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Innate Immune Responses during Respiratory Infection with a Bacterial Pathogen Induce Allergic Airway Sensitization

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Abstract

Background—The original hygiene hypothesis predicts that infections should protect against asthma, but does not account for increasing evidence that certain infections may also promote asthma development. A mechanistic reconciliation of these findings has not yet emerged. In particular, the role of innate immunity in this context is unclear.

Objective—To test whether bacterial respiratory infection causes airway sensitization towards an antigen encountered in parallel, and to elucidate the contribution of innate immune responses.

Methods—Mice were infected with different doses of *Chlamydia pneumoniae* (CP) followed by exposure to human serum albumin (HSA) and challenge with HSA two weeks later. Airway inflammation, immunoglobulins and lymph node cytokines were assessed. Furthermore, adoptive transfer of dendritic cells (DCs) and depletion of regulatory T-cells (Tregs) was employed.

Results—CP-pneumonia induced sensitization towards HSA resulting in eosinophilic airway inflammation after HSA-challenge. Airway sensitization depended upon severity and timing of infection: low-dose infection and antigen exposure within 5 days of infection induced allergic sensitization, while high-dose infection or antigen exposure 10 days after infection did not. Temporal and dose-related effects reflected DC activation, and could be reproduced by adoptive transfer of HSA-pulsed lung DCs from infected mice. MyD88 deficiency in DCs abolished antigen sensitization, and depletion of Tregs prolonged the time window in which sensitization could occur.

Conclusions—We conclude that moderate but not severe pulmonary bacterial infection can induce allergic sensitization to inert inhaled antigens by a mechanism that requires MyD88-dependent DC activation and is controlled by Tregs.

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Keywords

Asthma; allergic sensitization; adjuvant; dendritic cell; *Chlamydia pneumoniae*; regulatory T cells; bacterial pneumonia; allergen

INTRODUCTION

The dramatic rise in the incidence of asthma in industrialized countries during the last two generations [1, 2] has been attributed to improved general hygiene and better control of infectious diseases [3, 4]. This "hygiene hypothesis" [5] suggests that by decreasing early exposure to infections, the balance between Th1 and Th2 immunity is skewed towards Th2 responses that promote allergic inflammatory responses to common environmental allergens [4]. However, there is increasing recognition that infectious agents could paradoxically promote the pathogenesis of asthma, a concept that deviates from the notion that infection invariably boosts Th1 immunity. This is perhaps best illustrated for viral respiratory tract infections, which can stimulate airway hyper-reactivity but may also promote an immune response to common environmental allergens [6-8]. Other more recent studies similarly suggest that atypical bacterial infections caused by agents such as *Mycoplasma pneumoniae* [9] and *Chlamydia pneumoniae* [9-11] might facilitate allergic airway disease. These observations suggest that although increased prevalence of asthma in developed nations [1, 2] may be generally linked to decreased incidence of infections [3], certain respiratory pathogens may actually enhance the development of asthma [12]. But on a molecular and cellular level, precisely how this might occur is not well understood.

In their established role as key cellular regulators of the innate immune system, dendritic cells (DCs) use pattern recognition receptors (PRRs) to respond to infectious agents [13, 14]. PRRs such as the Toll-like receptors (TLRs) generally initiate and promote Th1 immunity, but there is growing awareness that TLRs could also be involved in Th2 skewing of the immune response under certain circumstances [15-17]. While the cellular pathways by which this response could be achieved are unknown, DCs direct effector T cell activities, but they also regulate the activity of $Foxp3^+CD4^+CD25^+$ regulatory T cells (Tregs) [18]. Tregs in turn suppress effector T cells as well as DCs and also inhibit the development of asthma [19]. One possible explanation for the promoting effect of immune stimulation on antigen sensitization is that infectious agents that modify key DC functions such as antigen presentation or cytokine production, like IL-6, could negate this Treg-directed suppressive pathway [20]. However, this possibility is speculative; within the context of infection and allergen sensitization, little is currently known regarding mechanisms by which TLR signaling and DC function might counter-regulate Treg function, and thereby impact development of asthma.

Here we report the results of a systematic investigation into the relationship between *Chlamydia pneumonia* (CP) infection and the induction of allergic airway sensitization towards human serum albumin (HSA), an antigen that usually does not elicit an allergic response. We show that CP infection in a murine asthma model induces allergic sensitization to HSA in a DC-dependent manner, and that sensitization depends on both the timing of the infection relative to allergen challenge and the severity of infection. The ability of DCs to trigger sensitization involves a MyD88-dependent signaling pathway that is regulated by the suppressive activity of $CD4^+CD25^+$ Tregs.

METHODS

Mice

Specific pathogen-free C57BL/6 mice 8 to 12 weeks of age were used throughout the study. MyD88$^{-/-}$ mice (provided by Shizuo Akira, Osaka University, Osaka, Japan, see Online Repository for further information) were backcrossed for at least 8 generations and bred at our facility. Caspase 1 KO mice (ICE, obtained from Chris Wilson, University of Washington Seattle) were bred at our facility. All experiments received prior approval from the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Infection with C. Pneumoniae

C. pneumoniae CM-1 (ATCC) was propagated in HEp-2 cells and stored suspended in 2-SPG buffer at −80°C as previously described [21]. *Chlamydia* stocks were determined to be free of *Mycoplasma* contamination by PCR [21]. For infection, mice were anaesthetized with isoflurane vapors and injected intranasally with 40 μl of either 5×10^6 or 0.5×10^6 inclusion forming units (IFU) of *C. pneumoniae* diluted in PBS. See Online Repository for further information.

Allergen Sensitization and Assessment of Eosinophilic Airway Inflammation

Human serum albumin (HSA-low endotoxin, Sigma) was used as an antigen throughout the study. Endotoxin contamination (determined by a chromogenic Limulus assay) was below 10 pg/mg. Mice previously infected with CP received 100 μg of HSA in PBS intranasally on 3 consecutive days (days 0 to 3) starting at various time points after infection as indicated in the text and figures. Control groups received PBS only. On days 15, 16, 19 and 20, mice were re-exposed to HSA by intranasal injection of 25 μg of protein. Mice were sacrificed on day 21. Challenge with HSA was performed as described above, and serum and lungs were harvested on day 21. The right lobes of the lungs were fixed in 10% formalin, paraffin-embedded, and hematoxylin and eosin (H&E)-stained sections were evaluated. The degree of inflammation was scored by blinded observers as described previously [21]. Goblet cells were detected by PAS staining. For assessment of eosinophilic airway inflammation, the left lobe was fixed in PBS/2% paraformaldehyde/0.2% picric acid and 7 mm cryosections were prepared. Eosinophils were detected by eosinophil peroxidase-specific staining as described previously [22]. Data were expressed as number of eosinophils per mm^2 lung section as well as number of goblet cells per mm bronchial basal membrane using Image Pro Plus 5.1 Software (Media Cybernetics).

Flow Cytometry and Immunohistochemistry

Total lung cell preparations from mice infected with CP were prepared by digesting lungs with 10 μg/ml of Blendzyme 3 (Roche), 20 μg/ml DNAseI (Roche) and antibiotics for 45 min. Cell suspensions were passed through 70 μm cell strainers and red blood cells were lysed. . Numbers of DCs in the lungs were determined by FACS with antibodies directed against CD11c and CD11b (Ebioscience). Tregs in the lungs were analyzed using Abs directed against CD4, CD25 and FoxP3 (EBioscience). Immunohistochemistry was performed using the Catalysed Signal Amplification (CSA) kit (Dako, Denmark). Anti-MIDC-8 (Serotec) as well as the isotype control were used at 1:10 and 1:5, respectively. See Online Repository for further information.

Determination of HSA-specific Immunoglobulins

Total IgE levels in serum were determined with ELISA (BD Bioscience). For HSA-specific IgG_1 and IgG_{2a} titers, plates were coated with 50 μg/ml HSA overnight, followed by blocking with PBS/1% BSA at 37°C for 30 min. Plates were incubated with serum samples

diluted in PBS/1% BSA at 37°C for 90 min, followed by detection of bound immunogobulin with biotinylated anti-mouse IgG_1 and IgG_{2a} antibodies, respectively (BD Bioscience) and streptavidin (Ebioscience). For HSA-specific IgE, HSA was biotinylated using the Fluoreporter©Biotin-XX kit (Invitrogen). Plates were coated with anti-mouse IgE antibody (BD Biosciences), blocked with BSA, and incubated with serum samples. Bound HSA-specific IgE was detected using biotinylated HSA and streptavidin. As a standard, pooled sera from mice immunized with HSA plus LPS was used and set arbitrarily at 1.0 U/ml.

Preparation of Lung Dendritic Cells

For lung DCs, lung cell suspensions were generated as described above, and DCs were enriched employing CD11c-coated microbeads (Miltenyi). Purity was checked by Flow cytometry using anti-CD11c and CD11b antibodies and was routinely above 90%. For adoptive transfer lung DCs were incubated overnight in the presence of 50 μg/ml HSA and antibiotics, followed by extensive washing and intratracheal injection into recipient mice.

Functional Characteristics of Dendritic Cells

Expression of MHC class II and the co-stimulatory molecules CD40, CD80 and CD86 were assessed by FACS. Numbers of DCs in the mediastinal lymph nodes was determined by Flow cytometry with staining for CD11c, CD11b and MHC class II. For analysis of presentation of HSA in the regional lymph nodes, mice were injected with 100 μg FITC-labeled HSA intratracheally [23]. After 24 h, mediastinal lymph nodes were removed and FITC-presenting DCs were identified as $FITC^+$ and $CD11c/CD11b^+$ by FACS.

Statistical Analyses

Independent experiments were conducted at least in triplicate except as otherwise noted. Results were summarized as mean ± SD and compared using 2-tailed unpaired Student's t tests. A p value of less than 0.05 was required to reject the null hypothesis.

RESULTS

Severity of Respiratory Infection with Live CP is an Important Determinant of Allergic Airway Sensitization

Groups of mice were infected with either 5×10^6 IFU of CP (high-dose or severe lung infection) or a 10-fold lower dose of 0.5×10^6 IFU (low-dose or moderate lung infection). Inoculation of high-dose bacteria led to severe pneumonia with neutrophils predominating, while the lower infectious dose induced moderate pneumonia with scattered peribronchial and perivascular infiltrates consisting predominantly of mononuclear cells (Fig. E1A and B in Online Repository). Levels of cytokines in the BAL including IL-6, TNF-α, IFN-γ and IL-5 were significantly higher in mice infected with the high-dose (severe infection) compared to low-dose (moderate infection, Fig. E1C, Online Repository). Starting at 5 days after infection, mice received either HSA or PBS intranasally for 3 consecutive days and were then subsequently challenged with HSA (Fig. 1A). Mice with moderate infection that were sensitized with HSA developed airway inflammation exhibiting features of an Th2-biased immune response with a marked increase in eosinophil and goblet cell numbers (Fig. 1B and C). Eosinophilic airway inflammation in these mice was associated with a significant increase in total IgE (data not shown) as well as HSA-specific IgE and IgG_1, but not IgG_{2a} (Fig. 1D). Airway inflammation was triggered by re-exposure to HSA, since mice sensitized with HSA but challenged with PBS appeared normal (Fig. 1B and C). In contrast to the findings with low-dose infection, mice with high-dose infection failed to develop either eosinophilic airway inflammation or goblet cell hyperplasia following HSA challenge (Fig

1B to D). Nevertheless, the latter group did produce HSA-specific IgE and IgG_1, albeit at lower levels compared to those seen in low-dose group (Fig. 1D).

Timing of Live CP Infection and Induction of Allergic Sensitization

We next asked whether airway sensitization during CP infection was dependent on the timing and stage of infection, and in particular whether pneumonia at a late stage still favored sensitization. Mice were infected with low-dose CP followed by HSA sensitization at day 10 (Fig. 1A). At this time point, the mice exhibited mild lung inflammation (Fig. E1A and B, Online Repository), but lacked detectable cytokines in the BAL fluid (Fig. E1B, Online Repository). In contrast to mice receiving HSA on day 5 after CP infection, mice sensitized with HSA on day 10 following infection failed to develop eosinophilic infiltrates, goblet cell hyperplasia (Figs. 1B and C) or increased HSA-specific antibodies (Fig. 1D). High-dose CP infection followed by HSA administration 10 days later also did not elicit eosinophilic airway inflammation (data not shown). Collectively, these data suggest that there is a time period in which a moderate degree of lung infection can exert adjuvant effects on allergen sensitization.

High-Dose CP infection Leads to Diminished Antigen Presentation

DCs play a major role in mediating airway sensitization by delivering inhaled antigen to the regional lymph nodes [24]. We therefore investigated whether differential results obtained by varying the dose and timing of infection could reflect differences in the numbers or activation state of different subsets of DCs in the lung. . However, FACS-Analysis of samples from all infected mice exhibited a similar increase in numbers of $CD11c^+/CD11b^+$ DCs in the lung (data not shown). Furthermore, we identified activated DCs by immunohistochemical staining for MIDC8 [25] and found those cells in roughly equal abundance in the lungs of all infected mice, irrespective of dose and timing of infectious challenge (Fig. 2A). While expression of co-stimulatory molecules CD80, CD86 and CD40 (Fig. E2B, Online Repository) were similar, MHC class II levels in DCs isolated from mice infected with high-dose CP were lower than in the low-dose infected groups (Fig. 2B). Furthermore, numbers of MHC class II^{high} DCs in the draining lymph nodes were significantly lower in high-dose infected mice than in low-dose infected mice (Fig. E2C, Online Repository). Strikingly, this was associated with a significantly lower delivery of FITC-labeled HSA by lymph node DCs from the high-dose group as compared to the low dose group (Fig. 2C). This observation was not due to increased apoptosis of lung DCs, since the percentages of lung DCs undergoing apoptosis were similar in the high-dose and low-dose infected mice (Fig E2D, Online Repository). These data suggest that high-dose infection compromises DC-maturation, allergen uptake and presentation by lung DCs.

Adoptive Transfer of DCs from CP-infected Lungs into Naïve Mice Induces Airway Sensitization

To directly determine the role of DCs in allergic airway sensitization, we performed adoptive transfer of pulmonary DCs from CP-infected mice into naïve mice. Mice were infected with low-dose CP. Five days later, lung DCs were isolated and incubated overnight with HSA or PBS in the presence of antibiotics. Additionally, $CD11c^+$ cells were isolated from uninfected mice and incubated with HSA. Isolated cells were checked for purity and MHC class II expression, which was increased in CP-infected mice (Fig. 3A). After extensive washing, DCs were adoptively transferred to naïve recipient mice by intratracheal installation, and mice were challenged with HSA 15 days later. HSA-presenting DCs from infected mice induced allergen sensitization with a significant increase in eosinophil and goblet cell numbers upon HSA challenge (Fig. 3B and C). In contrast, PBS-pulsed DCs isolated from infected mice or DCs from uninfected mice failed to induce airway sensitization (Fig. 3B and C). *In vitro* re-stimulation of mediastinal lymph node cells

isolated from the recipient mice with HSA led to preferential IL-5 release (Fig. 3D), consistent with the interpretation that DCs from CP-infected mice induced Th2 skewing of the immune response in recipient naïve mice. Although still showing elevated expression of MHC class II, pulmonary DCs obtained from CP-infected mice at day 10 were no longer able to reconstitute allergic sensitization when pulsed ex-vivo with HSA and adoptively transferred to recipient mice (Fig. 3B to D). This was associated with a diminished expression of IL-6 in these DCs as compared to cells isolated from mice 5 days after infection with CP (Fig. 3E). Furthermore, we found significantly greater numbers of Tregs in the lungs 10 days after low-dose infection as compared to 5 days (Fig 3F).

Tregs Control the Timing during Which CP Induces Allergic Sensitization

BAL cytokines levels were significantly lower at day 10 as compared to day 5 (Online Repository. Fig. E1), and lung DCs isolated from day 10 mice expressed less IL-6 mRNA than day 5 mice (Fig. 3E). Additionally, numbers of Tregs in the lungs of infected mice were elevated at 10 days vs. 5 days after infection (Fig. 3F). These observations, together with prior data indicating that a MyD88-dependent IL-6 pathway allows DCs to overcome the normally suppressive function of Tregs [20], suggest that the explanation for day 5 vs. day 10 differences might lie in the interaction between DCs and Tregs. To test this hypothesis, Tregs were depleted using anti-CD25 (PC61) antibody 8 days after low-dose CP infection (Fig. 4A). We then sensitized these animals with HSA on day 10. A single dose of PC61 antibody injected i.p. could deplete Tregs for at least 4 days (Fig. E3, Online Repository), ensuring that Treg numbers were suppressed during the sensitization period. Consistent with our hypothesis, Treg-depleted mice but not animals receiving an isotype control Ab became sensitized, developed eosinophilic airway inflammation and mounted an HSA-specific IgE response (Fig. 4B to D). In the absence of CP infection, depletion of Tregs before administration of HSA did not induce airway sensitization, demonstrating that the adjuvant signal provided by CP infection was necessary to induce allergen sensitization (Fig. 4B to D). These data suggest that Treg depletion could effectively extend the time window during which CP infection could exert an adjuvant effect.

Airway Sensitization by CP Requires MyD88-Dependent Signaling

Up-regulation of co-stimulatory molecules, cellular maturation and cytokine release by antigen presenting cells require TLR/MyD88-mediated signaling [26], suggesting that the MyD88 pathway might be essential for CP-induced allergen sensitization. In order to test this, MyD88-deficient mice were infected with low-dose live CP followed by HSA sensitization on day 5 and subsequent HSA challenge. These mice did not develop eosinophilic airway inflammation (Fig. 5A and B), and CP-induced allergic sensitization was TLR-MyD88 specific as Caspase 1-deficient mice did not differ from wild type mice (data not shown). In line with the absence of antigen sensitization during infection with CP, expression of co-stimulatory molecules on airway DCs in $MyD88^{-/-}$ mice remained unchanged during CP-induced pneumonia as compared to wild type mice (Fig. 5C).

DISCUSSION

Although the original hygiene hypothesis predicts that some infections inhibit the development of atopic diseases, there is increasing recognition that infectious agents could paradoxically promote the pathogenesis of asthma. We show here that respiratory infection with *Chlamydia pneumoniae*, a common respiratory pathogen, creates a pro-inflammatory environment in the lung that can exert an adjuvant effect on allergen sensitization. We found that DCs were centrally involved in triggering allergic sensitization, since either adoptive transfer of allergen-presenting DCs from infected mice or bone marrow-derived DCs infected with CP and challenged with HSA *ex vivo* could induce eosinophilic airway

inflammation in recipient mice. Furthermore, MyD88 expression by adoptively transferred donor DCs was required, but MyD88-expression was not necessary in recipient animals for sensitization (see Online Repository, Fig. E4B to D). Therefore, intact MyD88-dependent signaling in DCs was sufficient to induce allergic airway sensitization after CP infection and exposure to allergen.

However, airway sensitization depended upon severity and timing of the infection: low-dose infection and antigen exposure within 5 days of infection induced allergic airway sensitization, while high-dose infection or antigen exposure 10 days after infection did not. The time window during which successful sensitization could be performed appears to be directly linked to an immunoregulatory pathway that involves Treg function: by depleting the $CD4^+CD25^+$ Treg subset, we were able to prolong the time window in which sensitization could occur. Recent studies suggest that Tregs normally inhibit development of allergic asthma [19], but in studies focusing on Th1-biased immune responses, the suppressive activity of Tregs on DCs is countered by DCs themselves through TLR-mediated IL-6 release triggered by exposure to microbial ligands [20, 27]. Indeed, we found increased IL-6 levels during the time window in which sensitization could occur. Collectively, our results appear most consistent with the interpretation that allergen sensitization during bacterial lung infection is controlled by both the activation status of airway DCs in a MyD88-dependent manner and by $CD4^+CD25^+$ Treg numbers and function.

Our data indicate that mild pulmonary infections favor the development of allergic sensitization and asthma, but severe infections do not. One possible explanation for the lack of allergic antigen sensitization is that high-dose infection could accelerate DC maturation and production of Th1-skewing cytokines by DCs. This is consistent with reports that low-dose LPS exposure enhances allergic sensitization, but high-dose LPS has the opposite effect [16]. This appears to be due to the fact that high-dose LPS exposure induces predominantly IgG_{2a} production and Th1 skewing, which prevents allergen sensitization [16, 17]. In fact, during high-dose infection, we observed a trend towards less pronounced Th2 helper cell responses, as manifested by lower levels of HSA-specific IgG_1 and IgE compared to low-dose infected mice (Fig. 2D). Furthermore, high-dose infections were accompanied by higher levels of IFN-γ in the BAL and increased production of IL-12 but not IL-10 by DCs (Online Repository. E1B and E2A). Most importantly, our data also show that high-dose infection is associated with decreased antigen presentation (lower MHC class II^{high} expression in lung DCs as well as lower numbers of FITC-HSA^+ DCs in the lymph nodes). This finding may be related to the increased numbers of neutrophils observed in high-dose infected mice, since neutrophil degranulation inhibits DC-maturation *in vitro* [28]. Therefore, allergic antigen sensitization in high-dose infected mice may be impaired by the cytokine pattern elicited in DCs, which favors both Th1-responses, and an overall impairment of antigen presentation. Our data are consistent with a model that MyD88-dependent cellular activation in the presence of microbial antigens is sufficient to induce airway sensitization. Thus, it is tempting to speculate that comparable mechanisms apply to other bacteria and viruses that have been linked to the pathogenesis of asthma, such as *M. pneumoniae* or RSV. Additionally, our data show that moderate, but not severe infection predisposes to the development of asthma, thus yielding a possible explanation to the fact that so far, agents causing atypical pneumonia that rarely require hospitalization have been linked to the onset and exacerbation of asthma [9-11].

Previous studies addressing the impact of *Chlamydia* on allergic airway inflammation in murine models have focused on exacerbation of pre-existing asthma by infecting previously sensitized mice *with C. trachomatis or C. muridarum* [29, 30]. These studies yielded conflicting results; *C. trachomatis* inhibited airway inflammation [30], but *C. muridarum* infection was associated with an increase in inflammatory parameters [29]. In contrast to these

studies we investigated the mechanisms causing allergen sensitization in previously healthy mice sensitized with allergen during a concomitant CP infection. Our findings are therefore consistent with previous studies [7,8], which collectively emphasize that timing of infection relative to allergen exposure critically determines whether allergen sensitization is promoted or suppressed. However, in addition, our results now suggest that there is a time window during which moderate but not severe respiratory infections may act as adjuvants that promote allergen sensitization and the pathogenesis of asthma.

In summary, our results demonstrate that bacterial respiratory infection can elicit an innate immune environment that promotes allergen sensitization and eosinophilic airway inflammation in a temporally-restricted and severity-dependent manner. The mechanism requires intact MyD88-dependent signaling in DCs, and is importantly controlled by Tregs. The net outcome of the functional interactions between CP-activated DCs and Tregs dynamically alters the magnitude of antigen sensitization. Our data demonstrate that under certain circumstances, infectious agents may promote the pathogenesis of allergic disease by acting as adjuvants that favor Th2 differentiation. In a controlled experimental model of bacterial infection and allergen challenge, we demonstrate that the same infectious agent can promote the development of allergic sensitization under certain conditions, but fails to do so in other contexts. The development of allergic asthma was promoted by moderate infection, but suppressed by severe infection of the lungs. Our data provide important novel mechanistic insights into why and how this occurs, and what molecular and cellular participants impact both the likelihood that allergic sensitization could occur, and the duration of the time window during which this was possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this manuscript

BAL	broncho-alveolar lavage
CP	Chlamydia pneumoniae
CPUV	UV-inactivated *Chlamydia pneumoniae*
DC	dendritic cell
HSA	human serum albumin
PRR	pattern recognition receptor
TLR	Toll-like receptor
Treg	regulatory T-cell

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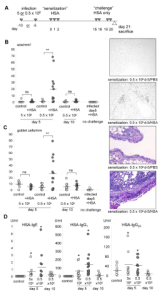

Figure 1. Low-Dose Live CP infection Induces Allergic Airway Sensitization to HSA

A: Groups of mice were infected as indicated. Starting with day 0, mice received either 3 consecutive intranasal injections of HSA (n=13 for low-dose, n=9 for high-dose) or PBS control (n=13 for low-dose, n=8 for high-dose). Some mice were infected with 0.5×10^6 CP and sensitization with HSA (day 0) was started 10 days after infection (n=9) or PBS control (n=10). Mice were challenged with HSA starting on day 15. One control group received PBS during the challenge period (n=10). **B:** Eosinophil numbers per lung section, with representative lung sections (100-fold magnification) shown at the right of the graph. **C:** Goblet cell numbers related to bronchial basal membrane length. Representative PAS stained sections (200-fold magnification) are shown. **D:** HSA-specific IgE, IgG_1 and IgG_{2a} titers of mice sensitized and challenged with HSA after infection. *p≤0.05, **p≤0.01, ns: not significant as compared to controls.

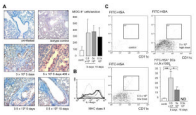

Figure 2. Characteristics of Pulmonary DCs in CP-infected Mice

A: Paraffin embedded sections were stained for MIDC8 to detect DCs. Total numbers of MIDC8$^+$ cells in 4 separate sections were counted (6 mice per group). **B:** Up-regulation of co-stimulatory molecules assessed by staining of lung leukocytes with antibodies against CD11c, CD11b and MHC class II. Shown are uninfected controls (grey), mice infected with high dose CP (dotted line) or low dose CP (bold line) 5 days prior to analysis, and low dose CP 10 days prior to analysis (thin line). Shown are representative results from 1 out of 3 experiments performed with n=5 mice. **C:** Numbers of FITC$^+$ DCs in mediastinal lymph nodes after infection and intratracheal injection of 100 μg of FITC-HSA 24 h before analysis. Cells were gated for CD11b. Shown are representative dot blots of cells (FITC-HSA x CD11c) as well as combined results out of 3 separate experiments (n=4 mice per experiment). ND = not determined. *p≤0.05.

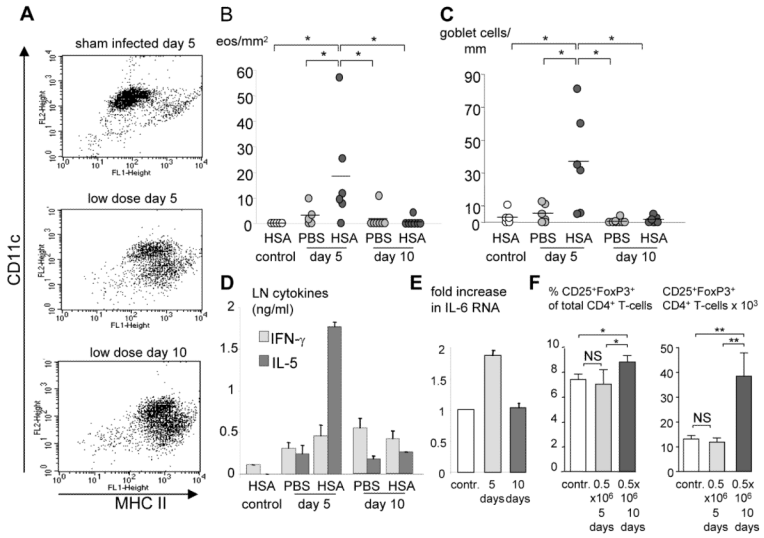

Figure 3. DCs from CP-Infected Mice Pulsed with HSA Can Adoptively Transfer Allergic Airway Sensitization

A: DCs were isolated from lungs of mice 5 or 10 days after infection with low dose CP or sham infection with PBS as described in Materials and Methods. DC expression of MHC class II expression by infected mice and controls was determined by FACS. Cells were incubated with either PBS or HSA overnight and transferred to recipient mice by intratracheal inoculation (500,000 cells/mouse) on day 0. Mice were challenged with HSA starting on day 15. **B:** Eosinophil numbers in lung sections from mice that received control DCs pulsed with HSA (n=5) or DCs from infected mice pulsed with either HSA (n=7) or PBS (n=8). **C:** Goblet cell numbers assessed by PAS staining. Shown are combined results from 3 separate experiments. *p≤0.05 **D:** Cytokine production by lymph node cells after *in vitro* restimulation with HSA. Shown are representative results from 1 out of 3 experiments performed. **E:** IL-6 RNA content of pulmonary DCs of mice 5 days and 10 days after infection.. IL-6 RNA was measured by TaqMan® real time rtPCR and compared to DCs of uninfected control mice. Combined data of n=4 mice per group are shown. **F:** Percentage of lung $CD4^+CD25^+FoxP3^+$ Tregs in naive wild type mice (n= 6-9 mice), 5 days after infection with either 5×10^5 or 5×10^6 CP, or 10 days after infection with 0.5×10^6 intranasally.

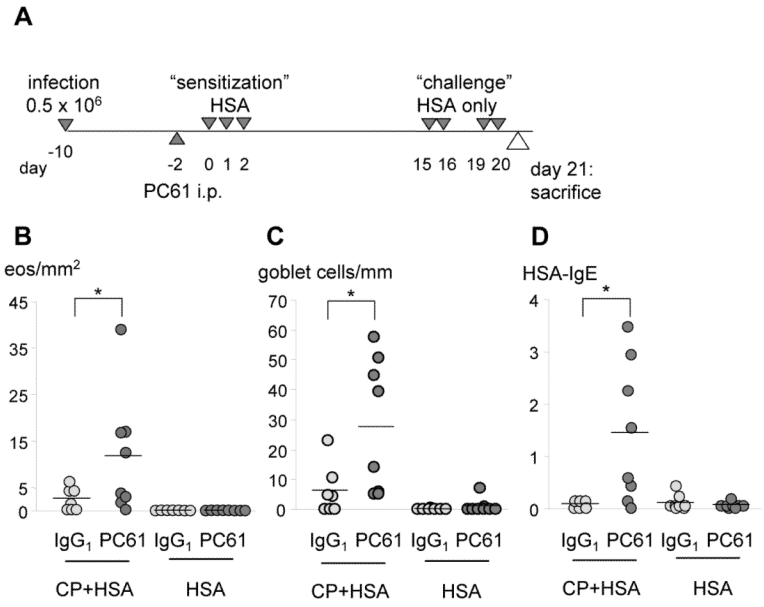

Figure 4. Tregs Regulate Airway Sensitization Induced by CP Infection

A: Infection, Treg depletion and sensitization/challenge protocol: mice were infected with 0.5×10^6 CP on day −10, followed by a single i.p. injection of 100 μg of PC61 antibody (n=8) or rat IgG_1 isotype control (n=7) at day −2. Mice were then exposed to HSA on 3 consecutive days, followed by HSA challenge 2 weeks later. Control mice were injected with PC61 or isotype control (n=8 and n=7, respectively) without prior infection, followed by HSA injection on days 0 to 2. **B** and **C:** Eosinophil numbers and goblet cell numbers. **D:** HSA-specific IgE titers. *p≤0.05.

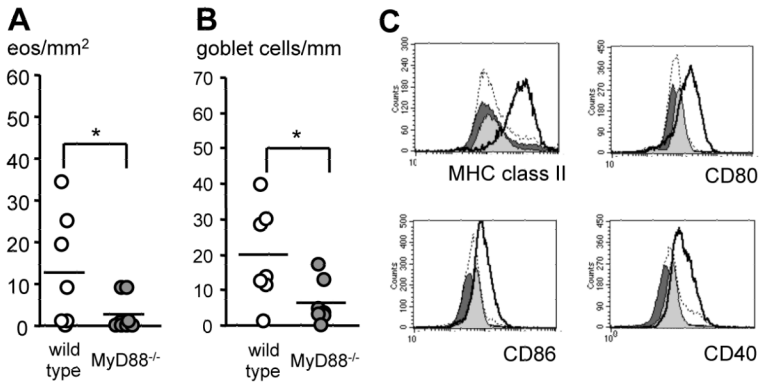

Figure 5. MyD88-Dependent Signaling in DCs is Required for Airway Sensitization

A and **B:** Eosinophil numbers and goblet cell numbers in lung sections from wild type mice (n=7) infected with 0.5×10^6 live CP, exposed to HSA on 3 consecutive days and challenged with HSA as shown in Fig. 1 A. Data obtained from $MyD88^{-/-}$ (n=8 mice are shown in comparison. *p≤0.05, **p≤0.01, ns= not significant. Wild type mice (n=7) and $MyD88^{-/-}$ mice (n=6) were infected with 0.5×10^6 CP and sacrificed after 5 days. **C:** Up-regulation of MHC class II, CD80, CD86 and CD40 in DCs of infected mice (bold line: wild type, dotted line: $MyD88^{-/-}$) vs. uninfected controls (grey: wild type, dark grey: $MyD88^{-/-}$) determined by FACS. DCs were identified as $CD11c^+/CD11b^+$.