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J Immunol published online 18 September 2013
<http://www.jimmunol.org/content/early/2013/09/18/jimmunol.1201232>

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Pneumococcal Components Induce Regulatory T Cells That Attenuate the Development of Allergic Airways Disease by Deviating and Suppressing the Immune Response to Allergen

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The induction of regulatory T cells (Tregs) to suppress aberrant inflammation and immunity has potential as a therapeutic strategy for asthma. Recently, we identified key immunoregulatory components of *Streptococcus pneumoniae*, type 3 polysaccharide and pneumolysin (T+P), which suppress allergic airways disease (AAD) in mouse models of asthma. To elucidate the mechanisms of suppression, we have now performed a thorough examination of the role of Tregs. BALB/c mice were sensitized to OVA (day 0) i.p. and challenged intranasal (12–15 d later) to induce AAD. T+P was administered intratracheally at the time of sensitization in three doses (0, 12, and 24 h). T+P treatment induced an early (36 h–4 d) expansion of Tregs in the mediastinal lymph nodes, and later (12–16 d) increases in these cells in the lungs, compared with untreated allergic controls. Anti-CD25 treatment showed that Treg-priming events involving CD25, CCR7, IL-2, and TGF- β were required for the suppression of AAD. During AAD, T+P-induced Tregs in the lungs displayed a highly suppressive phenotype and had an increased functional capacity. T+P also blocked the induction of IL-6 to prevent the Th17 response, attenuated the expression of the costimulatory molecule CD86 on myeloid dendritic cells (DCs), and reduced the number of DCs carrying OVA in the lung and mediastinal lymph nodes. Therefore, bacterial components (T+P) drive the differentiation of highly suppressive Tregs, which suppress the Th2 response, prevent the Th17 response and disable the DC response resulting in the effective suppression of AAD. *The Journal of Immunology*, 2013, 191: 000–000.

Asthma is an allergic airways disease (AAD) that is characterized by airway inflammation and hyperresponsiveness (AHR) to nonspecific stimuli (1, 2). The prevalence of asthma in westernized countries has doubled over the past three decades but now appears to have plateaued. Improvements in hygiene and vaccination regimes together with decreased exposure to infectious agents may be responsible for the increase in prevalence.

Asthma results from a dysregulation in immunity that is underpinned by alterations in effector T cell populations including Th1, Th2, Th17, and NKT cells (1, 2). Th2 and Th17 cells are particularly important in promoting the development, progression, and exacerbation of disease (3). These effector T cells produce numerous inflammatory cytokines and chemokines that induce

eosinophil influx, mucus hypersecretion, and AHR. Because so many factors are involved in asthma pathogenesis, therapeutic strategies that target a single effector T cell response or inflammatory signal are unlikely to be successful (1). This suggests the need for more global anti-inflammatory therapeutic approaches. One such approach is to suppress the dysregulated immune response through the induction of regulatory T cells (Tregs) that inhibit a broad array of proinflammatory factors and pathways (2).

Tregs are essential for maintaining homeostasis and preventing aberrant immune responses (2). They are characterized by the expression of the transcription factor FOXP3, which is essential for their suppressive effects (2). Tregs may be natural and constitutively present or inducible and develop from naive T cells. The induction of FOXP3 results in stable differentiation of the Treg phenotype preventing conversion or reversion into an effector T cell (4, 5). To suppress and control proinflammatory responses of immune cells Tregs use a number of different regulatory mechanisms including contact- or soluble factor-mediated suppression, deprivation of inflammatory cell nutritional requirements, modulation of Ag presentation, and deviation of T cell development away from the production of effector T cells (2). The roles of immune suppression versus deviation are poorly understood.

Asthmatics are known to have reduced numbers of Tregs and those that they do have exhibit reduced functional capacity (6, 7). Because Tregs have the capacity to regulate inflammation in asthma, harnessing or enhancing the suppressive effects of these cells may provide an effective therapeutic strategy (2). We have previously shown that live or killed *Streptococcus pneumoniae* (i.e., the pneumococcus) as well as pneumococcal vaccines suppress the hallmark features of AAD in mouse models of asthma. Thus, antigenic components of *S. pneumoniae* have the potential to be used as immunoregulatory therapies for asthma (8–10). We have also

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Received for publication May 2, 2012. Accepted for publication August 20, 2013.

This work was supported by the Hill family, the Asthma Foundation New South Wales, the Cooperative Research Centre for Asthma and Airways, the Australian Research Council, the National Health and Medical Research Council, and the University of Newcastle.

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Abbreviations used in this article: AAD, allergic airway disease; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; i.n., intranasal; mDC, myeloid dendritic cell; MHC, MHC class II; MLN, mediastinal lymph node; T+P, type 3 polysaccharide and pneumolysin; Treg, regulatory T cell.

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demonstrated that suppression of AAD by *S. pneumoniae* is mediated by the induction of Tregs. To develop a more defined and optimized *S. pneumoniae*-based immunoregulatory therapy for AAD, we have identified the key immunoregulatory components of the bacteria that are required for suppression of AAD (11). Two *S. pneumoniae* components, type 3 polysaccharide and pneumolysin (T+P), when coadministered before, during, or after sensitization suppressed the hallmark features of AAD including eosinophil infiltration of the airways, lung tissue, and blood; IL-5 and IL-13 release from mediastinal lymph node (MLN) and splenic T cells; mucus hypersecretion; and AHR. It remains unknown how these bacterial components induce Tregs or how these cells mediate the suppression of AAD.

In this study, we investigated the profile of induction of Tregs by *S. pneumoniae* components (T+P) and the mechanisms by which these cells and components suppress AAD.

Materials and Methods

Animals

Six- to 8-wk-old female BALB/c mice were obtained from the Animal Breeding Facility at the University of Newcastle (Callaghan, NSW, Australia). BALB/c DO11.10 TCR transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free and controlled environmental conditions. Procedures were approved by the Animal Ethics Committee of the University of Newcastle.

Allergic airways disease

Induction of AAD was performed (Fig. 1A) as described previously (8, 9, 12–14). Mice were sensitized to OVA (i.p.; day 0; 50 µg; Sigma-Aldrich, St. Louis, MO) with rehydrogel (1 mg; Reheis, Berkeley Heights, NJ) in sterile saline (200 µl). Mice were challenged by intranasal (i.n.) droplet application of OVA (days 12–15; 10 µg in 50 µl sterile saline) under isoflurane anesthesia. Where indicated mice received FITC-labeled OVA challenge (Invitrogen, Carlsbad, CA). Control mice received saline sensitization and OVA challenge. AAD was assessed on day 16.

Immunoregulatory therapy

During sensitization to OVA, mice were treated with T+P; type 3 polysaccharide (2 µg; American Type Culture Collection) and pneumolysin (LPS free; 40 ng; Prof. J. Paton, University of Adelaide, Adelaide, SA, Australia) in sterile saline (30 µl; three doses 12 h apart) by intratracheal administration under i.v. alfaxan anesthesia (Fig. 1A). Where indicated, mice received CD25-depleting Ab (i.p.; day –3; 100 µg Ab prepared in house from clone PC61; in 200 µl saline) or anti-TGF-β Ab (i.p.; days 0–3; 25 µg; clone 1d11; eBioscience, San Diego, CA; in 200 µl saline). Controls received isotype control Ab.

Cell preparation

Single-cell suspensions were prepared from MLNs by pushing through 70-µm sieves and from lungs and spleens using a gentle MACs homogenizer (Miltenyi Biotec, Auburn, CA), according to the manufacturer's protocol (8).

Flow cytometry

A total of 1×10^6 cells/well in 96-well U-bottom plates were stained for CD4, CD25 (BD Pharmingen, San Diego, CA), CTLA-4, CD103, CD11c, CD11b, F480, CD80, CD86, MHC class II (MHCII), ICOS (BioLegend, San Diego, CA), and PDCA (Miltenyi Biotec) using fluorescently labeled mAbs or isotype controls. Cells were permeabilized and stained intracellularly for Foxp3, according to the manufacturer's protocol (eBioscience) (8). For detection of IL-6 and IL-17, cells were stimulated with LPS (100 ng/ml) for 20 h or PMA (0.1 µg/ml) and ionomycin (1 µg/ml) for 6 h, respectively. Brefeldin A (8 µg/ml) was added during the final 2 h of each culture. Samples and controls were analyzed using a FACSCanto flow cytometer controlled by FACSDiVa software (version 4.1.1; BD Biosciences, Mississauga, ON, Canada).

Assessment of airway inflammation

Bronchoalveolar lavage (BAL) was performed as previously described, and differential leukocyte counts were determined from a total of 250 cells (15, 16).

T cell cytokine release

A total of 1×10^6 cells/well in 96-well U-bottom plates were cultured in RPMI 1640 medium supplemented with 10% FCS, HEPES (20 mM), penicillin/streptomycin (10 µg/ml), L-glutamine (2 mM), 2-ME (50 µM), and sodium pyruvate (1 mM). Cells were stimulated with either OVA (200 µg/ml), OVA peptide (10 µg/ml), anti-CD3 and anti-CD28 (5 µg/ml), or LPS (100 ng/ml) and cultured for 4 d (MLNs and lungs) or 6 d (spleen) (5% CO₂, 37°C). Supernatants were collected and stored at –20°C until analysis. Cytokine concentrations in cell culture supernatants were determined by ELISA (BD Pharmingen, San Diego, CA) (8).

Airway hyperresponsiveness

AHR was assessed as described previously (13–15). Briefly, anesthetized and tracheotomized mice were cannulated and connected to inline aerosol and ventilator apparatus. Changes in airway function following challenge with increasing doses of aerosolized methacholine (1.25, 2.5, 5, and 10 mg/ml) were assessed by analysis of pressure and flow waveforms, and transpulmonary resistance and dynamic compliance were determined.

Real-time PCR

For analysis of gene expression, total RNA was prepared from whole lungs or MLNs by TRIzol extraction or from cell culture using a Purelink kit (Invitrogen), and cDNA was generated. Real-time RT-PCR was performed as described previously (15). The following primer sequences were used to assess gene expression: IL-2, 5'-CCTGAGCAGGATGGAGAATTACA-3' (forward) and 5'-TCCAGAACATGCCGACAG-3' (reverse); TGF-β, 5'-CCCGAAGCGGACTACTATGCTAAA-3' (forward) and 5'-GGTAACGC-CAGGAATTGTTGCTAT-3' (reverse); CCR7, 5'-TGGTGGCTCTCCTTGTCATT-3' (forward) and 5'-GTGGTATTCTCGCCGATGTAGT-3' (reverse); IL-6, 5'-AGAAAACAATCTGAAACTTCCAGAGAT-3' (forward) and 5'-GAAGACCAGAGGAAATTTCAATAGG-3' (reverse); IL-25, 5'-CTGAA-GTGGAGCTCTGCATCTGT G-3' (forward) and 5'-GTCCATGTGGGAG-CCTGTCTGTAG-3' (reverse); Foxp3, 5'-GGCCCTTCTCCAGGACAGA-3' (forward) and 5'-GCTGATCATGGCTGGGTGTG-3' (reverse); IL-10, 5'-C-ATTTGAATTCCTGGGTGAGAAG-3' (forward) and 5'-GCCTTGATAG-CACCTTGGTCTTGG-3' (reverse); Ebi3, CCGCTCCCCTGGTTACACTG-3' (forward) and 5'-GTGGCAATGAAGGACGTGGA-3' (reverse); p35, 5'-GCCAGGTGTCTTAGCCAGTCC-3' (forward) and 5'-GCCAGGCAACTC-TCGTTCTTG-3' (reverse); IL-9, 5'-CATCCTTGCTCTGTTTGTCTCTT-3' (forward) and 5'-TCTTGATTCTGTGGCATTTGGT-3' (reverse); and TLR2, 5'-TGTAGGGGCTTCACTTCTCTGCTT-3' (forward) and 5'-AGACTCTGAGCAGAACAGCGTTT-3' (reverse).

CCR7 levels

CCR7 levels were assayed by Western blot analysis (R&D Systems, Minneapolis, MN) and quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, MD).

In vitro culture

A total of 1×10^6 DO11.10 splenocytes/well in 96-well U-bottom plates were cultured in supplemented RPMI 1640 medium (4 d, 5% CO₂, 37°C). OVA peptide (10 µg/ml; Invitrogen) and/or T+P (6 µg and 120 ng, respectively) were added as indicated.

Proliferation assay

CD4⁺CD25⁺ cells were isolated (>90% pure) using CD11c depletion followed by CD4⁺CD25⁺ Treg isolation (AutoMACs; Miltenyi Biotec). CD4⁺CD25⁺ cells were further purified by CD4⁺ selection (94% pure) (10). CD4⁺CD25⁺ cells (5×10^4) and varying numbers of CD4⁺CD25⁺ cells were cultured in RPMI 1640 medium (200 µl, 10% FCS; 72 h, 37°C) with anti-CD28 (1 µg/ml; BD Pharmingen) and plate-bound anti-CD3 (1 µg/ml). Cells were pulsed for the final 18 h of culture with [³H]thymidine (Amersham International, Little Chalfont, Buckinghamshire, U.K.) and enumerated using a microbeta counter.

Data analysis

Data were analyzed using GraphPad Prism (GraphPad Software, CA) and are represented as the mean ± SEM. One-way ANOVA with Dunnett's posttest was used to determine significance between data with multiple comparisons. Unpaired Student *t* test was used to determine differences between two groups. One-way repeated measures ANOVA and Bonferroni's posttest were used to determine significance for AHR data. A *p* value <0.05 was considered statistically significant.

Results

T+P induces an early expansion of Tregs in the MLNs and a later increase in the lung in AAD

A well-established model of AAD was used (Fig. 1A), where mice were sensitized to OVA i.p. (day 0) and challenged i.n. on days 12–15 to induce AAD (8, 9, 13–15). T+P was administered intratracheal at the time of sensitization, in three doses (0, 12, and 24 h; Fig. 1A). To determine the capacity of T+P to induce Tregs, we performed a time-course analysis (0 and 36 h, and 4, 8, 12, and 16 d) of Tregs in the MLNs and lungs using flow cytometry.

In the MLNs, T+P treatment (T+P/OVA groups) increased the percentage of CD4⁺ cells that were CD25⁺Foxp3⁺ and the total number of CD4⁺CD25⁺Foxp3⁺ cells between 36 h and 4 d compared with the untreated (OVA groups) controls (Fig. 1B). T+P treatment did not alter the percentage or number of Tregs in the MLNs at later time points or the percentage or number of CD25[−]Foxp3⁺ cells in the MLNs (data not shown).

In the lung, T+P treatment increased the percentage of CD4⁺ cells that were CD25⁺Foxp3⁺ and the total number of CD4⁺CD25⁺Foxp3⁺ cells between 12 and 16 d compared with untreated controls (Fig. 1C). T+P treatment also increased the percentage of CD4⁺ cells that were CD25[−]Foxp3⁺ and the total number of CD4⁺CD25[−]Foxp3⁺ cells between 12 and 16 d (Fig. 1D). T+P treatment did not alter the percentage or number of Tregs at earlier time points.

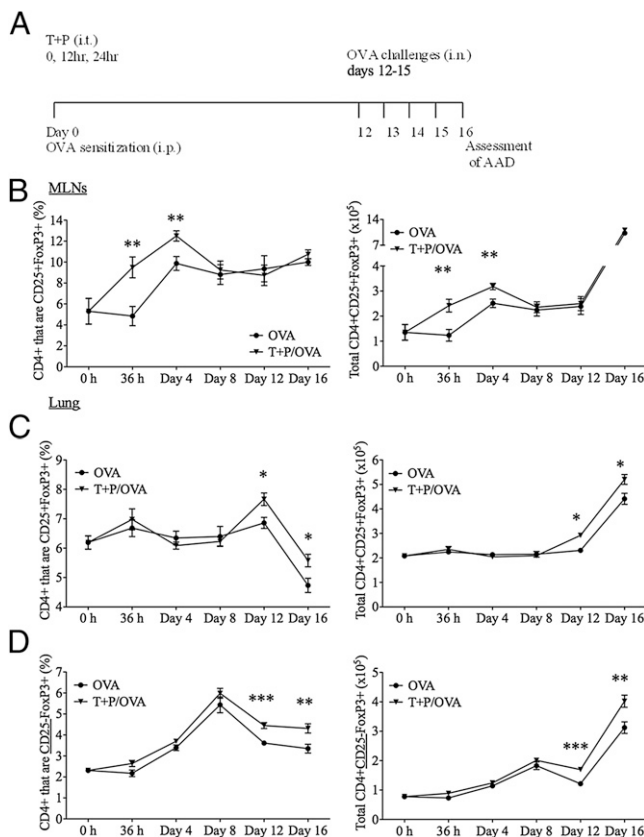


FIGURE 1. Time-course analysis of T+P-induced Tregs in the MLNs and lung. **(A)** AAD was induced by i.p. OVA sensitization and intranasal (i.n.) OVA challenge. Controls received saline sensitization and OVA challenge. T+P treatment was administered at the time of OVA sensitization in three doses every 12 h. Features of AAD were assessed 24 h after the final OVA challenge. The percentage of CD4⁺ cells that were CD25⁺Foxp3⁺ and total number of CD4⁺CD25⁺Foxp3⁺ cells in the MLNs **(B)** and the lung **(C)**. **(D)** The percentage of CD4⁺ cells that were CD25[−]Foxp3⁺ and total number of CD4⁺CD25[−]Foxp3⁺ cells in the lung. Data represent mean $\pm$ SEM from six to eight mice. * p < 0.05, ** p < 0.01, *** p < 0.001, significant differences.

These results demonstrate that T+P enhances the early expansion of Tregs in the MLNs and later increases of these cells in the lung.

Anti-CD25 Ab depletes the number of CD4⁺ cells that are CD25⁺Foxp3⁺ in the MLNs and lungs that are induced by T+P treatment in AAD

To investigate the role of early and late T+P-induced Tregs in mediating the suppression of AAD, we first established a protocol to deplete CD25⁺ Tregs using anti-CD25 (PC61) Ab. Anti-CD25 Ab was administered either 3 d prior to T+P treatment (day −3) or 3 d prior to OVA challenge (day +9). Tregs were assessed in MLNs and lungs on days 4 and 16.

In the MLNs, administration of anti-CD25 Ab on day −3 depleted the percentage of CD4⁺ cells that were CD25⁺Foxp3⁺ on day 4 (Fig. 2A). Administration on either day −3 or +9 depleted the percentage of CD4⁺ cells that were CD25⁺Foxp3⁺ on day 16.

In the lung, administration on day −3 depleted the percentage of CD4⁺ cells that were CD25⁺Foxp3⁺ on day 4 (Fig. 2B). Administration on either day −3 or +9 depleted the percentage of CD4⁺ cells that were CD25⁺Foxp3⁺ on day 16.

Further assessment of Treg populations in the lung showed that administration of anti-CD25 Ab on day −3 had no effect on the percentage of CD4⁺ cells that were CD25[−]Foxp3⁺ in the lung on day 4 but suppressed these cells on day 16 (Fig. 2C). Administration on day +9 had no effect on these cells at either time point.

The induction of Tregs by T+P in the early phase is CD25 dependent and required for the suppression of Th2 responses and AAD

We then used these protocols to investigate whether T+P-induced Tregs were required for the suppression of Th2 responses and the subsequent development of AAD. The development of AAD was characterized by increases in the numbers of eosinophils in the BAL, OVA-induced IL-5, IL-13, and IFN- γ release from MLN T cells, and AHR (isotype-treated OVA groups; Fig. 3). As expected, T+P treatment suppressed: the recruitment of eosinophils into the BAL; OVA-induced IL-5 and IL-13 release from MLN T cells; and AHR (isotype T+P/OVA groups). Of note, T+P treatment had no effect on IFN- γ levels (data not shown), which indicates that suppression of Th2 responses by increased Th1 cells did not occur, as we have discussed in our other studies (8–11). Administration of anti-CD25 on day −3 reversed the effect of T+P treatment on the following: the recruitment of eosinophils into the BAL; OVA-induced IL-5 and IL-13 release from MLN T cells; and AHR (anti-CD25 −3 T+P/OVA). However, anti-CD25 treatment on day +9 had no effect on these features of AAD (anti-CD25 +9 T+P/OVA). These data suggest that the Treg population induced early, but not late, after T+P treatment was CD25 dependent and was required for the suppression of Th2 responses and AAD.

T+P induced IL-2/IL-2R interactions may contribute to the suppression of AAD

CD25 is the IL-2 α receptor, and our results that show that anti-CD25 treatment reverses the protective effects of T+P treatment on AAD indicate that altered IL-2 interactions are involved in protection. Thus, we next assessed how T+P-mediated suppression of AAD affects IL-2 interactions by quantifying IL-2 gene expression in the lung. T+P treatment increased IL-2 gene expression on day 4 only (Fig. 4A). Therefore, because IL-2 expression was increased and that blocking IL-2/IL-2R interactions with anti-CD25-depleted Tregs and reversed the effects of T+P, IL-2 is likely to be important for the induction of Tregs by T+P and the subsequent suppression of AAD.

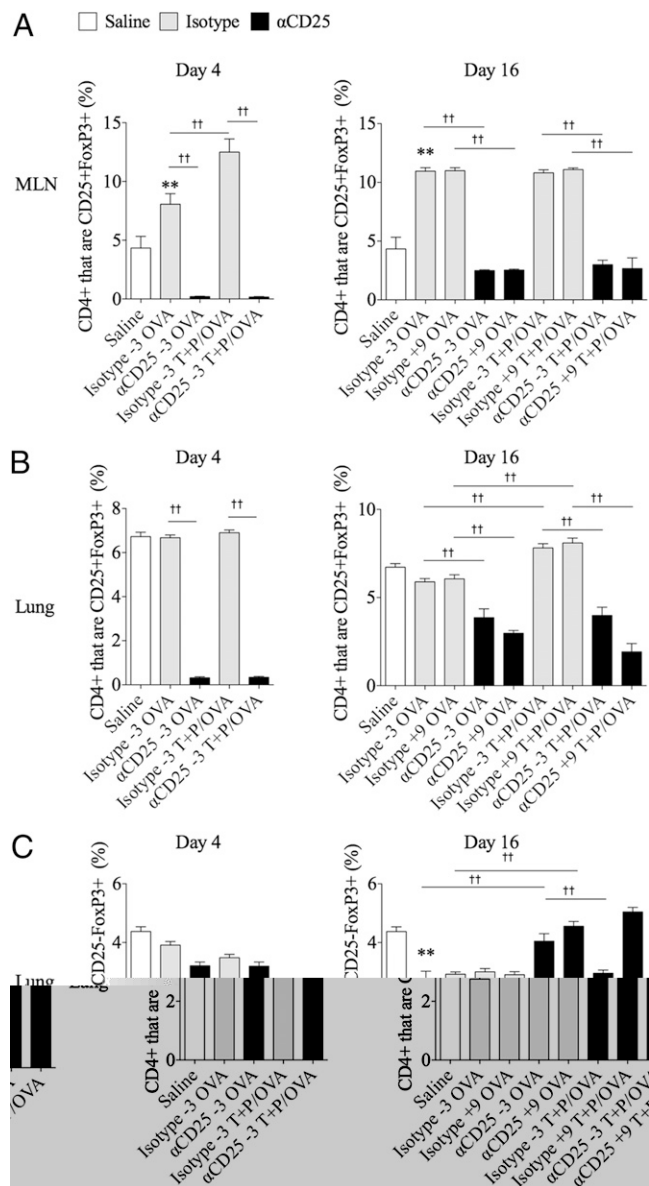


FIGURE 2. The effects of anti-CD25 Ab administration on Treg populations in the MLNs and lung when administered day -3 (-3) or day 9 (+9). The percentage of CD4⁺ cells that were CD25⁺Foxp3⁺ and total number of CD4⁺CD25⁺Foxp3⁺ cells in the MLNs (**A**) and lungs (**B**) on days 4 and 16. (**C**) The percentage of CD4⁺ cells that were CD25⁻Foxp3⁺ and total number of CD4⁺CD25⁻Foxp3⁺ cells in the lung on days 4 and 16. Data represent mean $\pm$ SEM from six to eight mice. ** p < 0.01, significant differences. †† p < 0.01.

T+P-induced TGF- β contributes to the suppression of AAD

TGF- β promotes the development of Tregs, is produced by Tregs, and suppresses proinflammatory responses (2). Thus, we next assessed the role of TGF- β in T+P-mediated suppression of AAD by quantifying TGF- β gene expression in the lung. T+P treatment increased TGF- β expression on day 4 only (Fig. 4B). Of note, IL-10 was not increased at this time point (data not shown); therefore, CD4⁺IL-10⁺ Tregs are not likely to be involved. We further investigated the role of TGF- β using anti-TGF- β -neutralizing Ab. Administration of anti-TGF- β -neutralizing Ab on days 0–3 had no significant effect on T+P-mediated suppression of eosinophil recruitment into the BAL (Fig. 4C). However, anti-TGF- β reversed the suppressive effect of T+P treatment on OVA-induced IL-5 and

IL-13 release from MLN T cells (Fig. 4D, 4E). In addition, anti-TGF- β partially reversed the suppressive effect of T+P treatment on AHR (Fig. 4F). Therefore, TGF- β is partially required for the complete suppression of AAD.

T+P induces CCR7 and its ligands in the lung

Because T+P-induced IL-2 and TGF- β in the lung were associated with the expansion of Tregs in the MLNs and because CCR7 expression is associated with Treg migration out of the lung and into the lymph nodes (17), we assessed CCR7 gene expression in the lung. T+P treatment increased CCR7 expression in the lung on day 4, and this increase was confirmed at the protein level (Fig. 4G). This increase in CCR7 may contribute to the movement of Tregs into the MLNs at this time point. CCL19 and CCL21 gene expression levels were also assessed and were increased at the 36 h time point (data not shown).

T+P suppresses Th17 effector cell responses in AAD

We next examined the effects of T+P on Th17 responses. In the presence of TGF- β , IL-6 promotes Th17 cell differentiation (1). Therefore, we assessed the expression of IL-6 in the lung. T+P treatment inhibited IL-6 gene expression throughout the time course investigated (Fig. 4H). CD11c⁺ cells are major sources of IL-6, and the reduction in IL-6 expression was associated with the attenuation of the total percentage of CD11c⁺IL-6⁺ cells in the lung in AAD compared with untreated controls (Fig. 4I). T+P-induced increases in TGF- β expression and decreases in IL-6 expression and total percentage of CD11c⁺IL-6⁺ cells were associated with reductions in the total percentage of CD4⁺IL-17⁺ cells, IL-25 (IL-17E) expression in the lung and IL-17A protein levels in AAD (Fig. 4J, 4K). These results suggest that T+P-induced Tregs suppress the induction and function of Th17 cells.

T+P suppresses the establishment of Th2 and Th17 effector cell responses ex vivo

To confirm our in vivo observations, we used an ex vivo system that used splenocytes from DO11.10 TCR transgenic mice. These mice have T cells that react specifically to the OVA 323–339 peptide (OVAp). DO11.10 splenocytes were cultured with or without T+P and/or OVAp, and the effects on cytokine release and gene expression were assessed.

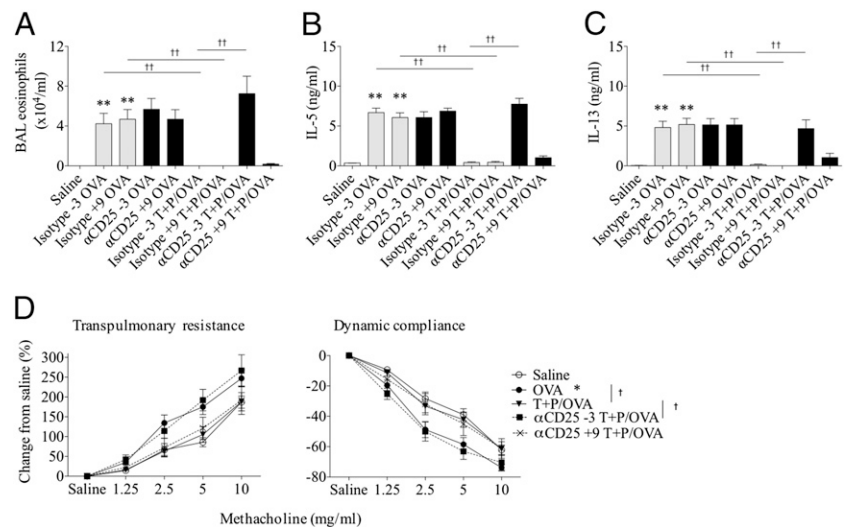
T+P treatment suppressed OVAp-induced IL-4, IL-5, IL-13, and IL-17 release from splenocytes but had no effect on IL-10, compared with untreated controls (Fig. 5A–E). T+P treatment also induced TGF- β release and the maintenance of the expression of Foxp3 (Fig. 5F, 5G). Therefore, there is an association between the suppression of Th2 and Th17 responses and enhanced TGF- β expression and sustained expression of Foxp3.

T+P-induced Tregs in the lungs in AAD have a highly suppressive phenotype

To determine the mechanism of suppression, we first assessed the effects of T+P on the gene and protein levels of the immunosuppressive cytokines IL-10 and TGF- β in the lungs in AAD (i.e., after both sensitization and challenge to induce AAD at day 16 of the experimental protocol). T+P treatment suppressed OVA-induced IL-10 gene expression in the lung and OVA-stimulated IL-10 release from isolated lung cells in AAD (Fig. 6A). T+P treatment did not alter OVA-induced TGF- β gene expression or protein release on day 16 (Figs. 4B, 6B).

We then assessed the effects of T+P on the suppressive phenotype of Tregs using markers associated with the functional capacity of these cells. T+P treatment enhanced the percentage of CD4⁺CD25⁺Foxp3⁺ cells that expressed CTLA-4 in the lung

FIGURE 3. The effects of anti-CD25 Ab administration on T+P-mediated suppression of AAD. **(A)** The number of eosinophils in BAL and OVA-induced IL-5 **(B)** and IL-13 **(C)** release from MLN T cells and AHR **(D)** in terms of airways resistance and dynamic compliance (dotted lines denote anti-CD25 Ab administration). Data represent mean $\pm$ SEM from six to eight mice. * $p < 0.05$, ** $p < 0.01$, significant differences relative to saline. † $p < 0.05$, †† $p < 0.01$ relative to untreated. Significances for resistance and compliance are given for the entire dose response curve.



compared with untreated controls (Fig. 6C). T+P also maintained the percentage of CD4⁺CD25⁺Foxp3⁺CD103⁺ cells (Fig. 6D). T+P treatment did not alter the expression of L-selectin, GITR, or PD-1 (data not shown).

Next, we assessed a panel of other markers that are associated with enhanced suppressive capacity of Tregs. T+P maintained Ebi3 gene expression and increased p35 gene expression, which together form the heterodimeric cytokine IL-35 (Fig. 6E). T+P treatment also increased IL-9 and TLR2 (small effect) gene expression (Fig. 6F, 6G). These results suggest that T+P-induced Tregs have an enhanced suppressive phenotype. Therefore, we assessed their suppressive capacity using a proliferation assay with cells isolated on days 4, 9, and 16. CD4⁺CD25⁺ cells isolated from the lung of T+P-treated mice suppressed CD4⁺CD25⁺ effector T cell proliferation to a greater extent compared with those from untreated mice (Fig. 6H; data not shown). In addition, CD4⁺CD25⁺ cells from T+P-treated mice suppressed CD4⁺CD25⁺ effector T cell release of IL-5 and IL-13 (data not shown).

T+P-suppressed CD86 expression on myeloid dendritic cells and the number of dendritic cells loaded with OVA

APCs are essential mediators of immune responses. Therefore, we investigated their role in T+P-mediated suppression of AAD. To do this, a time-course analysis (36 h and days 4, 8, 12, and 16) of plasmacytoid dendritic cells (DCs) (PDCA⁺CD11c⁺CD11b⁺), myeloid DCs (mDCs) (CD11c⁺CD11b⁺), and macrophages (F480⁺CD11c⁺), and their expression of MHCII, CD80, and CD86 was performed on MLN and lung cells using flow cytometry. T+P had minimal effects on the numbers or surface marker expression on these cells (data not shown). The only significant result was a decrease in CD86 expression on mDCs in the lung in AAD (day 16), compared with untreated controls (Fig. 7A). This suggests that APC maturation status is unaffected by T+P treatment but that CD86 expression on mDCs may play a role in T+P-mediated suppression of AAD. We also assessed ICOS expression on CD4⁺ T cells. T+P treatment did not alter the level of ICOS expression on CD4⁺ T cells (data not shown).

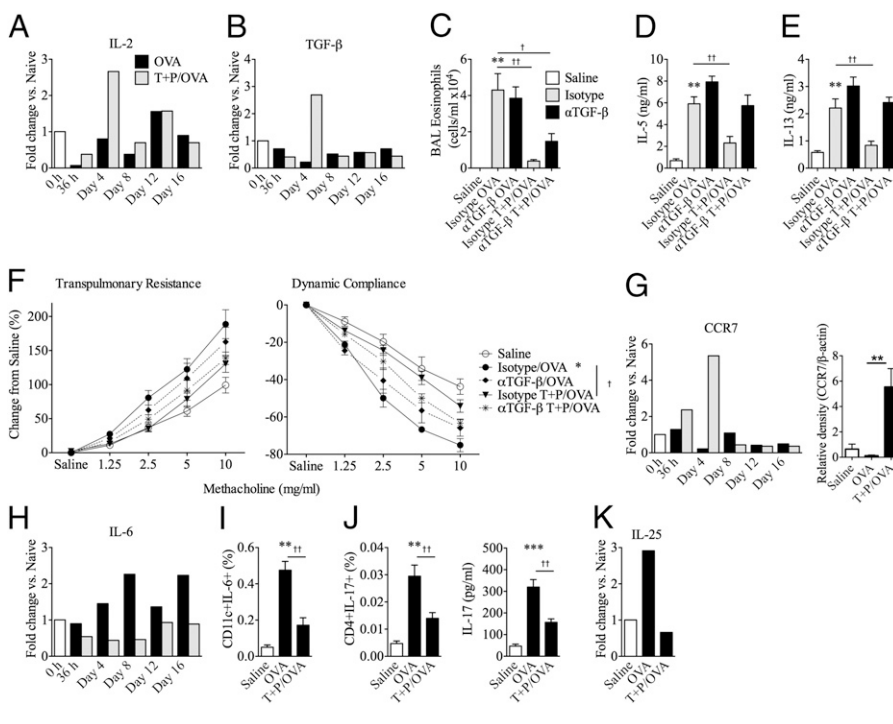
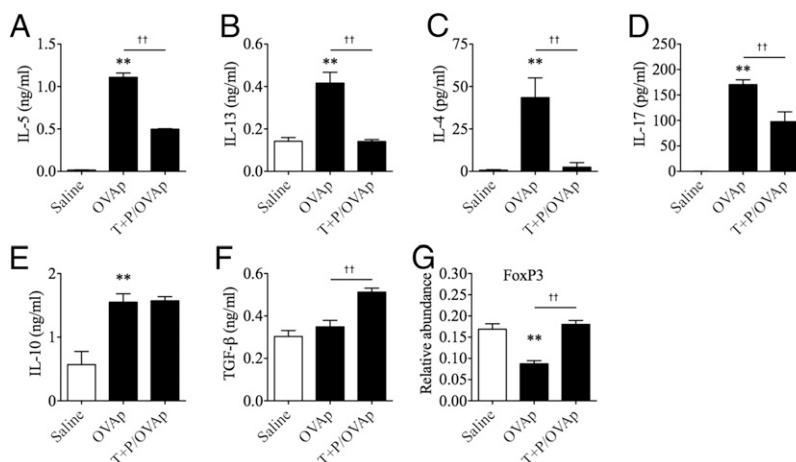


FIGURE 4. The effect of T+P on IL-2 **(A)** and TGF- β **(B)** mRNA expression in the lung. The effect of anti-TGF- β Ab administration on T+P-mediated suppression of the number of eosinophils in BAL **(C)**, OVA-induced IL-5 **(D)** and IL-13 **(E)**, and AHR **(F)** (dotted lines denote anti-TGF- β administration). The effect of T+P on CCR7 mRNA **(G)** and protein expression in the lung. The effect of T+P on IL-6 mRNA expression **(H)**, the percentage of CD11c⁺IL-6⁺ cells **(I)**, CD4⁺IL-17⁺ cells and IL-17A protein levels **(J)**, and IL-25 mRNA expression **(K)** in the lung in AAD. For mRNA expression, data are representative of six pooled RNA samples, and data were confirmed in a repeat experiment. Where error bars are provided, data represent mean $\pm$ SEM from six to eight mice. * $p < 0.05$, ** $p < 0.01$, significant differences relative to saline. † $p < 0.05$, †† $p < 0.01$ relative to untreated. Significance for resistance and compliance are given for the entire dose response curve.

FIGURE 5. The effects of T+P treatment on OVA peptide (OVAp)-induced IL-5 (A), IL-13 (B), IL-4 (C), IL-17 (D), IL-10 (E), and TGF- β (F) release and Foxp3 mRNA (G) expression in OVAp-stimulated OVAp-TRC transgenic splenocytes in vitro. Data represent mean $\pm$ SEM of triplicate wells. ** $p < 0.01$, significant differences. †† $p < 0.01$.



Next, we assessed the number of DCs carrying OVA by administering FITC-labeled OVA during the challenge phase. T+P reduced the number of CD11c⁺MHCII⁺ cells carrying OVA in the MLNs and lungs (on day 16) compared with untreated controls (Fig. 7B, 7C).

Discussion

In this study, we show that treatment of mice with the *S. pneumoniae* components, T+P, drives Treg differentiation, which is required to suppress the development of AAD (Figs. 1–3). The

T+P-induced Treg were promoted by IL-2 and TGF- β in the lung (Fig. 4) and directed to the lymph node by CCR7-mediated chemotaxis. In addition, T+P suppressed the production of IL-6, which prevented the development of Th17 cells (Fig. 4). Our results were confirmed using OVA-TCR transgenic T cells ex vivo (Fig. 5). The T+P-induced Tregs did not suppress via IL-10 or TGF- β ; instead, Tregs had an increased expression of markers associated with an enhanced suppressive phenotype (Fig. 6). This phenotype resulted in Tregs that had increased suppressive capacity (Fig. 6H). T+P-induced Tregs also reduced the number of

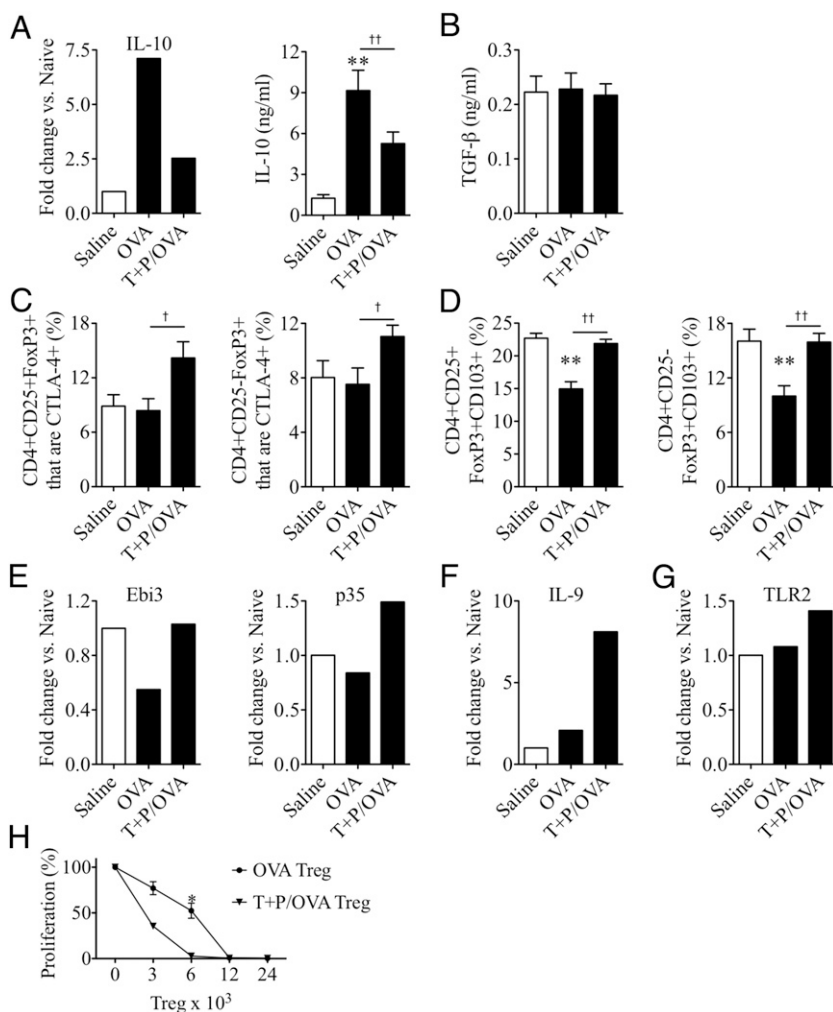


FIGURE 6. The effects of T+P treatment on Treg functional markers. (A) IL-10 mRNA expression in the lung and OVA-induced IL-10 release from lung T cells. (B) OVA-induced TGF- β release from lung T cells. (C) The percentage of CD4⁺CD25⁺Foxp3⁺ and CD4⁺CD25⁺Foxp3⁺ cells that were CTLA-4⁺. (D) The percentage of CD4⁺CD25⁺Foxp3⁺CD103⁺ and CD4⁺CD25⁺Foxp3⁺CD103⁺ cells. Ebi3 and p35 (E), IL-9 (F), and TLR2 mRNA (G) expression in the lung. (H) Proliferation capacity of CD4⁺ T cells in the presence of increasing numbers of Tregs from the lungs of untreated or T+P-treated mice. Where error bars are provided, data represent mean $\pm$ SEM from six to eight mice. For mRNA expression, data are representative of six pooled RNA samples, and data were confirmed in a repeat experiment. * $p < 0.05$, ** $p < 0.01$, significant differences are relative to saline. † $p < 0.05$, †† $p < 0.01$ relative to untreated.

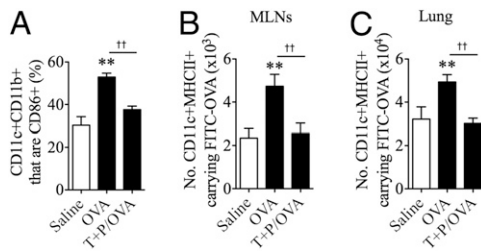


FIGURE 7. The effects of T+P treatment on the percentage of CD11c⁺ CD11b⁺ cells that are CD86⁺ in the lung (**A**). The number of CD11c⁺ MHCII⁺ DCs carrying FITC-labeled OVA in the MLNs (**B**) and lung (**C**). Data represent mean $\pm$ SEM of triplicate wells. ** $p < 0.01$, significant differences. ** $p < 0.01$.

DC containing Ag (Fig. 7). Thus, T+P drives the differentiation of highly suppressive Tregs, which suppress the Th2 response, prevent the Th17 response and disable the DC response resulting in the suppression of AAD.

We have previously shown that a currently available human vaccine and *S. pneumoniae* components (including T+P) suppress AAD through the induction of Tregs (10, 11). In this study, we extend these studies by profiling Treg development and providing insights into the nature of the enhanced Treg responses and the mechanisms of protections against AAD.

T+P treatment induced an early expansion of Tregs in the MLNs by 36 h, which continued to day 4 (Fig. 1B). Under Treg-inducing conditions in vitro naive T cells take up to 5 d to differentiate into a functional Foxp3⁺ phenotype (18). Therefore, we hypothesize that there may be two different populations of Tregs in the MLNs during the early phase. The T+P-induced population detected after 36 h may be an innate-like population that has expanded in the MLNs. The T+P-induced population detected after 4 d is likely to have developed in association with increases in IL-2 and TGF- β (Fig. 4A, 4B). Because CCL19 and CCL21 are induced in the lung by T+P after 36 h, it is likely that naive T cells expressing the ligand (CCR7⁺) are recruited from the bloodstream into the lung by these chemokines. This explains the increase in CCR7⁺ expression in the lung on day 4 (Fig. 4G). Once recruited, CCR7⁺ cells migrate out of the lung and into the lymph nodes (19, 20). At the same time, there was an increase in Treg-promoting factors (IL-2 and TGF- β). IL-2 signaling via CD25 is essential for the induction of TGF- β and expansion of CD4⁺CD25⁺Foxp3⁺ cells (21). Therefore, it is likely that together, CCR7, IL-2, and TGF- β promote the migration and differentiation of Tregs, which increased in the MLN on day 4. Hopefully in the future, advanced technology will enable us to track the development of Tregs in vivo, which will increase our understanding of their production and function.

Anti-CD25 administration on day -3 reversed the effect of T+P on the development of AAD, confirming that Treg were required for suppression of AAD. Indeed, anti-CD25 administration on day -3, but not day 9, depleted the population of T+P-induced CD25⁺ Tregs, which were required for the suppression of AAD (Figs. 2, 3). This indicates that the differentiation of T+P-induced Tregs, which are required for suppression, occurs prior to day 9.

As expected, administration of anti-CD25 on day +9 did not affect the number of T+P-induced CD25⁺ Tregs. This population may mediate the suppression of AAD in the absence of CD25⁺ Tregs (Fig. 2C). T+P administration induced a trend toward the downregulation of CD25 on the Foxp3 population on day 9 in the lung, but there was no change in the MLN (data not shown but similar to day 8 Fig. 1C). Downregulation of CD25 would mean that the anti-CD25 Ab does not deplete these Tregs. Others have

shown that CD25⁺ Tregs are known to retain their suppressive function (22). Thus, the CD25⁺ Treg population, which is not depleted by anti-CD25 administration, could account for T+P-mediated suppression of AAD. However, when anti-CD25 is administered on day -3, there are still CD25⁺ Tregs present (which remain to day 9, although they are reduced in number; Fig. 2C). These cells are not adequate for suppression of AAD because anti-CD25 administration on day -3 still prevents the effects of T+P. Thus, the production of CD25⁺ Tregs (day 4) is an essential component that underpins the mechanism of T+P-mediated suppression of AAD.

Anti-CD25 depletion of Tregs may also deplete CD25⁺ effector T cells, because CD25⁺ is also a marker of T cell activation (22). However, anti-CD25 administration had no effect on untreated allergic (anti-CD25 OVA) controls and completely restored AAD in treated (T+P/OVA groups) indicating that effector T cells were not affected (Figs. 1, 2). This method of Treg depletion prevents the interaction of IL-2 with its receptor and inhibits the CD25 signaling pathway, which prevents the induction of Foxp3 and results in cell death. Whether Tregs migrate to the lung on days 12 and 16, or a new population is formed, is unknown and further investigations are required. Nevertheless, Tregs expanded in response to OVA (day 12 versus day 16; Fig. 1C) and may therefore be OVA-specific. However, in other studies, we have shown that *S. pneumoniae*-induced Tregs also suppress anti-CD3/CD28-stimulated Th2 cell responses (8, 10). Therefore, T+P treatment may prime Tregs to respond both specifically or non-specifically to Ag challenge. Notably, although the changes in percentage and number of Tregs are small relative to other methods of immune deviation using bacterial products, Tregs are potentially immunosuppressive and even small increases can have substantial effects. Thus, it is likely that it is the combination of increased numbers and suppressive capacity of T+P-induced Tregs that suppresses AAD.

IL-10 and TGF- β are immunosuppressive cytokines that may be released by Tregs to mediate suppression; however, whether they are released by Tregs is dependent on the immunological context (2). IL-10 is a pleiotropic cytokine and is also produced by Th2 cells in AAD. In our study, in AAD (day 16), T+P suppressed IL-10 expression and TGF- β remained unchanged (Fig. 6A, 6B), which makes them unlikely candidates for mediating suppression. Numerous studies have shown that Treg-mediated suppression of AAD may or may not require IL-10 or TGF- β and may occur through other mechanisms such as cell-cell contact (8, 19, 23–25). Instead, it is likely that T+P are modifying several Treg pathways or populations in concert to increase their number and suppressive activity that suppresses AAD.

Of note, administration of anti-TGF- β in our model showed that TGF- β was partially required for T+P-mediated suppression of AAD. Other studies have demonstrated an important role for TGF- β in Treg-mediated suppression. For example, TGF- β activates of Notch-HES1-mediated Treg suppression, CD4⁺LAP⁺Foxp3⁺ cells have been shown to suppress AAD and TGF- β release from macrophages is important for generating Tregs (26–28). Thus, it is possible that TGF- β is mediated its effect in our model via one of these mechanisms.

We showed that the T+P-induced Treg population (on day 16) contained a high proportion of CTLA-4- and CD103-expressing cells (Fig. 6C, 6D). Tregs expressing these factors have a greater suppressive phenotype and enhanced functional capacity (29, 30). CTLA-4 is involved in the maintenance of T cell and CD4⁺Foxp3⁺ T cell homeostasis and is required for the development and activity of Tregs (31, 32). Deficiency in CTLA-4 impairs the suppressive function of Tregs, including their capacity to downregulate

CD80 and CD86 expression on DCs (30). Thus, Tregs require CTLA-4 to suppress APC activation of T cells. CD103 is another marker associated with the functional capacity of Tregs and occurs on highly potent and functionally distinct populations of Tregs, which are specialized for crosstalk with epithelial environments (31). Other studies have shown that CD103 expression plays an important role in the retention of Tregs at the epithelium through the recognition of cadherin during infection (32). Furthermore, CD103⁺ Tregs (CD25^{+/−}) that express CTLA-4 suppress T cell proliferation *in vitro* and were protective in a mouse model of colitis (31). Hence, the T+P-mediated increase in Tregs expressing CTLA-4 and CD103 may be responsible for the increased suppressive capacity of the Treg population.

IL-35 is another, recently discovered, soluble factor that mediates close-contact suppression of effector cell responses by Tregs (33). It is a heterodimeric cytokine composed of EBV-induced gene 3 (Ebi3) and p35 (IL-12a) and is highly expressed by Foxp3⁺ but not resting CD4⁺ cells. Ebi3^{−/−} and IL-12a^{−/−} mice have Tregs with reduced suppressive capacity, which confirms the importance of IL-35 in Treg-mediated suppression (33). Our results showed that T+P treatment sustained Ebi3 and increased p35 gene expression suggesting that IL-35 may also be involved in T+P-induced Treg-mediated suppression of AAD. IL-9 is also a pleiotropic cytokine that is recognized for its role in Th2-mediated disease (34). However, IL-9 has also been reported to enhance the function of Foxp3⁺ Tregs (35). In our study T+P increased IL-9 expression in the lung, which may be involved in enhancing the function of Tregs because its expression was associated with increases in other markers of functional capacity and the suppression of effector T cell responses. The understanding of the relationship between Tregs and IL-9 is in its infancy and requires further investigation. TLR2 expression on Tregs has also been implicated in controlling the expansion and function of Tregs and engagement of this receptor promotes Treg survival (36, 37). It is possible that T+P-induced increases in TLR2 expression may contribute to Treg function and stability. Taken together, these increases in markers associated with Treg-suppressive phenotype and functional capacity may be important for the suppression of effector cell responses in AAD. The upregulation of these Tregs in the lung during the late phase and the lack of later increases in immunosuppressive cytokines IL-10 and TGF- β suggests that T+P-mediated suppression of AAD during the challenge phase occurs through contact-mediated mechanisms. However, increases in numerous Treg-suppressive markers during the time course indicate that many independent pathways of suppression may lead to the increase in suppressive capacity and combine to attenuate AAD.

Our results show that T+P treatment blocks increases in IL-6 (Fig. 4H). This may promote the induction of Tregs in favor of the development of Th17 cells. Other studies have shown that blocking membrane-bound IL-6R, with anti-IL-6R Ab induces Tregs and attenuates AAD (38). Because the Th17 cell cytokines IL-25 and IL-17 contribute to the inflammatory milieu in AAD (13, 39), and these factors were suppressed by treatment (Fig. 4J, 4K), T+P may be effective in suppressing Th17-mediated asthma pathogenesis.

Our *in vivo* data were supported by *ex vivo* studies, which confirmed that T+P-treatment suppressed Th2 and Th17 effector cell responses and maintained Foxp3 expression (Fig. 5). Foxp3 has been shown to control NFAT and cause a diversion of differentiating effector T cells into Tregs (40). This study supports our data, which show that Foxp3 expression is inversely associated with the release of Th2 cytokines and IL-17 by effector T cells.

Pulmonary CD11c⁺CD11b⁺ mDCs play an important role in promoting Th2 responses in AAD (20). mDC activity is enhanced by the expression of costimulatory (CD80 and CD86) and activation (MHCII) molecules. In our study, T+P attenuated the expression of CD86 on mDCs. This does not suggest that other costimulation or activation markers are not required, and it is possible that there is a phase-dependent role for CD80 and CD86 as described in a mouse model of allergic rhinitis (41). Further investigation is needed to comprehensively determine the role of costimulation and activation markers at different stages of the response. CD86 on mDCs is recognized by CD28 (stimulatory) and CTLA-4 (inhibitory) receptors on T cells (42). Thus, it is likely that T+P-induced expression of CTLA-4 on Tregs reduces the capacity of DCs to prime T cells, which would suppress AAD. It is possible that the T+P-mediated reduction in CD86 expression on mDCs is promoted by CTLA-4⁺ Tregs. An alternative hypothesis is that *S. pneumoniae* products are maturing DCs, which makes them less endocytic. However, T+P did not change the level of CD80, CD86, or MHCII expression when assessed at 36 h, or 4, 8, or 12 d and therefore does not mature DCs. T+P-induced Tregs and suppression of AAD were associated with reductions in the number of DCs containing OVA in the MLNs and lung. This may be because T+P-induced Tregs induce Fas–Fas ligand-mediated or perforin-dependent killing of DCs in the lung, leading to reduced numbers of DCs carrying OVA in the MLNs (20, 41). Therapeutic inhibition of DCs in AAD has been reported previously and proposed as a potential treatment for asthma (43). Reductions in both CD86 and the number of DCs carrying OVA are likely to contribute to T+P-mediated suppression of AAD.

We have previously shown that T+P treatment also suppresses AAD if delivered therapeutically (11). T+P was administered in established disease and prior to a subsequent challenge. This demonstrated that T+P has potential for use as a therapy for asthma. Of note, nasal colonization events or administration of T+P intranasally are not sufficient to prevent AAD. In other studies, we have found that T+P must be administered into the lower airways (intratracheally) to induce its effects, whereas upper airway delivery (intranasal) is not protective (data not shown).

In summary, we have shown that a novel immunoregulatory therapy based on *S. pneumoniae* components, T+P, induces Tregs that suppress AAD. We have determined that the promotion of Treg involving CCR7, IL-2, CD25, and TGF- β is required for effective suppression of AAD because this primes Treg populations with enhanced suppressive capacity. These T+P-induced Treg do not suppress via IL-10 and TGF- β , but instead up regulate a number of pathways which suppress effector cell responses. In addition to suppressing Th2 responses, T+P-induced Tregs mediate the diversion away from Th17 differentiation by restricting IL-6 levels and also reduce the number of DCs containing OVA. Utilizing these bacterial components to induce Tregs may be a novel therapeutic strategy and global anti-inflammatory approach for the effective prevention and/or treatment of asthma. These effects may be applicable to Treg-induced suppression of other diseases.

Thus our model continues to provide a valuable tool for examining the development of Tregs, their mechanisms of suppression and their therapeutic potential in asthma and potentially other inflammatory diseases.

Disclosures

The authors have no financial conflicts of interest.

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