D)** neutrophils (N = 3-5 mice). Bars show mean +/- SEM. P* < 0.005 for synergy between Pam2 and ODN by linear regression. P = PBS. H = HDM.

Figure 4.

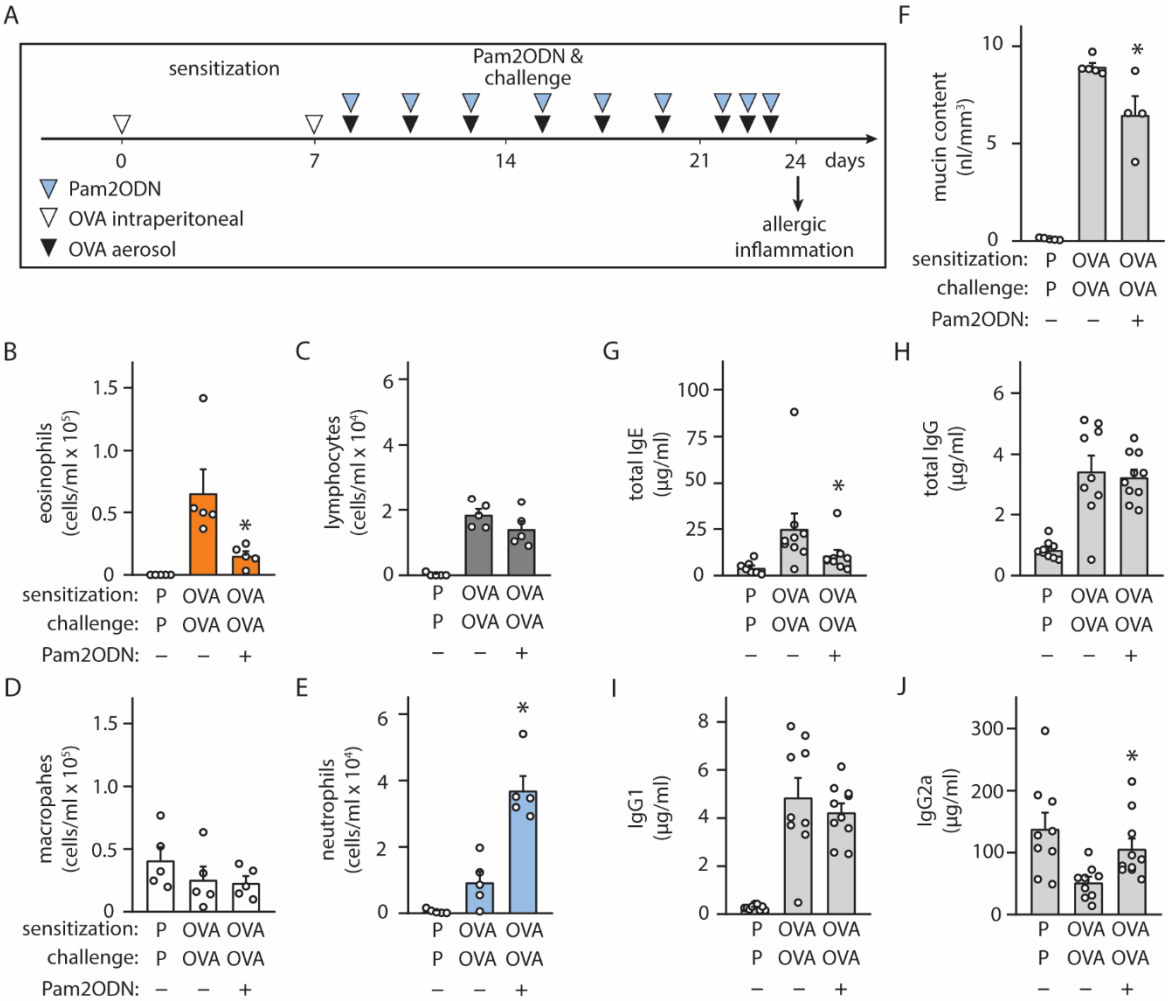

Figure 4. Exposure to Pam2-ODN attenuates allergic inflammation to OVA. (A) OVA experimental paradigm with sensitization of 20 µg OVA by intraperitoneal injection and aerosol challenges of 2.5% OVA. **(B-E)** Quantification of leukocytes in lung lavage for **(B)** eosinophils, **(C)** lymphocytes, **(D)** macrophages, and **(E)** neutrophils (N = 4-5 mice). **(F)** Quantification of intracellular mucin content by image analysis of airway stained with PAFS (N = 4-5 mice). **(G-J)** Quantification of serum immunoglobulin concentrations by ELISA for **(G)** IgE, **(H)** IgG, **(I)** IgG1, and **(J)** IgG2a (N = 8-9 mice). Bars show mean +/- SEM. P* < 0.05 by unpaired students' T test for OVA vs OP-OVA. P = PBS. OVA = Ovalbumin.

Figure 5.

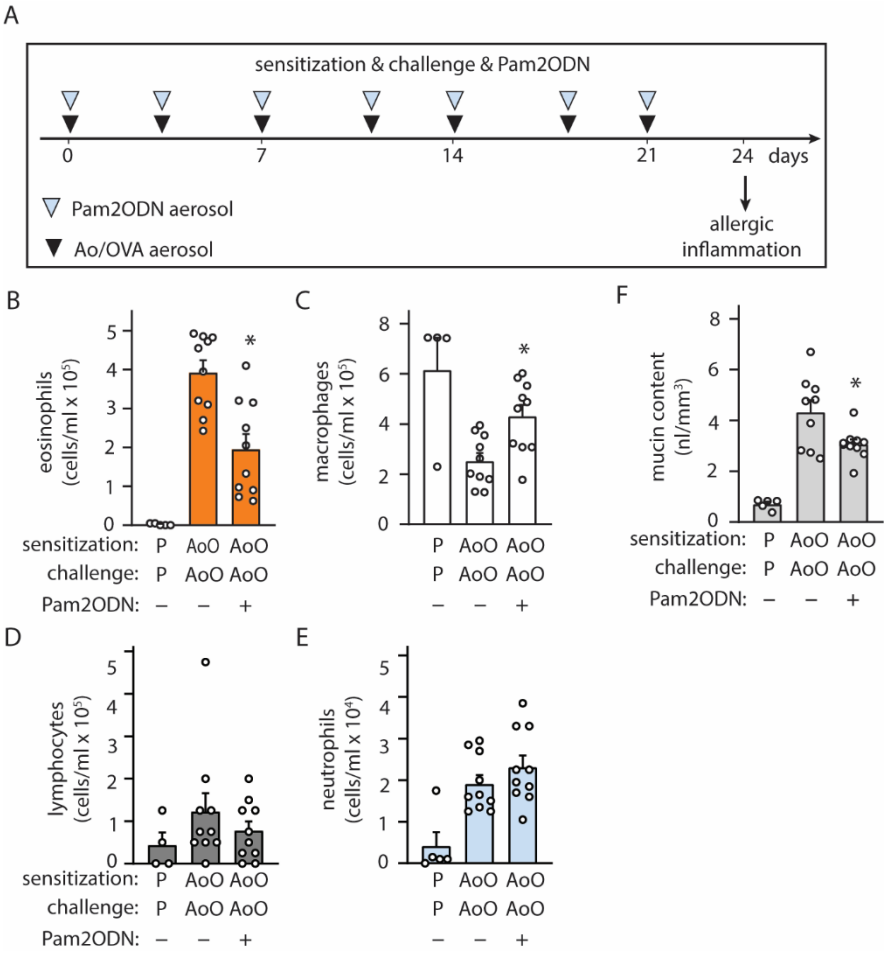

Figure 5. Exposure to Pam2-ODN prevents allergic inflammation to Aspergillus oryzae. (A) Ao-OVA experimental paradigm with aerosol challenges of 10 µg Ao and 2.5% OVA. **(B-E)** Quantification of leukocytes in lung lavage for **(B)** eosinophils, **(C)** lymphocytes, **(D)** macrophages, and **(E)** neutrophils (N = 4-9 mice). **(F)** Quantification of intracellular mucin content by image analysis of airway stained with PAFS (N = 4-9 mice). Bars show mean +/- SEM. P* < 0.05 by unpaired students' T test for OVA vs OP-OVA.