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This information is current as
of April 3, 2012

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J Immunol; Prepublished online 28 March 2012;
doi:10.4049/jimmunol.1101299

<http://www.jimmunol.org/content/early/2012/03/28/jimmunol.1101299>

Supplementary Data

<http://www.jimmunol.org/content/suppl/2012/03/28/jimmunol.1101299.DC1.html>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Components of *Streptococcus pneumoniae* Suppress Allergic Airways Disease and NKT Cells by Inducing Regulatory T Cells

Alison N. Thorburn,^{*,†} Paul S. Foster,^{†,‡} Peter G. Gibson,^{*,‡} and Philip M. Hansbro^{†,‡}

Asthma is an allergic airways disease (AAD) caused by dysregulated immune responses and characterized by eosinophilic inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR). NKT cells have been shown to contribute to AHR in some mouse models. Conversely, regulatory T cells (Tregs) control aberrant immune responses and maintain homeostasis. Recent evidence suggests that *Streptococcus pneumoniae* induces Tregs that have potential to be harnessed therapeutically for asthma. In this study, mouse models of AAD were used to identify the *S. pneumoniae* components that have suppressive properties, and the mechanisms underlying suppression were investigated. We tested the suppressive capacity of type-3-polysaccharide (T3P), isolated cell walls, pneumolysin (Ply) and CpG. When coadministered, T3P + Ply suppressed the development of: eosinophilic inflammation, Th2 cytokine release, mucus hypersecretion, and AHR. Importantly, T3P + Ply also attenuated features of AAD when administered during established disease. We show that NKT cells contributed to the development of AAD and also were suppressed by T3P + Ply treatment. Furthermore, adoptive transfer of NKT cells induced AHR, which also could be reversed by T3P + Ply. T3P + Ply-induced Tregs were essential for the suppression of NKT cells and AAD, which was demonstrated by Treg depletion. Collectively, our results show that the *S. pneumoniae* components T3P + Ply suppress AAD through the induction of Tregs that blocked the activity of NKT cells. These data suggest that *S. pneumoniae* components may have potential as a therapeutic strategy for the suppression of allergic asthma through the induction of Tregs and suppression of NKT cells. *The Journal of Immunology*, 2012, 188: 000–000.

The prevalence of asthma continues to increase in Westernized countries (1, 2). Th2 cells are critical for the pathogenesis of asthma through the release of cytokines (IL-4, IL-5, and IL-13) that promote eosinophil influx, mucus hypersecretion, and airway hyperresponsiveness (AHR) (3). NKT cells also are now recognized for their importance in asthma pathogenesis (4). NKT cells are a small but potent population of lymphocytes that express both T and NK cell markers and release high concentrations of Th2 cytokines (5). The CD4⁺ IL-4– and IL-13–producing NKT cell subset is indispensable for the development of AHR in some mouse models of allergic airways disease (AAD) (6). The induction of both Th2 cells and NKT cells may be controlled by the function of regulatory T cells (Tregs), which suppress aberrant immune responses and maintain homeostasis (7). Asthma patients are known to have fewer and less functional Tregs, and as a result, asthma is a state of dysregulated immunity (8, 9).

Some infectious agents predispose to and/or exacerbate the severity of AAD, whereas others are protective (7, 10–16). Infectious agents that are protective may prevent or suppress AAD by modulating the immune system and are under investigation for therapeutic use. However, there are major limitations that need to be addressed before the potential of infectious agents can be transferred into therapeutic use (7). These include the removal of infectious ability and simplification of the composition, while preserving the immunoregulatory capacity. Furthermore, elucidation of the mechanisms of action is required to understand the implications and limitations of the therapy.

In humans, *Streptococcus pneumoniae* immunization has been associated with decreased asthma-related hospitalizations in both children and the elderly, which may be the result of immune modulation (17–19). We have previously used mouse models to demonstrate that live and killed *S. pneumoniae* suppressed the development of features of AAD (20, 21). Therefore, *S. pneumoniae* has potential to be harnessed as an immunoregulatory therapy for AAD (22).

We hypothesized that particular *S. pneumoniae* components must be essential for the suppression of AAD and that their identification may enable the development of a simplified therapy. We selected four of the most immunostimulatory *S. pneumoniae* components and identified the maximally effective yet least complex immunoregulatory combination. We investigated the immunoregulatory and suppressive properties of this combination in different mouse models of AAD. Their mechanism of action was characterized by assessing the effects on NKT cells and the involvement of Tregs.

Materials and Methods

Animals

Adult female BALB/c or C57BL/6 mice were maintained under specific pathogen-free and controlled environmental conditions. Procedures were

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Received for publication May 10, 2011. Accepted for publication February 6, 2012.

This work was supported by the Hill Family, Asthma Foundation New South Wales, the Cooperative Research Centre for Asthma and Airways, the National Health and Medical Research Council (Grants 401238 and 569219), the Australian Research Council (Grant 110101107), and the University of Newcastle.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AAD, allergic airways disease; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; α -GalCer, α -galactosylceramide; HDM, house dust mite; i.n., intranasal; MLN, mediastinal lymph node; Ply, pneumolysin; T3P, type-3-polysaccharide; Treg, regulatory T cell.

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approved by the animal ethics committee of The University of Newcastle or Monash University. All of the experiments were performed in BALB/c mice with the exception of those represented in Supplemental Fig. 1.

Allergic airways disease

AAD was induced as described previously (11, 23). For OVA-induced AAD, mice were sensitized (i.p., day 0; 50 µg [and day 7 for C57BL/6 mice]; Sigma-Aldrich, St. Louis, MO) with Rehydrogel (1 mg; Reheis, Berkeley Heights, NJ) in sterile saline (200 µl) and challenged with OVA (intranasal [i.n.]; days 12–15; 10 µg in 50 µl sterile saline). To recapitulate established disease, mice received OVA i.p. (day 0) followed by two sets of challenges (days 11–13 and 33–34). For house dust mite (HDM)-induced AAD, mice were sensitized to HDM (*Dermatophagoides pteronyssinus*) extract (i.n.; days 0, 1, and 2; 50 µg; Greer Labs, Lenoir, NC) in sterile saline (50 µl) and challenged with HDM (i.n.; days 14–17; 5 µg in 50 µl sterile saline). Where indicated, mice received anti-CD25 Ab (i.p.; day –3; 100 µg in 200 µl saline, PC61).

S. pneumoniae and components

Ethanol-killed *S. pneumoniae* (type 3 strain; American Type Culture Collection NCO1265) was prepared as described previously (21). Cell walls were purified as described previously with minor modifications (24).

Mice were administered killed *S. pneumoniae* (2×10^5 CFU), type-3-polysaccharide (T3P; 2 µg; American Type Culture Collection), cell walls (equivalent of 2×10^5 CFU), pneumolysin [Ply; LPS-free; 40 ng; a detoxified form of pneumolysin with a Trp to Phe substitution at amino acid 433; hemolytic activity reduced by >800-fold, 1.4×10^3 hemolytic units/mg compared with pneumolysin at 1.2×10^6 hemolytic units/mg; University of Adelaide, Adelaide, SA, Australia (25)], and/or CpG oligodinucleotides (10 µg; 5'-TCCATGACGTTCTACGTT-3'; Geneworks, Thebarton, SA, Australia) in sterile saline (30 µl) by intratracheal administration under alfaxan i.v. anesthesia. No overt symptoms of illness or disease or additional pathology were observed at any time in mice treated with *S. pneumoniae* components.

Cellular inflammation

Preparation and enumeration of blood and bronchoalveolar lavage (BAL) leukocytes were performed as described previously (11).

T cell cytokines

Cells (5×10^6 per ml) from mediastinal lymph nodes (MLNs) or spleens were cultured in RPMI 1640-supplemented media with OVA (200 µg/ml) or anti-CD3/CD28 (5 µg/ml) for 4 or 6 d (5% CO₂, 37°C), respectively. IL-5 and IL-13 concentrations in cell culture supernatants were determined by ELISA (BD Pharmingen, San Diego, CA) (20).

Airway hyperresponsiveness

AHR was assessed as described previously (15).

Lung histology

Lungs were perfused, inflated, fixed, embedded, sectioned, and stained to enumerate airway mucus-secreting cells and tissue eosinophils as described previously (20).

Flow cytometry

A total of 1×10^6 cells per well in 96-well U-bottom plates were stained for α-galactosylceramide (α-GalCer)-loaded CD1d, TCRβ, CD4, and CD25 (BD Pharmingen, San Diego, CA) (20, 26). Cells were permeabilized and stained for Foxp3 according to the manufacturer's protocol (eBioscience, San Diego, CA). Cells were analyzed using a FACSCanto flow cytometer controlled by FACSDiva software (version 4.1.1; BD Biosciences, San Jose, CA).

Adoptive transfer

Splenocytes were obtained from naive mice and enriched for CD4⁺ cells using microbead-conjugated Abs (AutoMACS Pro; Miltenyi Biotec, Auburn, CA). CD1d⁺ cells then were isolated from the CD4⁺-enriched population using α-GalCer-loaded CD1d-PE tetramer and anti-PE microbeads. CD1d⁺TCRβ⁺ cells were purified subsequently by flow cytometry (FACSARIA, BD Biosciences). CD1d⁺TCRβ⁺ cells were administered by i.v. injection (day 10; 3×10^5 in 100 µl saline).

IDO

IDO levels were assayed by Western blot (R&D Systems, Minneapolis, MN) and quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, MD).

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA) and are represented as mean ± SEM. One-way ANOVA with Dunnett's post test was used to determine significance between data with multiple comparisons. One-way repeated measures ANOVA and Bonferroni's post test was used to determine significance for AHR data. A *p* value <0.05 was considered statistically significant.

Results

Identification of *S. pneumoniae* immunoregulatory components

The immunoregulatory capacities of four major *S. pneumoniae* components, T3P, cell walls, Ply, and CpG oligodinucleotides, were tested in a widely used OVA-induced model of AAD (11, 20, 21). Different combinations of components were administered at the time of sensitization to OVA, and the effects on the induction of AAD were assessed (Fig. 1A).

Increased eosinophil numbers in BAL and OVA-induced IL-5 and IL-13 release from MLN T cells were used as indicators of AAD for screening purposes (Fig. 1B–D). Killed *S. pneumoniae* (positive control) suppressed eosinophil numbers in BAL and OVA-induced IL-5 and IL-13 release from MLN T cells. When all four components (T3P + cell walls + Ply + CpG) were administered, eosinophil numbers in BAL and OVA-induced IL-5 and IL-13 release from MLN T cells also were suppressed.

Potent suppression of eosinophil numbers in BAL and OVA-induced IL-5 and IL-13 release from MLN T cells was achieved whenever T3P and Ply were administered together. Neither T3P alone nor Ply alone attenuated these features of AAD.

CpG attenuated eosinophil numbers in BAL but had no effect on OVA-induced IL-5 and IL-13 release from MLN T cells. When CpG and T3P were administered together, these features of AAD were suppressed, however, not to the same magnitude as observed with the T3P and Ply combination.

Cell walls alone also suppressed these features of AAD; however, their action was dampened in combination with most other components.

Identification of *S. pneumoniae* components that suppress AHR

Combinations of components that suppressed the development of eosinophils in BAL and OVA-induced IL-5 and IL-13 release from MLN T cells were investigated for their capacities to suppress AHR.

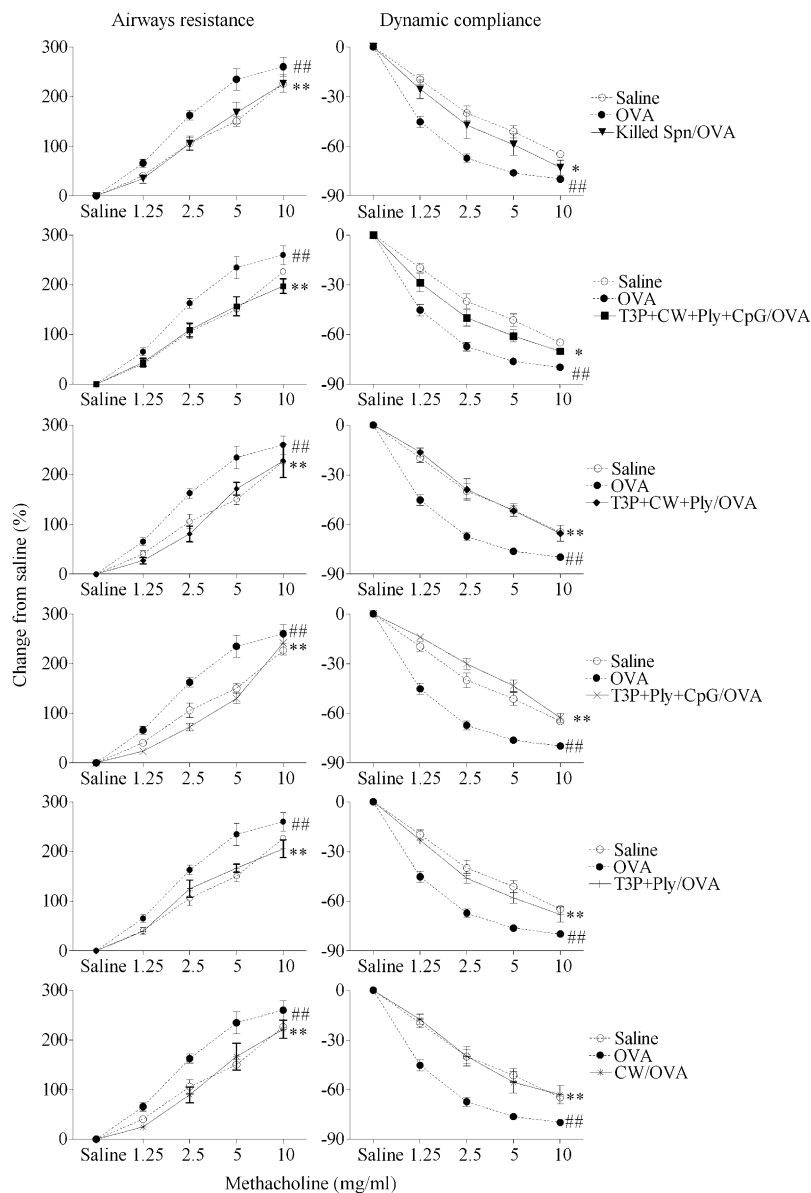
The development of AHR in AAD also was characterized by increased transpulmonary resistance and decreased dynamic compliance compared with those in saline-sensitized mice (Fig. 2). Killed *S. pneumoniae* suppressed the development of AHR. Component combinations that attenuated eosinophil influx and Th2 cytokine release (Fig. 1) also were effective at suppressing AHR. When T3P and Ply were administered together, with or without other components, they completely inhibited the development of AHR.

The administration of cell walls alone also inhibited the development of AHR and showed potential as an immunoregulatory therapy for AAD (Fig. 2). However, cell walls are complex mixtures of carbohydrates, peptidoglycan, and proteins. Therefore, extensive additional research would be required to identify the active component(s). Consequently, the combination of T3P + Ply was chosen as a potential immunoregulatory therapy for AAD and was the focus of subsequent studies.

T3P + Ply immunoregulatory therapy suppresses the development of additional features of AAD

We then examined the capacity of T3P + Ply immunoregulatory therapy to suppress the induction of additional features of AAD. Administration of T3P + Ply during sensitization potently suppressed the levels of eosinophils in the blood and lung tissue,

FIGURE 2. Selected *S. pneumoniae* components suppress AHR. Components that suppressed eosinophil influx into the airways and Th2 cytokine release in AAD were tested for their capacities to suppress AHR, in terms of transpulmonary resistance and dynamic compliance. Data represent mean $\pm$ SEM from six to eight mice. Significant differences for the entire dose-response curves between saline-sensitized (Saline) and OVA-sensitized (OVA) controls are shown as $^{###}p < 0.01$ and between OVA-sensitized and component-treated OVA-sensitized mice as $^{*}p < 0.05$ and $^{**}p < 0.01$.



CD4⁺CD25⁺Foxp3⁺ Tregs in the lung compared with those in untreated mice. Notably, T3P + Ply also increased the level of IDO in the lung, which is likely to play a role in the induction of these Tregs (Supplemental Fig. 3).

To determine whether Tregs were required for the attenuation of AAD by T3P + Ply, anti-CD25 (PC61) Ab was delivered before T3P + Ply administration. Anti-CD25 Ab depleted the number of CD4⁺CD25⁺Foxp3⁺ Tregs in the lungs to the level of the saline group (data not shown). Anti-CD25 administration reversed the protective effects of T3P + Ply on the level of eosinophils in BAL and blood, OVA-induced IL-5 and IL-13 release from MLN T cells, and AHR compared with those of the relevant isotype controls (Fig. 7C–G). These data demonstrate that T3P + Ply-induced Tregs are required to suppress the development of AAD.

T3P + Ply-induced Tregs are required to suppress the induction of NKT cells

We then investigated whether Tregs were required for the suppression of NKT cells and therefore AHR. Anti-CD25 depletion of Tregs reversed the capacity of T3P + Ply to suppress the induction of NKT cells in the lymphocytic infiltrates (Fig. 8).

Discussion

This study has identified a specific combination of two *S. pneumoniae* components that may have potential as an immunoregulatory therapy for asthma. Using different models of AAD, we showed that the combination of *S. pneumoniae* capsular polysaccharide and pneumolysin (T3P + Ply) suppressed the development of all of the hallmark features of AAD, including eosinophils in BAL, blood, and tissue; Th2 cytokine (IL-5 and IL-13) release from MLN and splenic T cells; the number of mucus-secreting cells around the airways; and AHR. Importantly, we showed that the administration of T3P + Ply during established disease also suppressed AAD. The suppressive effects of T3P + Ply were associated with a reduction in the numbers and function of NKT cells. Indeed, the adoptive transfer of NKT cells induced AHR, which was reversed by T3P + Ply treatment. This provided a direct link among the suppressive effects of T3P + Ply, suppression of NKT cells, and attenuation of AHR. We also showed that T3P + Ply induced increases in the numbers of Tregs, which were required for the attenuation of AAD. Finally, we showed that T3P + Ply-induced Tregs were necessary to suppress the induction of NKT cells. Although we did not detect proinflammatory cytokine production

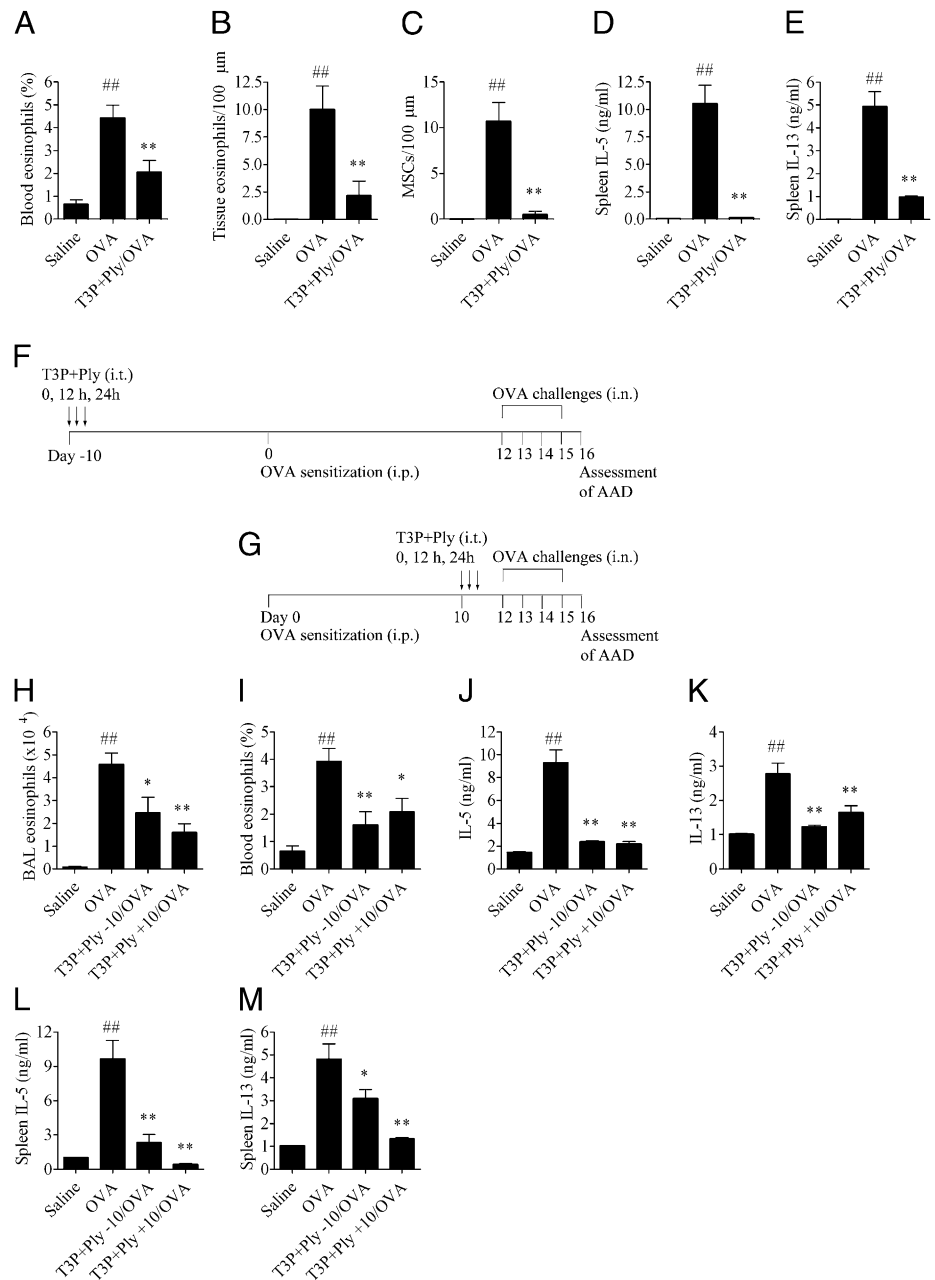


FIGURE 3. T3P + Ply suppresses the development of additional features of AAD. (**A–E**) The effects of intratracheal T3P + Ply treatment on the levels of eosinophils in (**A**) blood and (**B**) airway tissue, (**C**) mucus secreting cells (MSCs) around the airways, and OVA-induced (**D**) IL-5 and (**E**) IL-13 release from splenic T cells. (**F** and **G**) Experimental protocol used for the administration of T3P + Ply (**F**) before and (**G**) after sensitization. (**H–M**) The effects of T3P + Ply administration before (T3P + Ply -10) and after (T3P + Ply +10) sensitization on eosinophils in (**H**) BAL and (**I**) blood, OVA-induced (**J**) IL-5 and (**K**) IL-13 release from MLN T cells, and OVA-induced (**L**) IL-5 and (**M**) IL-13 release from splenic T cells. Data represent mean $\pm$ SEM from six to eight mice. Significant differences between saline-sensitized (Saline) and OVA-sensitized (OVA) controls are shown as ^{##} $p < 0.01$. Significant differences between OVA-sensitized and component-treated OVA-sensitized mice are shown as ^{*} $p < 0.05$ and ^{**} $p < 0.01$.

upon treatment with pneumolysoid or ethanol-killed *S. pneumoniae*, others have detected such responses. Pneumolysoid B (PdB, otherwise known as F433), which is the same as the Ply used in our study, has residual cytotoxic activity and induces CD4 T cell proliferation and the release of low levels of IFN- γ and TNF- α (27). Others have detected cytokine release from murine macrophages stimulated with ethanol-killed *S. pneumoniae* (28), and heat-killed *S. pneumoniae* elicited weak neutrophilic airway inflammation and cytokine responses mainly from macrophages (29). These studies indicate that pneumolysin derivatives that completely lack cytotoxic and proinflammatory activities should be used in human studies.

T3P + Ply had synergistic effects, because neither capsular polysaccharide (T3P) nor Ply alone suppressed AAD. Given that Ply is an immunogenic protein, it is likely to provide adjuvant properties to T3P. CpG also had synergistic effects with T3P, however, not to the same magnitude as Ply. Furthermore, the use of CpG oligodeoxynucleotides as an immunotherapy also may be prob-

lematic, because they induce IFN- γ , which has damaging effects on the airways and may increase the severity of AAD (30). Cell walls contain noncapsular polysaccharide and components that also may act as adjuvants (31). Notably, when combined with cell walls, T3P + Ply was not effective. It is likely that complex and competing immune responses are involved that prevent the induction of Tregs. At this stage, the use of cell walls alone as an immunotherapy for AAD is problematic due to their reversible action and complex composition. T3P + Ply is effective and relatively simple. Therefore, the combination of capsular polysaccharides and pneumolysoid are the most likely components for the development of immunoregulatory therapies for asthma. Other pneumolysoids are now available that have undetectable hemolytic activities that could be used in human therapies.

Our results indicate the potential mechanisms of action of T3P + Ply. Polysaccharides alone are known to induce T cell-independent responses that are poorly immunogenic (32). Without T cell help, the development of Ab affinity maturation, isotype switching, and

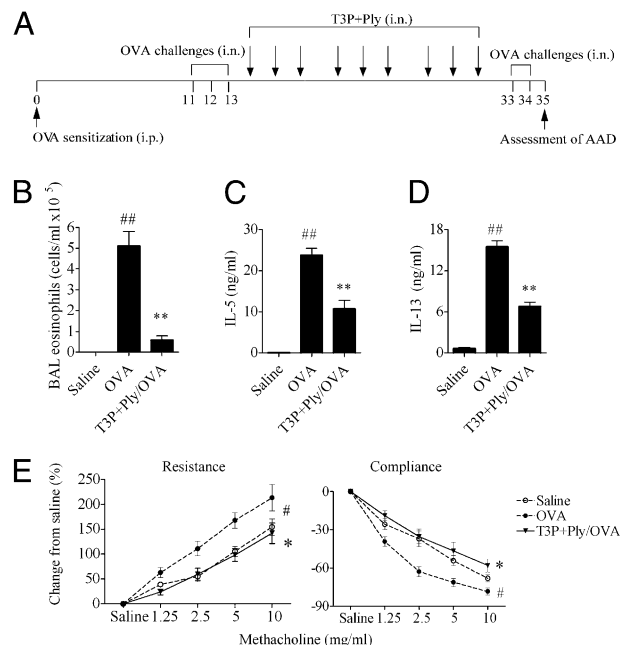


FIGURE 4. T3P + Ply attenuate AAD when administered during established disease. **(A)** Experimental protocol: AAD was established by i.p. OVA sensitization followed by an initial i.n. OVA challenge and then a second challenge to recapitulate AAD. T3P + Ply were delivered intratracheally three times per week commencing day 14 (totaling nine doses). **(B–E)** The effects of T3P + Ply treatment on (B) eosinophils in BAL, OVA-induced (C) IL-5 and (D) IL-13 release from MLN T cells, and (E) AHR. Data represent mean $\pm$ SEM from six to eight mice. Significant differences between saline-sensitized (Saline) and OVA-sensitized (OVA) controls are shown as $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. Significant differences between OVA-sensitized and component-treated OVA-sensitized mice are shown as $^*p < 0.05$ and $^{**}p < 0.01$. Significances for resistance and compliance are given for the entire dose-response curve.

immunological memory is limited. However, in the presence of an adjuvant, polysaccharides may elicit a T cell-dependent response and enhance Ab production (33). Others have shown that immunological protection against *S. pneumoniae* infection is T cell-dependent (34). Furthermore, the currently available *S. pneumoniae* 7-valent protein-conjugated vaccine is much more effective in protecting against pneumococcal infection compared with the 23-polysaccharide vaccine, which lacks the diphtheria toxoid adjuvant. Collectively, these observations indicate that T3P + Ply may suppress AAD through mechanisms of action that are T cell-dependent. Our subsequent studies show that these mechanisms are mediated by the induction of Tregs that suppress AAD that involve the reduction of NKT cells and associated AHR.

In our NKT cell experiments, it is possible that the adoptive transfer of NKT cells from naive mice into saline-sensitized mice and the associated increase in AHR may be due to the activation of NKT cells *ex vivo*. NKT cells were isolated using α -GalCer-loaded CD1d, which may activate the NKT cells and cause the associated increase in AHR. This caveat must be considered, because the adoptively transferred NKT cells may not represent a steady-state population. Nevertheless, the capacity for T3P + Ply treatment to suppress NKT cell numbers and function and therefore AHR has been clearly demonstrated.

Apart from *S. pneumoniae*, other infectious agents such as helminths, lactobacilli, and *Mycobacterium* have been shown to induce Tregs and suppress AAD in mouse models (35–37). Mycobacterial cell wall components (lipoglycans lipoarabinomannan, and phosphatidylinositol mannan) also have been shown to suppress AAD;

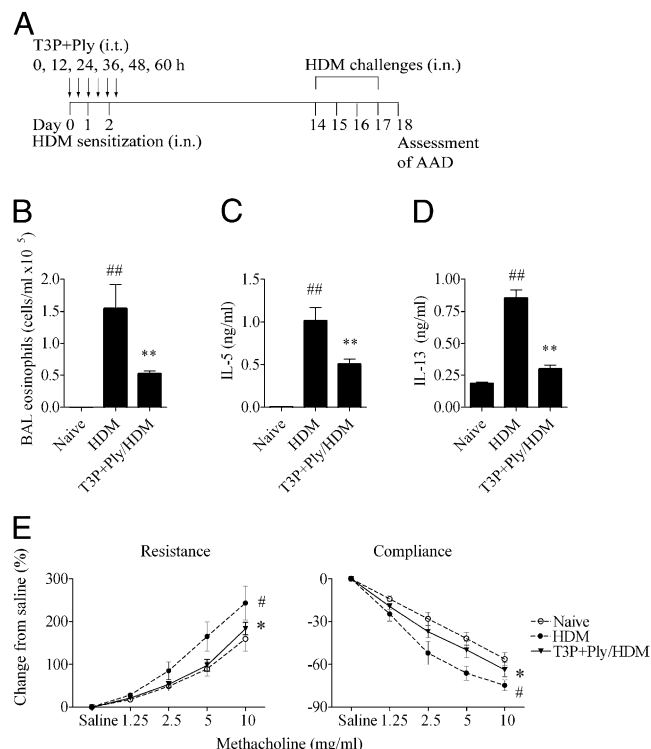


FIGURE 5. T3P + Ply suppress the development of HDM-induced AAD. **(A)** Experimental protocol: AAD was induced by three i.n. administrations of HDM followed by four i.n. challenges. Component treatment was administered intratracheally throughout the sensitization phase every 12 h for six doses. **(B–E)** Features of AAD were assessed 24 h after the final HDM challenge: (B) eosinophils in the BAL, anti-CD3/anti-CD28-stimulated (C) IL-5 and (D) IL-13 release from MLN T cells, and (E) AHR. Data represent mean $\pm$ SEM from six mice. Significant differences between saline-sensitized (Saline) and OVA-sensitized (OVA) controls are shown as $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. Significant differences between OVA-sensitized and component-treated OVA-sensitized mice are shown as $^*p < 0.05$ and $^{**}p < 0.01$. Significances for resistance and compliance are given for the entire dose-response curve.

however, their effects on AHR have not been determined (38). Furthermore, mycobacterial phosphatidylinositol mannoside has been reported to bind CD1d and activate human and mouse NKT cells, which may be a problem for the therapeutic use of mycobacterial components (39). Clinical applications of heat-killed *Mycobacterium vaccae* and its delipidated, deglycolipidated, and arabinogalactan-depleted derivative have not been successful in clinical trials for allergic diseases including asthma (40, 41). Interestingly, *S. pneumoniae* induces Tregs to a much greater extent than other Gram-positive bacteria (34). Therefore, *S. pneumoniae* may have specific attributes that make it a good choice for the development of asthma immunotherapies. Furthermore, *S. pneumoniae* has been shown recently to have NKT cell glycolipid agonist ligands (42). However, the identification of relatively simple active components, T3P + Ply, enables the potential development of immunoregulatory therapies for the attenuation of AAD, while excluding *S. pneumoniae*-inducing glycolipids.

Tregs, and therefore immunoregulation, may be induced by IDO, the rate-limiting enzyme for tryptophan catabolism produced by activated dendritic cells (43). In humans, *S. pneumoniae* infection has been associated with high IDO levels (44). Our results demonstrate that T3P + Ply induced IDO in the lungs, and it is likely that the activation of this pathway leads to the induction of Tregs in our studies. Notably, T3P + Ply also may affect the expression of co-

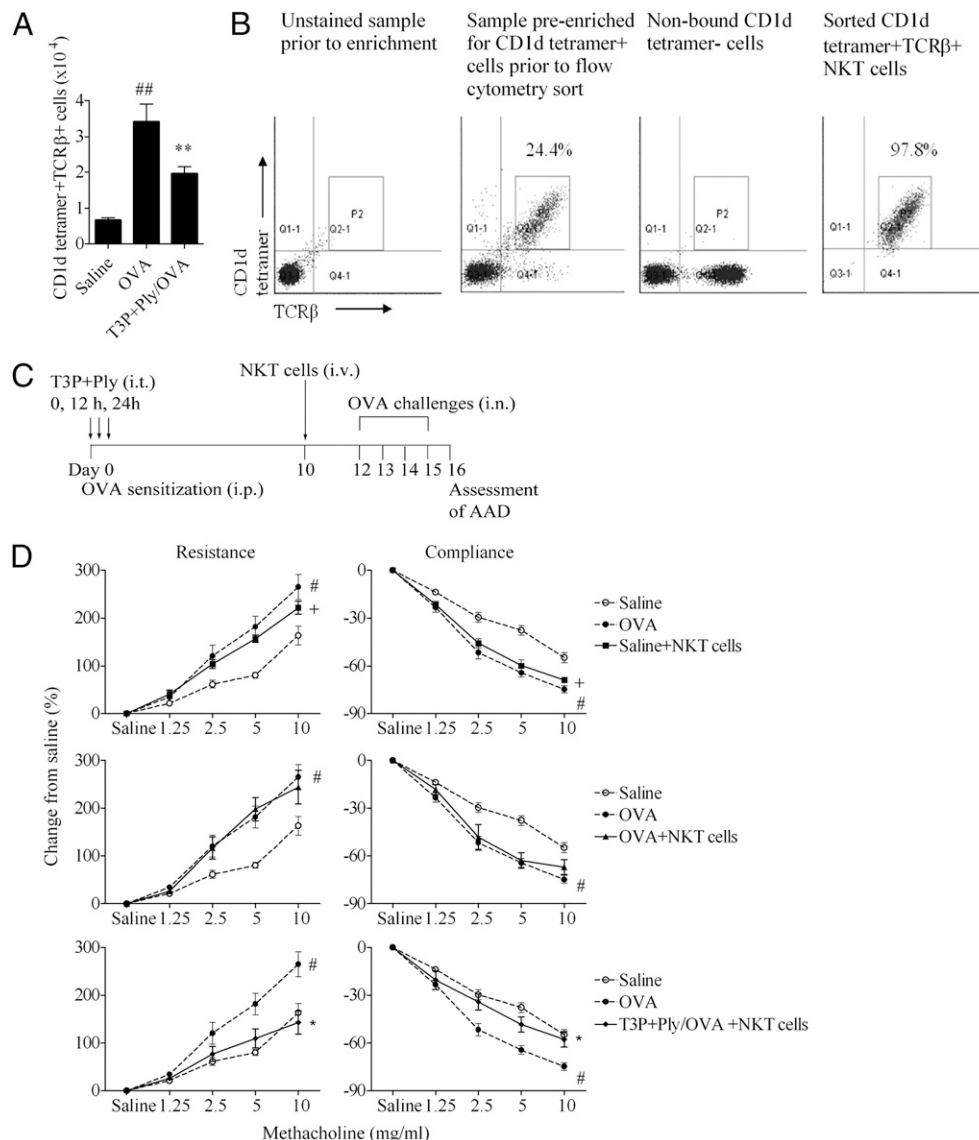


FIGURE 6. T3P + Ply suppress NKT cells and NKT cell-induced AHR in AAD. **(A)** Total CD1d⁺TCRβ⁺ (NKT) cells in the lungs after the induction of OVA-induced AAD. **(B)** Representative flow cytometry data for the isolation of CD1d⁺TCRβ⁺ cells showing unstained, presorted, control cells that did not bind to the CD1d⁺ tetramer beads and final populations. **(C)** Experimental protocol for adoptive transfer: Similar to Fig. 1 but mice received 3×10^5 CD1d⁺TCRβ⁺ (NKT) cells i.v. on day 10. **(D)** The effect of adoptive transfer of NKT cells and T3P + Ply treatment on AHR. Data represent mean $\pm$ SEM from six to eight mice. Significant differences between saline-sensitized (Saline) and OVA-sensitized (OVA) controls are shown as # $p < 0.05$ and ## $p < 0.01$. Significant differences between OVA-sensitized and T3P + Ply-treated OVA-sensitized mice are shown as * $p < 0.05$ and ** $p < 0.01$. Significant differences between Saline and Saline + NKT cells are shown as + $p < 0.05$. Significances for resistance and compliance are given for the entire dose-response curve.

stimulatory and activation molecules on APCs and therefore suppress the induction of AAD. TLR2 signaling also has been shown to expand the number of Tregs independently of APCs (45). Because T3P is a polysaccharide and therefore may engage with TLR2, the involvement of this pathway warrants further investigation. Notably, in the presence of TLR2 agonists, pneumolysin can activate macrophage responses (46). Whether this synergistic effect underpins T3P + Ply-mediated suppression of AAD remains to be determined. The importance of these pathways and mechanisms in T3P + Ply-mediated suppression of AAD requires further investigation.

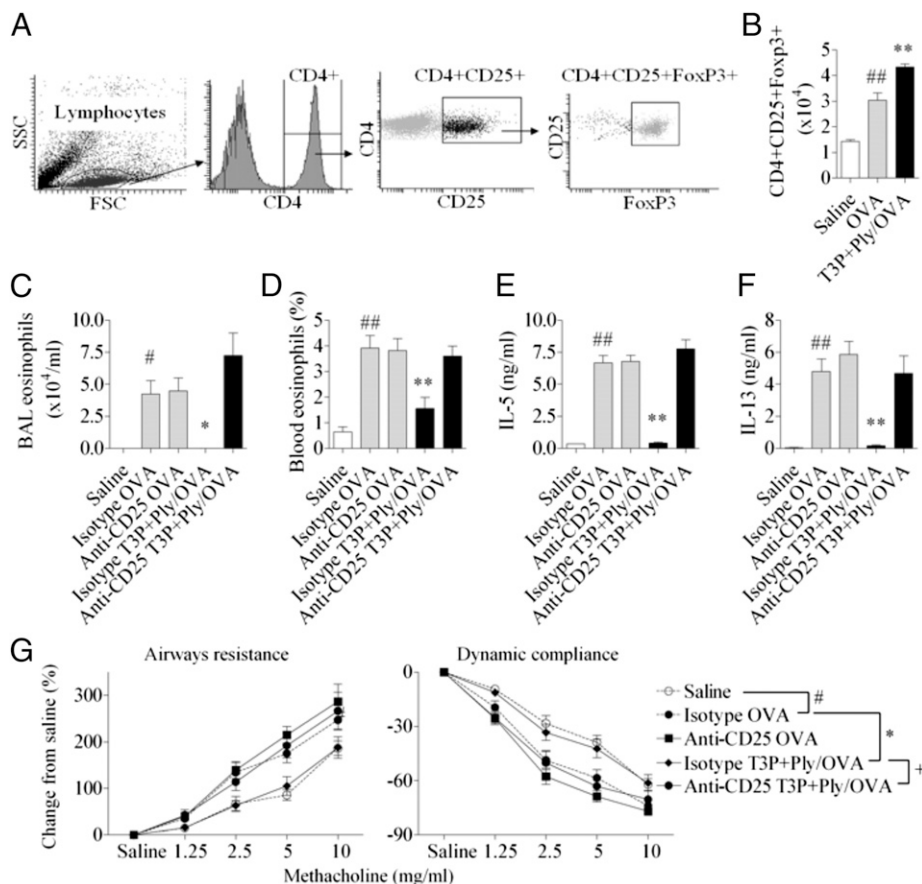
The recognition of asthma as a Th2-driven disease has led to the development of a number of Th2-targeted therapies; however, they have not been very successful yet (47, 48). Because NKT cells are required for the development of AHR, NKT cells may be important targets for a successful therapy. NKT cells are known to release Th1, Th2, and Th17 cytokines and chemokines, which enhance allergic inflammation and AHR (48, 49). NKT cells also

activate alveolar macrophages to produce IL-13 and IL-25, which are involved in the pathogenesis of asthma (48, 50). The capacity of T3P + Ply to suppress the development of NKT cells in the lung highlights its potential as an immunotherapy.

Importantly, to our knowledge, this is the first study to report the suppression of the numbers and function of NKT cells by inducible Tregs in an in vivo model of AAD. The induction of Tregs by T3P + Ply was clearly required to suppress the development of AAD, because the depletion of Tregs by the administration of anti-CD25 reversed the protective effects of T3P + Ply. This protocol depletes the number of CD4⁺ cells that are CD25⁺ for up to 24 d (data not shown) (51). This occurs through the neutralization of CD25 (IL-2R α) and blockade of the signaling cascade induced by IL-2, which compromises the induction of Foxp3 and the development of Tregs.

Unlike conventional T cells, NKT cells require cell–cell contact for their proliferation and function to be suppressed by Tregs,

FIGURE 7. T3P + Ply induces Tregs that are required for the suppression of AAD. **(A)** Representative flow cytometry data demonstrating the gating strategy for CD4⁺CD25⁺Foxp3⁺ cells. **(B)** Total CD4⁺CD25⁺Foxp3⁺ cells in the lungs after intratracheal T3P + Ply treatment and the induction of OVA-induced AAD. **(C–G)** The effects of anti-CD25 administration on the suppressive effects of T3P + Ply treatment on eosinophils in (C) BAL and (D) blood, OVA-induced (E) IL-5 and (F) IL-13 release from MLN T cells, and (G) AHR. Data represent mean $\pm$ SEM from six to eight mice. Significant differences between saline-sensitized (Saline) and OVA-sensitized (OVA) controls are shown as $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. Significant differences between OVA-sensitized and T3P + Ply-treated OVA-sensitized mice are shown as $^*p < 0.05$ and $^{**}p < 0.01$. Significant differences between isotype and anti-CD25 administration on T3P + Ply treatment are shown as $^+p < 0.05$. Significances for resistance and compliance are given for the entire dose-response curve.



which has been demonstrated in transwell studies (52). Neutralization of IL-10 and TGF- β showed that these cytokines were not required for the suppression of NKT cells; however, ICAM-1, which plays an important role in cell-cell contact, was necessary. Moreover, the suppression of NKT cells did not involve the inhibition of APCs. Together, this evidence suggests that Treg ligands, such as galectin-1, which induces cell death of the target cell, may be involved in Treg-mediated suppression of NKT cells (53). If cell-cell contact is necessary for Treg-mediated suppression of NKT cells, then this suggests that Tregs need to be in the same location as NKT cells in order for effective suppression to

occur. In our study, the increase in T3P + Ply-induced Tregs and associated decrease in NKT cells in the lungs, but not MLNs, provide circumstantial evidence that cell contact-mediated suppression in the lungs may be occurring.

An interesting relationship between NKT cells and Tregs in asthma is beginning to emerge. A study by Nguyen et al. (54) demonstrated that NKT cells from asthma patients secreted higher levels of perforin and granzyme B and killed Tregs more effectively compared with those of healthy controls. This suggests that asthmatic NKT cells have increased toxicity toward Tregs, which may contribute to the dysregulated immune response in asthma patients. In addition to higher total numbers of NKT cells, asthma patients also have been reported to have lower numbers of Tregs (8). It therefore is possible that there is an imbalance between Tregs and NKT cells that promotes allergic inflammation and pathogenesis in asthmatic individuals. T3P + Ply-induced Tregs and the associated suppression of NKT cells have potential to restore this imbalance, thereby providing an effective therapy for asthma.

Recently, a CD1d-dependent antagonist, dipalmitoyl phosphatidylethanolamine polyethylene glycol, has been shown to inhibit NKT cells and suppress AHR (55). However, dipalmitoyl phosphatidylethanolamine polyethylene glycol had no effect on the development of OVA-specific Th2 responses. Hence, the induction of Tregs, which also suppress other effector cells such as Th2 cells, may be a more global and effective strategy than directly targeting NKT cells.

Together, our results indicate that T3P + Ply may be a feasible therapy for humans. Our observations that T3P + Ply suppresses responses to both OVA and HDM suggest that it may be effective against multiple Ags. We demonstrate that T3P + Ply suppresses hallmark features of AAD when administered before, during, or

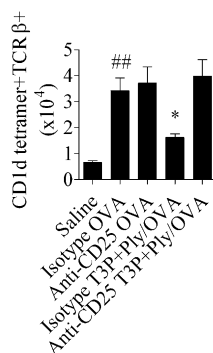


FIGURE 8. T3P + Ply-induced Tregs are required for the suppression of NKT cells. Total CD1d⁺TCR β ⁺ (NKT) cells in the lungs after the induction of OVA-induced AAD, intratracheal administration of T3P + Ply, and treatment with anti-CD25. Data represent mean $\pm$ SEM from six to eight mice. Significant differences between saline-sensitized (Saline) and OVA-sensitized (OVA) controls are shown as $^{\#\#}p < 0.01$. Significant differences between OVA-sensitized mice and T3P + Ply treated OVA-sensitized mice are shown as $^*p < 0.05$.

after sensitization or in established disease. This indicates that treatment in humans may be effective prophylactically before they have asthma, as they develop asthma, or as a treatment when they already have asthma.

In summary, we have shown that *S. pneumoniae* components T3P + Ply reverse the development of hallmark features of AAD. T3P + Ply suppresses the number of NKT cells in the lungs through the induction of Tregs. The ability of Tregs to suppress multiple effector arms of the immune response suggests that this therapy has many advantages over current therapies and may have potential as a therapeutic approach for asthma.

Acknowledgments

We thank Prof. Dale Godfrey and Kon Kyparissoudis for providing the α -GalCer-loaded CD1d tetramer and helpful advice on the design of experiments and preparation of the manuscript.

Disclosures

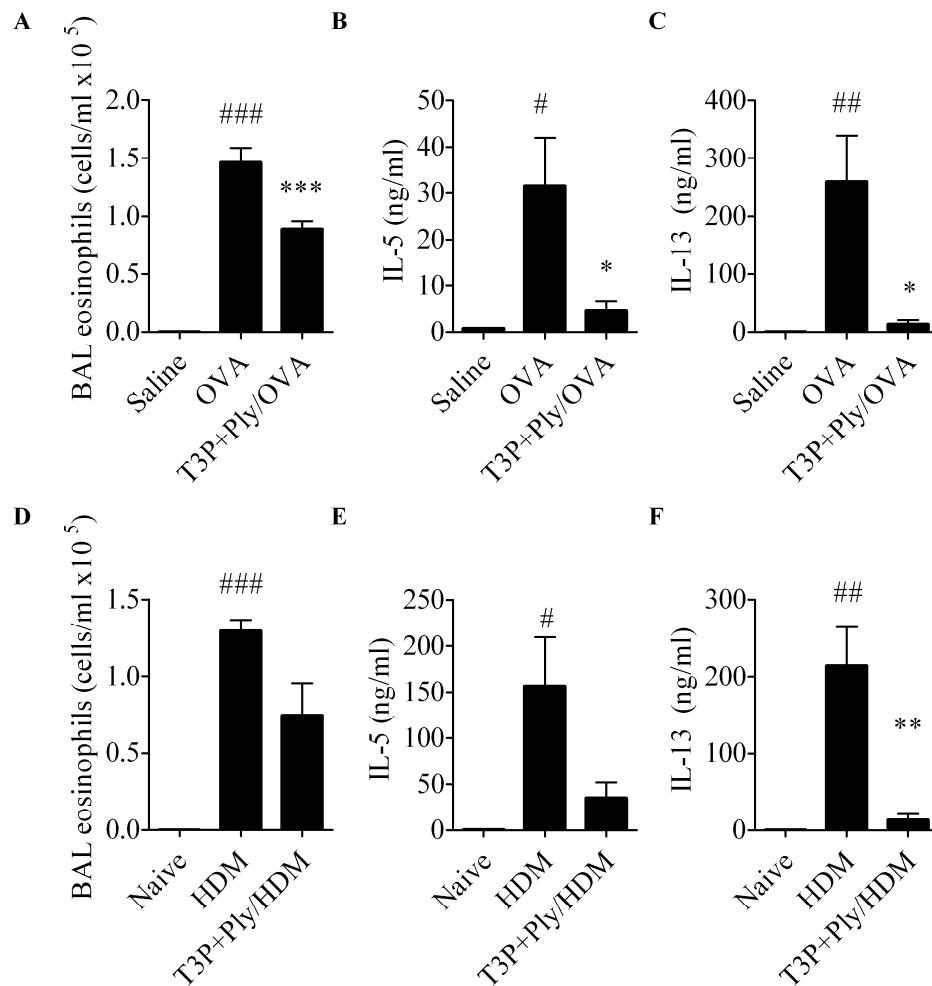
The authors have no financial conflicts of interest.

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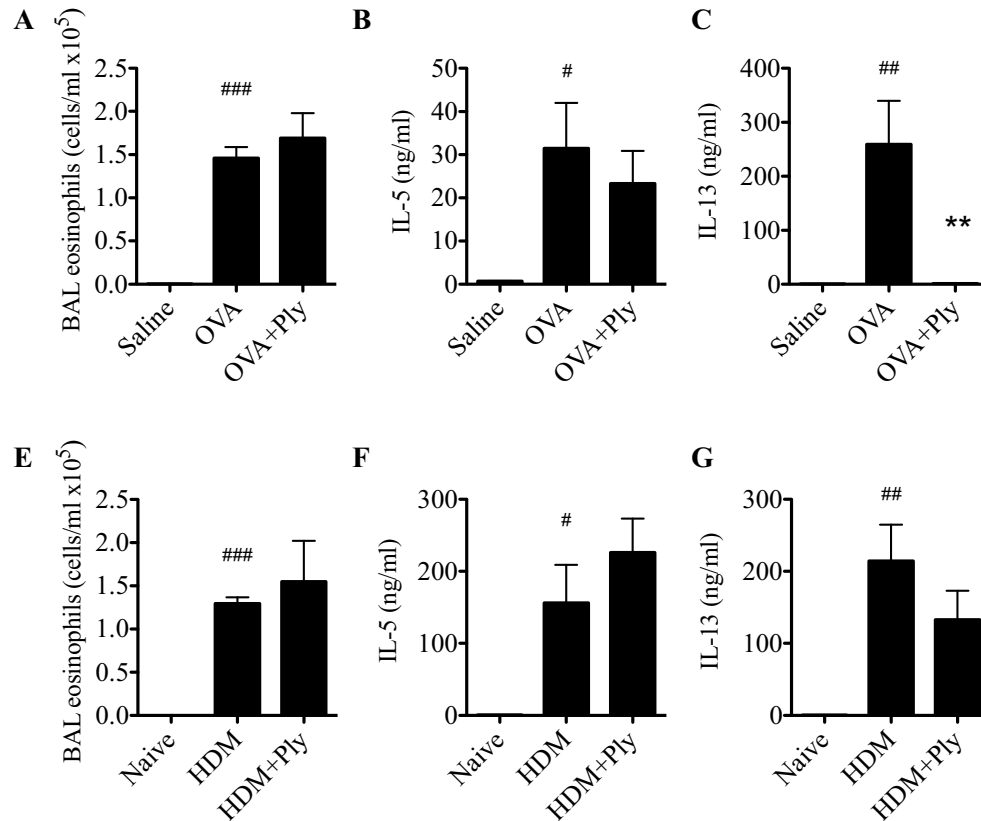
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Supplementary Data



Supplementary Figure 1. T3P+Ply suppress the development of features of AAD in C57BL/6 mice. For ovalbumin (OVA)-induced AAD mice were sensitised intraperitoneally and challenged intranasally (i.n.) with OVA. For house dust mite (HDM)-induced AAD, mice were sensitised and challenged i.n. with HDM. T3P+Ply treatment was administered intratracheally at the time of sensitization, in 3 doses every 12 h. Features of AAD were assessed 24 h after the final challenge. The effect of T3P+Ply in OVA-induced AAD on (A) eosinophils in BAL and OVA-induced (B)

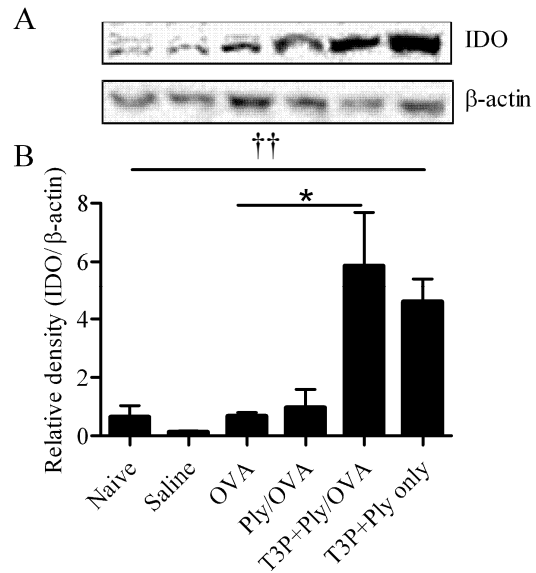
IL-5 and (C) IL-13 release from mediastinal lymph node T cells. The effect of T3P+Ply in HDM-induced AAD on (D) eosinophils in BAL and HDM-induced (E) IL-5 and (F) IL-13 release from mediastinal lymph node T cells. Data represent mean $\pm$ SEM from 6 mice. Significant differences between non-allergic (Saline-sensitized or Naive) and OVA- or HDM-sensitized controls are shown as # $p<0.05$, ## $p<0.01$ or ### $p<0.001$. Significant differences between OVA- or HDM-sensitized and T3P+Ply treated OVA- or HDM-sensitized mice are shown as * $p<0.05$, ** $p<0.01$ or *** $p<0.001$.



Supplementary Figure 2. Ply does not substantially affect AAD. For ovalbumin (OVA)-induced AAD mice were sensitised intraperitoneally and challenged intranasally (i.n.) with OVA. For house dust mite (HDM)-induced AAD, mice were sensitised and challenged i.n. with HDM. Ply was administered intratracheally at the time of sensitization, in 3 doses every 12 h. Features of AAD were assessed 24 h after the final challenge. The effect of Ply in OVA-induced AAD on (A) eosinophils in BAL and OVA-induced (B) IL-5 and (C) IL-13 release from mediastinal lymph node T cells. The effect of Ply in HDM-induced AAD on (D) eosinophils in BAL and HDM-induced (E) IL-5 and (F) IL-13 release from mediastinal lymph node T cells. Data represent mean $\pm$ SEM from 6 mice. Significant differences between non-allergic (Saline-sensitized or Naive) and OVA- or HDM-sensitized controls are shown as # $p < 0.05$, ## $p < 0.01$ or ### $p < 0.001$. Significant differences between OVA- or

HDM-sensitized controls and Ply treated OVA- or HDM-sensitized mice are shown as

** $p < 0.01$.



Supplementary Figure 3. T3P+Ply induces increases in indolamine 2,3-dioxygenase (IDO) levels. (A) Western blot for IDO and β -actin (loading control). (B) Quantification of western blot by densitometric analysis. Data represent mean $\pm$ SEM from 4 mice. Significant differences between OVA-sensitized and T3P+Ply treated OVA-sensitized mice are shown as * $p < 0.05$. Significant differences between naïve and T3P+Ply treated mice are shown as $\dagger\dagger$ $p < 0.01$