Innate immune responses during respiratory tract infection with a bacterial pathogen induce allergic airway sensitization

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Background: The original hygiene hypothesis predicts that infections should protect against asthma but does not account for increasing evidence that certain infections might 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: We sought to test whether bacterial respiratory tract infection causes airway sensitization toward an antigen encountered in parallel and to elucidate the contribution of innate immune responses.
Methods: Mice were infected with different doses of *Chlamydia pneumoniae*, followed by exposure to human serum albumin (HSA) and challenge with HSA 2 weeks later. Airway inflammation, immunoglobulins, and lymph node cytokines were assessed. Furthermore, adoptive transfer of dendritic cells (DCs) and depletion of regulatory T (Treg) cells was performed.
Results: *C pneumoniae*–induced lung inflammation triggered sensitization toward HSA, resulting in eosinophilic airway inflammation after HSA challenge. Airway sensitization depended on the severity and timing of infection: low-dose infection and antigen exposure within 5 days of infection induced allergic sensitization, whereas 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 means of adoptive transfer of HSA-pulsed lung DCs from infected mice. MyD88 deficiency in DCs abolished antigen sensitization, and depletion of Treg cells 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 through a mechanism that requires MyD88-dependent DC activation and is controlled by Treg cells. (J Allergy Clin Immunol 2008;122:595-602.)

The dramatic increase in the incidence of asthma in industrialized countries during the last 2 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 T_H1 and T_H2 immunity is skewed toward T_H2 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 T_H1 immunity. This is perhaps best illustrated for viral respiratory tract infections, which can stimulate airway hyperreactivity but can 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] might be generally linked to decreased incidence of infections,[3] certain respiratory pathogens might 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 to respond to infectious agents.[13,14] Pattern recognition receptors, such as the Toll-like receptors (TLRs), generally initiate and promote T_H1 immunity, but there is growing awareness that TLRs could also be involved in T_H2 skewing of the immune response under certain circumstances.[15-17] Although the cellular pathways by which this response could be achieved are unknown,

> *Abbreviations used*
> BAL: Bronchoalveolar lavage
> BMDC: Bone marrow–derived dendritic cell
> DC: Dendritic cell
> FITC: Fluorescein isothiocyanate
> HSA: Human serum albumin
> IFU: Inclusion forming units
> TLR: Toll-like receptor
> Treg: Regulatory T

DCs direct effector T-cell activities, but they also regulate the activity of Foxp3$^+$CD4$^+$CD25$^+$ regulatory T (Treg) cells.[18] Treg cells 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 cell–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 counterregulate Treg cell function and thereby affect the development of asthma.

Here we report the results of a systematic investigation into the relationship between *C pneumoniae* infection and the induction of allergic airway sensitization toward human serum albumin (HSA), an antigen that usually does not elicit an allergic response. We show that *C pneumoniae* 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$^+$ Treg cells.

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 the Methods section in this article's Online Repository at www.jacionline.org for further information) were backcrossed for at least 8 generations and bred at our facility. Caspase 1 knockout mice (ICE; obtained from Chris Wilson, University of Washington, Seattle, Wash) 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, Manassas, Va) was propagated in HEp-2 cells and stored suspended in 2-sucrose phosphate glutamate buffer at −80°C, as previously described.[21] *C pneumoniae* stocks were determined to be free of *Mycoplasma* species contamination by means of PCR.[21] For infection, mice were anesthetized 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 the Methods section in this article's Online Repository for further information.

Allergen sensitization and assessment of eosinophilic airway inflammation
HSA (low endotoxin, Sigma, St Louis, Mo) was used as an antigen throughout the study. Endotoxin contamination (determined by using a chromogenic Limulus assay) was less than 10 pg/mg. Mice previously infected with *C pneumoniae* received 100 μg of HSA in PBS intranasally on 3 consecutive days (days 0-3), 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 means of intranasal injection of 25 μg of protein. Mice were killed on day 21. Challenge with HSA was performed as described above, and sera and lungs were harvested on day 21. The right lobes of the lungs were fixed in 10% formalin, and paraffin-embedded and hematoxylin and eosin–stained sections were evaluated. The degree of inflammation was scored by blinded observers, as described previously.[21] Goblet cells were detected by means of periodic acid–Schiff 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 means of eosinophil peroxidase–specific staining, as described previously.[22] Data were expressed as the number of eosinophils per square millimeter lung section, as well as the number of goblet cells per millimeter of bronchial basal membrane by using Image Pro Plus 5.1 Software (Media Cybernetics, Bethesda, Md).

Flow cytometry and immunohistochemistry
Total lung cell preparations from mice infected with *C pneumoniae* were prepared by digesting lungs with 10 μg/mL Blendzyme 3 (Roche, Mannheim, Germany), 20 μg/mL DNAseI (Roche), and antibiotics for 45 minutes. Cell suspensions were passed through 70-μm cell strainers, and red blood cells were lysed. Numbers of DCs in the lungs were determined by means of FACS with antibodies directed against CD11c and CD11b (eBioscience, San Diego, Calif). Treg cells in the lungs were analyzed with antibodies directed against CD4, CD25, and Foxp3 (eBioscience). Immunohistochemistry was performed with the Catalysed Signal Amplification kit (Dako, Glostrup, Denmark). Anti-MIDC-8 (Serotec, Oxford, United Kingdom) and the isotype control were used at 1:10 and 1:5 dilutions, respectively. See the Methods section in this article's Online Repository for further information.

Determination of HSA-specific immunoglobulins
Total IgE levels in serum were determined with ELISA (BD Bioscience, San Jose, Calif). For HSA-specific IgG1 and IgG2a titers, plates were coated with 50 μg/mL HSA overnight, followed by blocking with PBS/1% BSA at 37°C for 30 minutes. Plates were incubated with serum samples diluted in PBS/1% BSA at 37°C for 90 minutes, followed by detection of bound immunoglobulin with biotinylated anti-mouse IgG1 and IgG2a antibodies, respectively (BD Biosciences), and streptavidin (eBioscience). For HSA-specific IgE, HSA was biotinylated with the FluoreporterBiotin-XX kit (Invitrogen, Carlsbad, Calif). Plates were coated with anti-mouse IgE antibody (BD Biosciences), blocked with BSA, and incubated with serum samples. Bound HSA-specific IgE was detected by 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 DCs
For lung DCs, lung cell suspensions were generated as described above, and DCs were enriched by using CD11c-coated microbeads (Miltenyi Biotech, Auburn, Calif). Purity was checked by means of flow cytometry with anti-CD11c and anti-CD11b antibodies and was routinely greater than 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 DCs
Expression of MHC class II and the costimulatory molecules CD40, CD80, and CD86 were assessed by means of FACS. Numbers of DCs in the mediastinal lymph nodes were determined by means of flow cytometry with staining for CD11c, CD11b, and MHC class II. For analysis of
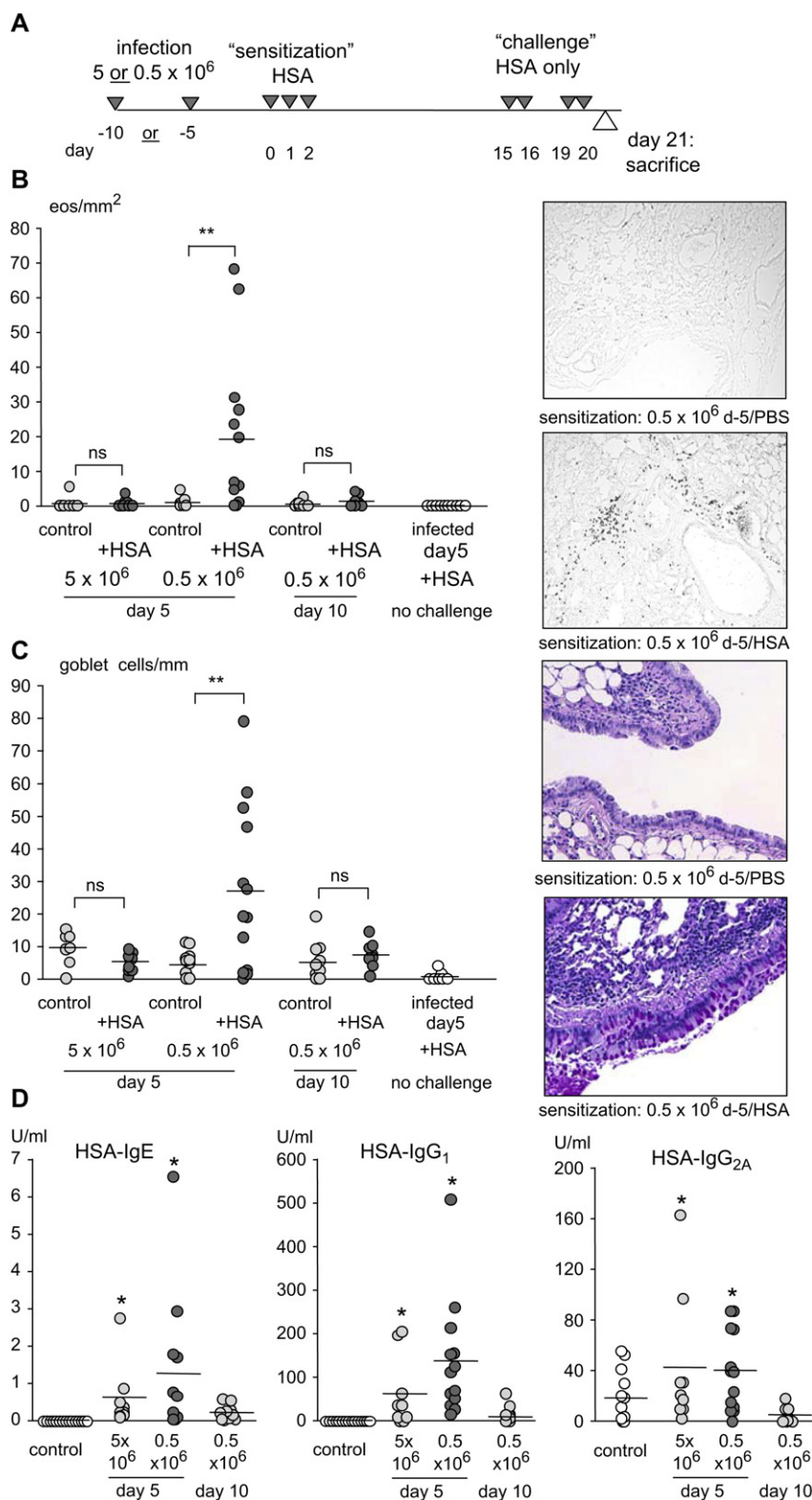
FIG 1. *(Continued).*

presentation of HSA in the regional lymph nodes, mice were injected with 100 μg of fluorescein isothiocyanate (FITC)–labeled HSA intratracheally.[23] After 24 hours, mediastinal lymph nodes were removed, and FITC-presenting DCs were identified as FITC+ and CD11c/CD11b+ using FACS.

Statistical analyses

Independent experiments were conducted at least in triplicate, except as otherwise noted. Results were summarized as means ± SD and compared by using 2-tailed unpaired Student *t* tests. A *P* value of less than .05 was required to reject the null hypothesis.

FIG 2. Characteristics of pulmonary DCs in *C pneumoniae*–infected mice. **A,** Paraffin-embedded sections were stained with MIDC-8 to detect DCs. Total numbers of MIDC-8$^+$ cells in 4 separate sections were counted (6 mice per group). **B,** Upregulation of costimulatory molecules assessed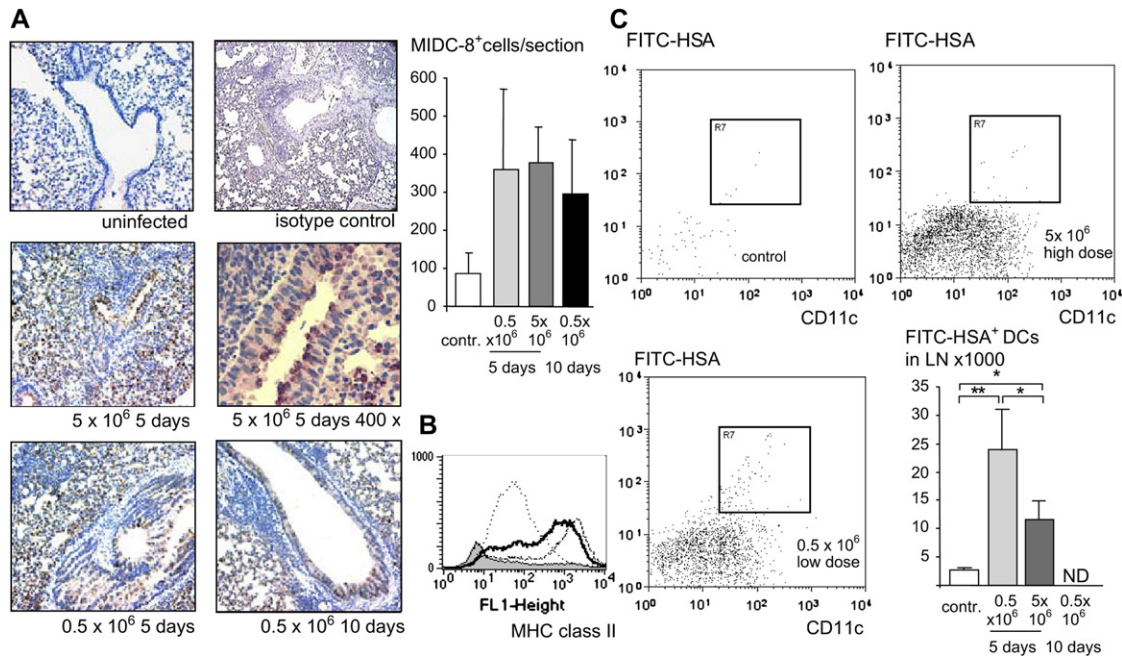 by means of staining of lung leukocytes with antibodies against CD11c, CD11b, and MHC class II. Shown are uninfected control animals *(gray)*, mice infected with high-dose *C pneumoniae (dotted line)* or low-dose *C pneumoniae (bold line)* 5 days before analysis, and mice infected with low-dose *C pneumoniae* 10 days before analysis *(thin line)*. These are representative results from 1 of 3 experiments performed with 5 mice. **C,** Numbers of FITC$^+$ DCs in mediastinal lymph nodes *(LN)* after infection and intratracheal injection of 100 μg of FITC–HSA 24 hours before analysis. Cells were gated for CD11b. Shown are representative dot blots of cells (FITC–HSA × CD11c), as well as combined results of 3 separate experiments (n = 4 mice per experiment). *$P \le .05$. ND, Not determined.

RESULTS

Severity of respiratory tract infection with live *C pneumoniae* is an important determinant of allergic airway sensitization

Groups of mice were infected with either 5×10^6 IFU of *C pneumoniae* (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, whereas the lower infectious dose induced moderate pneumonia with scattered peribronchial and perivascular infiltrates consisting predominantly of mononuclear cells (see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). Levels of cytokines in the bronchoalveolar lavage (BAL) fluid, including IL-6, TNF-α, IFN-γ, and IL-5, were significantly higher in mice infected with the high dose (severe infection) compared with those infected with the low dose (moderate infection; see Fig E1, *B*). 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 1, *A*). Mice with moderate infection that were sensitized with HSA had airway inflammation, exhibiting features of a T$_H$2-biased immune response with a marked increase in eosinophil and goblet cell numbers (Fig 1, *B* and *C*). Eosinophilic airway inflammation in these mice was associated with a significant increase in total IgE levels (data not shown), as well as HSA-specific IgE and IgG1, but not IgG2a, levels (Fig 1, *D*). Airway inflammation was triggered by means of re-exposure to HSA because mice sensitized with HSA but challenged with PBS appeared normal (Fig 1, *B* 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 after HSA challenge (Fig 1, *B-D*). Nevertheless, the latter group did produce HSA-specific IgE and IgG1, albeit at lower levels compared with those seen in low-dose group (Fig 1, *D*).

FIG 1. Low-dose live *C pneumoniae* 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 and n = 9 for high dose) or PBS control (n = 13 for low dose and n = 8 for high dose). Some mice were infected with 0.5×10^6 *C pneumoniae*, 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 *(eos)* numbers per lung section, with representative lung sections (100-fold magnification) are shown at the right of the graph. **C,** Goblet cell numbers related to bronchial basal membrane length. Representative periodic acid–Schiff–stained sections (200-fold magnification) are shown. **D,** HSA-specific IgE, IgG1, and IgG2a titers of mice sensitized and challenged with HSA after infection. *$P \le .05$, **$P \le .01$. *ns,* Not significant compared with control animals.
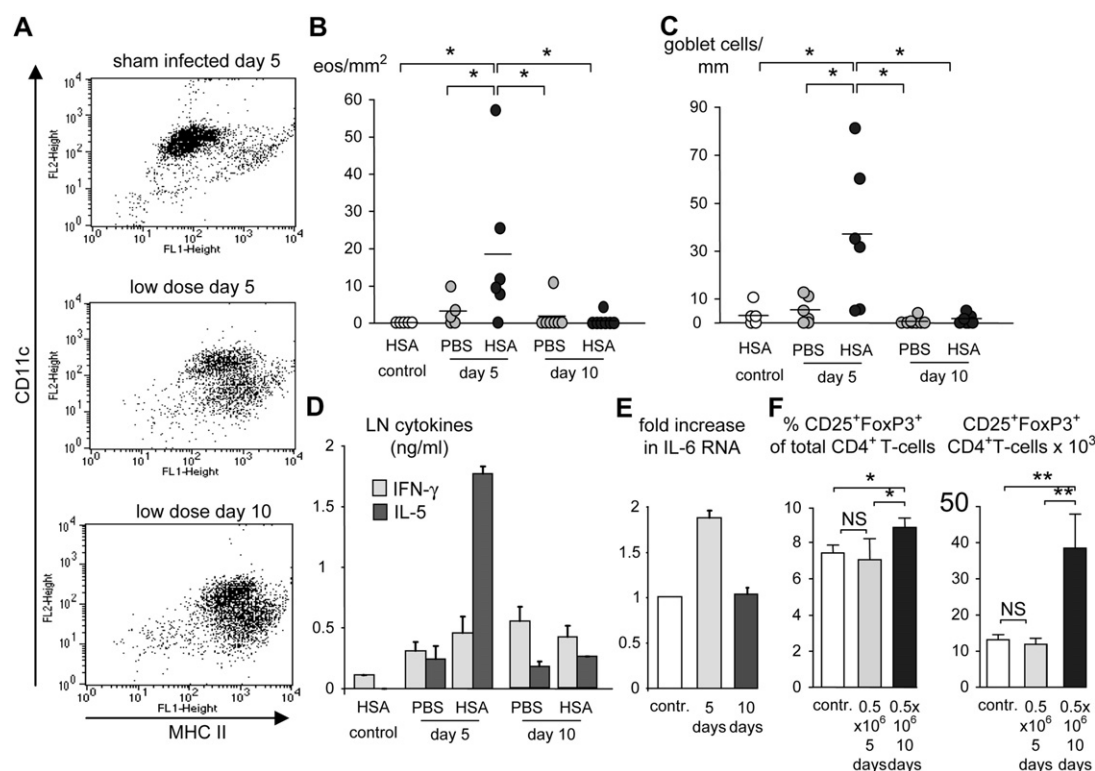
FIG 3. DCs from *C pneumoniae*–infected mice pulsed with HSA can adoptively transfer allergic airway sensitization. **A**, DCs were isolated from lungs of mice 5 or 10 days after infection with low-dose *C pneumoniae* or sham infection. DC expression of MHC class II expression by infected mice and control animals was determined by means of FACS. Cells were incubated with either PBS or HSA overnight and transferred to recipient mice by means of intratracheal inoculation (500,000 cells per mouse) on day 0. Mice were challenged with HSA starting on day 15. **B**, Eosinophil *(eos)* 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 means of periodic acid–Schiff staining. Shown are combined results from 3 experiments. *$P \leq .05$ **D**, Cytokine production by lymph node *(LN)* cells after *in vitro* restimulation with HSA. Shown are representative results from 1 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 using TaqMan real-time RT-PCR and compared with that seen in DCs of uninfected control mice. Combined data of 4 mice per group are shown. **F**, Percentage of lung CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in naive wild-type mice (n = 6-9 mice) 5 days after infection with either 5×10^5 or 5×10^6 *C pneumoniae* or 10 days after infection with 0.5×10^6 *C pneumoniae* administered intranasally.

Timing of live *C pneumoniae* infection and induction of allergic sensitization

We next asked whether airway sensitization during *C pneumoniae* 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 *C pneumoniae* followed by HSA sensitization at day 10 (Fig 1, *A*). At this time point, the mice exhibited mild lung inflammation (see Fig E1, *A* and *B*) but lacked detectable cytokines in the BAL fluid (see Fig E1, *B*). In contrast to mice receiving HSA on day 5 after *C pneumoniae* infection, mice sensitized with HSA on day 10 after infection did not have eosinophilic infiltrates, goblet cell hyperplasia (Fig 1, *B* and *C*), or increased HSA-specific antibodies (Fig 1, *D*). High-dose *C pneumoniae* 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 *C pneumoniae* 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 the numbers of CD11c$^+$/CD11b$^+$ DCs in the lung (data not shown). Furthermore, we identified activated DCs by means of immunohistochemical staining for MIDC-8[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 2, *A*). Although expression of the costimulatory molecules CD80, CD86, and CD40 (see Fig E2, *B*, in this article's Online Repository at www.jacionline.org) was similar, MHC class II levels in DCs isolated from mice infected with high-dose *C pneumoniae* were lower than in the low-dose groups (Fig 2, *B*). Furthermore, numbers of MHC class II^{high} DCs in the
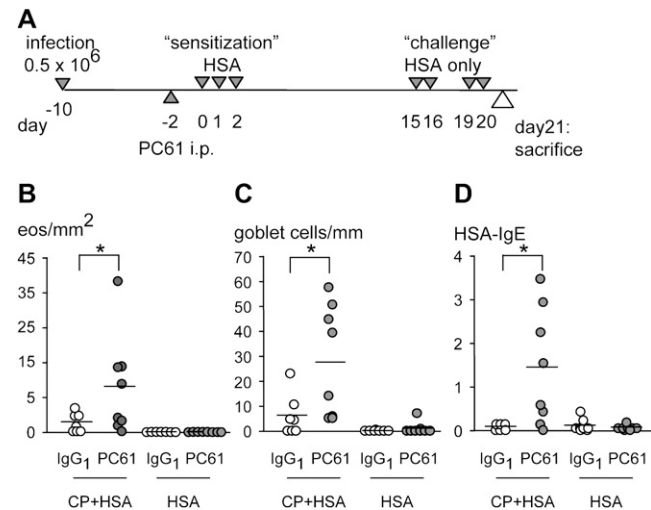
FIG 4. Treg cells regulate airway sensitization induced by *C pneumoniae* (CP) infection. **A**, Infection, Treg cell depletion, and sensitization/challenge protocol: mice were infected with 0.5×10^6 *C pneumoniae* on day −10, followed by a single intraperitoneal injection of 100 μg of PC61 antibody (n = 8) or rat IgG1 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 (**eos**) numbers and goblet cell numbers. **D**, HSA-specific IgE titers. *$P \leq .05$.

draining lymph nodes were significantly lower in high-dose infected mice than in low-dose infected mice (see Fig E2, *C*). Strikingly, this was associated with a significantly lower delivery of FITC-labeled HSA by lymph node DCs from the high-dose group compared with that seen in the low-dose group (Fig 2, *C*). This observation was not due to increased apoptosis of lung DCs because the percentages of lung DCs undergoing apoptosis were similar in the high-dose and low-dose infected mice (see Fig E2, *D*). These data suggest that high-dose infection compromises DC maturation, allergen uptake, and presentation by lung DCs.

Adoptive transfer of DCs from *C pneumoniae*–infected lungs into naive mice induces airway sensitization

To directly determine the role of DCs in allergic airway sensitization, we performed adoptive transfer of pulmonary DCs from *C pneumoniae*–infected mice into naive mice. Mice were infected with low-dose *C pneumoniae*. 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 *C pneumoniae*–infected mice (Fig 3, *A*). After extensive washing, DCs were adoptively transferred to naive recipient mice by means of 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 on HSA challenge (Fig 3, *B* and *C*). In contrast, PBS-pulsed DCs isolated from infected mice or DCs from uninfected mice failed to induce airway sensitization (Fig 3, *B* and *C*). *In vitro* restimulation of mediastinal lymph node cells isolated from the recipient mice with HSA led to preferential IL-5 release (Fig 3, *D*), which is consistent with

the interpretation that DCs from *C pneumoniae*–infected mice induced T_H2 skewing of the immune response in recipient naive mice. Although still showing increased expression of MHC class II, pulmonary DCs obtained from *C pneumoniae*–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 3, *B-D*). This was associated with a diminished expression of IL-6 in these DCs compared with that seen in cells isolated from mice 5 days after infection with *C pneumoniae* (Fig 3, *E*). Furthermore, we found significantly greater numbers of Treg cells in the lungs 10 days after low-dose infection compared with 5 days after infection (Fig 3, *F*).

Treg cells control the timing during which *C pneumoniae* induces allergic sensitization

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

Airway sensitization by *C pneumoniae* requires MyD88-dependent signaling

Upregulation of costimulatory 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 *C pneumoniae*–induced allergen sensitization. To test this hypothesis, MyD88-deficient mice were infected with low-dose live *C pneumoniae* followed by HSA sensitization on day 5 and subsequent HSA challenge. These mice did not have eosinophilic airway inflammation (Fig 5, *A* and *B*), and *C pneumoniae*–induced allergic sensitization was TLR–MyD88 specific because 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 *C pneumoniae*, expression of costimulatory molecules on airway DCs in MyD88^{−/−}
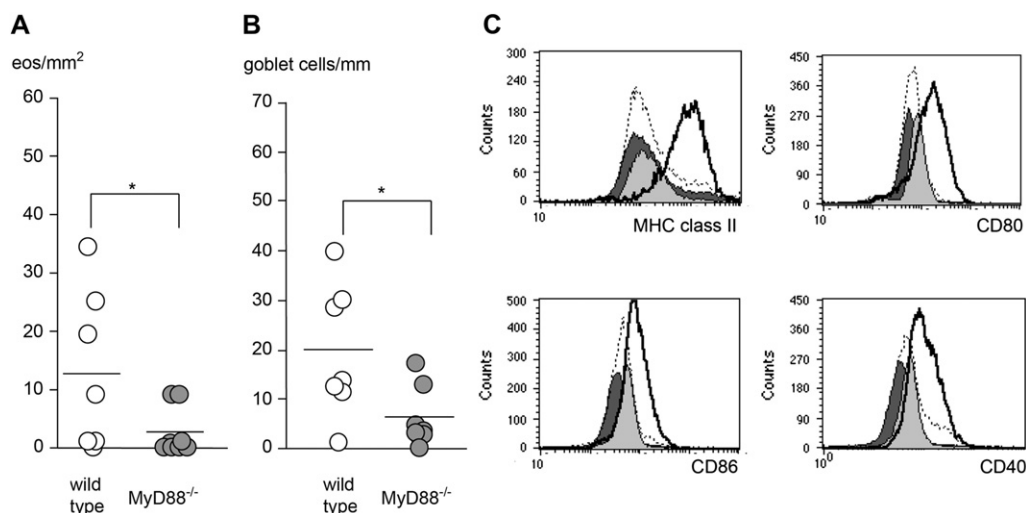
FIG 5. MyD88-dependent signaling in DCs is required for airway sensitization. **A and B**, Eosinophil *(eos)* numbers and goblet cell numbers in lung sections from wild-type mice (n = 7) infected with 0.5×10^6 live *C pneumoniae*, exposed to HSA on 3 consecutive days, and challenged with HSA, as shown in Fig 1, *A*. Data obtained from MyD88$^{-/-}$ mice (n = 8 mice are shown in comparison). *$*P \leq .05$, $**P \leq .01$. ns*, Not significant. Wild-type mice (n = 7) and MyD88$^{-/-}$ mice (n = 6) were infected with 0.5×10^6 *C pneumoniae* and killed after 5 days. **C**, Upregulation of MHC class II, CD80, CD86, and CD40 in DCs of infected mice *(bold line*, wild-type mice; *dotted line*, MyD88$^{-/-}$ mice) versus uninfected control animals *(gray*, wild-type mice; *dark gray*, MyD88$^{-/-}$ mice) determined by means of FACS. DCs were identified as CD11c$^+$/CD11b$^+$.

mice remained unchanged during *C pneumoniae*–induced pneumonia compared with wild-type mice (Fig 5, *C*).

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 tract infection with *C pneumoniae*, a common respiratory pathogen, creates a proinflammatory environment in the lung that can exert an adjuvant effect on allergen sensitization. We found that DCs were centrally involved in triggering allergic sensitization because either adoptive transfer of allergen-presenting DCs from infected mice or bone marrow–derived DCs infected with *C pneumoniae* 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 Fig E4, *B-D*, in this article's Online Repository at www.jacionline.org). Therefore intact MyD88-dependent signaling in DCs was sufficient to induce allergic airway sensitization after *C pneumoniae* infection and exposure to allergen.

However, airway sensitization depended on the severity and timing of the infection: low-dose infection and antigen exposure within 5 days of infection induced allergic airway sensitization, whereas 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 cell function: by depleting the CD4$^+$CD25$^+$ Treg cell subset, we were able to prolong the time window in which sensitization could occur. Recent studies suggest that Treg cells normally inhibit the development of allergic asthma,[19] but in studies focusing on T_H1-biased

immune responses, the suppressive activity of Treg cells 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 cell 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 T_H1-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 IgG2a production and T_H1 skewing, which prevents allergen sensitization.[16,17] In fact, during high-dose infection, we observed a trend toward less pronounced T_H2 cell responses, as manifested by lower levels of HSA-specific IgG1 and IgE compared with those seen in low-dose infected mice (Fig 2, *D*). Furthermore, high-dose infections were accompanied by higher levels of IFN-γ in the BAL fluid and increased production of IL-12, but not IL-10, by DCs (see Fig E1, *B*, and E2, *A*). 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 might be related to the increased numbers of neutrophils observed in high-dose infected mice because neutrophil degranulation inhibits DC maturation *in vitro*.[28] Therefore allergic antigen sensitization in high-dose infected mice might be impaired by the cytokine pattern elicited in DCs, which favors both T_H1 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 respiratory syncytial virus. 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 thus 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 effect of *Chlamydia* species on allergic airway inflammation in murine models have focused on exacerbation of pre-existing asthma by infecting previously sensitized mice with *Chlamydia trachomatis* or *Chlamydia 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 *C pneumoniae* infection. Our findings are therefore consistent with previous studies,[7,8] which collectively emphasize that the 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 tract infections can act as adjuvants that promote allergen sensitization and the pathogenesis of asthma.

In summary, our results demonstrate that bacterial respiratory tract 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 Treg cells. The net outcome of the functional interactions between *C pneumoniae*–activated DCs and Treg cells dynamically alters the magnitude of antigen sensitization. Our data demonstrate that under certain circumstances, infectious agents can promote the pathogenesis of allergic disease by acting as adjuvants that favor T_H2 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 affect both the likelihood that allergic sensitization could occur and the duration of the time window during which this is possible.

Key messages

- **Moderate, but not severe, pulmonary infection with *C pneumoniae* induces allergic airway sensitization.**
- **Sensitization is mediated by MyD88-dependent activation of DCs and is inhibited by Treg cells.**

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METHODS
Mice

Mice bred at our facility were routinely tested for the following pathogens with negative results: murine hepatitis virus (MHV), murine minute virus (MMV), murine parvovirus 1-3 (MPV), influenza virus (NS1), murine norovirus (MNV), Sendai virus, Theiler's murine encephalomyelitis virus (TMEV), epizootic diarrhea of infant mice virus (EDIM), murine pneumonia virus of mice (PVM), reovirus 3, lymphocytic choriomeningitis (LCM) virus, *Mycoplasma pulmonis*, *Helicobacter* species, *Ectromelia* species, pinworms, and fur mites.

Bone marrow–derived DCs

Bone marrow–derived dendritic cells (BMDCs) were generated by incubating bone marrow cells with 10 ng/mL recombinant mouse GM-CSF (Biosource, Bethesda, Md) for 6 days, with medium changes at days 3 and 5. DCs were harvested at day 6 and purified with CD11c microbeads, as described above. Purity assessed by means of flow cytometry was routinely around 98%.

Cytokine detection

For some experiments, BAL fluid was collected with 0.5 mL of PBS, and cytokine levels were determined by means of ELISA (IL-6 and TNF-α, BD Biosciences; IFN-γ and IL-5, eBioscience). Intracellular content of IL-10 and IL-12p40 was determined by using the phycoerythrin-labeled anti-IL-10 and anti-IL-12p40 antibodies (eBioscience) with the Cytofix/Cytoperm intracellular staining kit (BD PharMingen, San Jose, Calif).

RESULTS
MyD88-dependent signaling in *C pneumoniae*–infected DCs is required for adoptive transfer of airway sensitization

BMDCs from wild-type or MyD88$^{-/-}$ mice, after *ex vivo* infection with *C pneumoniae* and incubation with HSA, were intratracheally administered to wild-type or MyD88$^{-/-}$ recipient mice to test whether MyD88 deficiency affects antigen presentation. Adoptive transfer of wild-type DCs incubated with *C pneumoniae* plus HSA could trigger eosinophilic airway inflammation and goblet cell hyperplasia in wild-type recipients following HSA challenge (Fig E4, *A-C*). In contrast, adoptive transfer of MyD88$^{-/-}$ DCs to wild-type naive mice did not trigger sensitization, which is consistent with the interpretation that the MyD88 pathway is essential for the initiation of DC-induced allergen sensitization (Fig E4, *A-C*). Conversely, intratracheal adoptive transfer of infected wild-type DCs pulsed *ex vivo* with HSA rescued allergic sensitization in MyD88$^{-/-}$ mice (Fig E4, *A-C*). MyD88 deficiency did not diminish *Chlamydia* species uptake, as evidenced by similar staining intensity of ingested bacteria in wild-type compared with MyD88$^{-/-}$ DCs (Fig E4. *D*). Thus MyD88 in DCs is sufficient for allergic sensitization to occur. Together, these data suggest that in the absence of MyD88, DCs can take up *Chlamydia* species but cannot induce the required T-cell responses that lead to allergen sensitization.
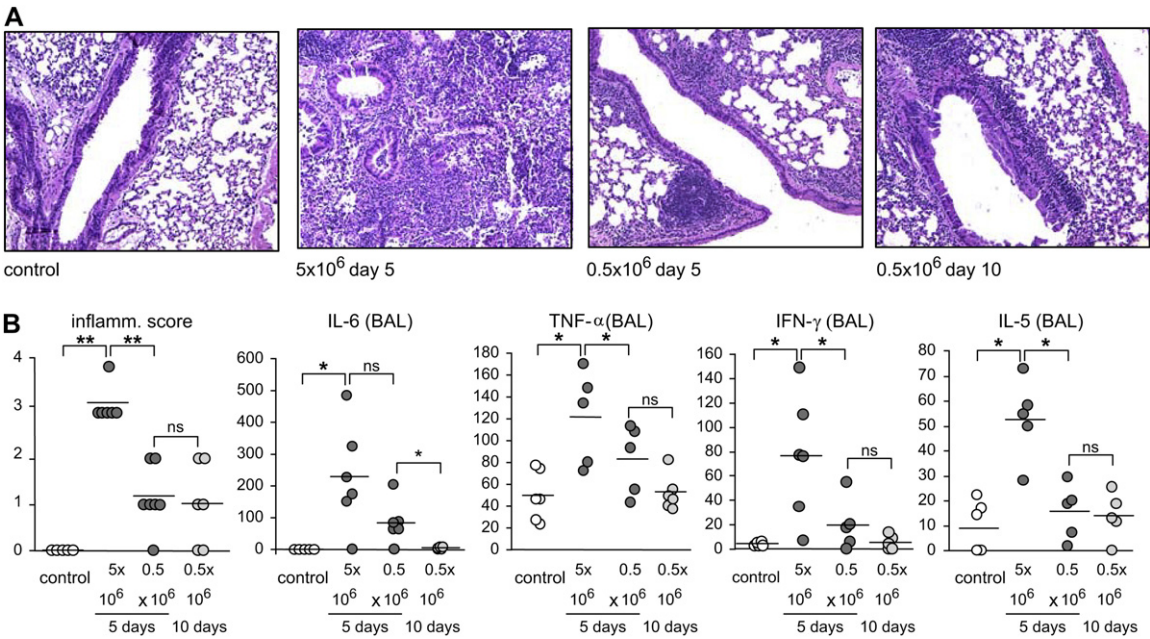
FIG E1. Characteristics of pneumonia caused by low-dose and high-dose *C pneumoniae* at day 5 or low-dose *C pneumoniae* at day 10. Mice (n = 6 per group) were infected by means of intranasal injection of either 0.5×10^6 or 5×10^6 IFU of *C pneumoniae* 5 days or 0.5×10^6 *C pneumoniae* 10 days before analysis. **A**, Representative hematoxylin and eosin–stained sections of paraffin-embedded lungs. **B**, Inflammatory scores obtained from hematoxylin and eosin–stained sections and levels of IL-6, TNF-α, IFN-γ, and IL-5 in the BAL fluid. *$P \leq .05$, **$P \leq .01$. *ns*, Not significant.
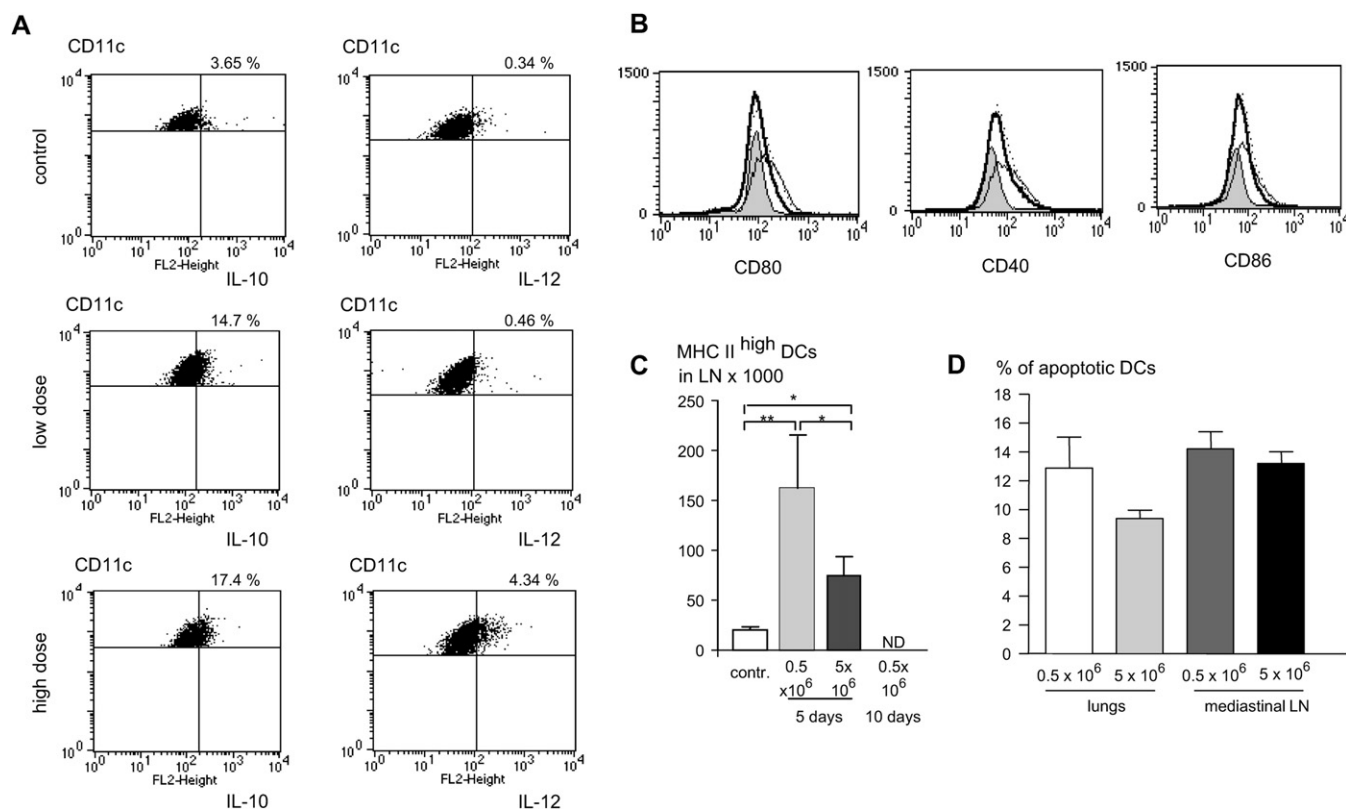
FIG E2. Cytokine production and expression of costimulatory molecules in DCs isolated from *C pneumoniae*–infected mice. Mice were infected with either 0.5×10^6 or 5×10^6 IFU of *C pneumoniae*, and lung leukocytes were prepared as described in the Methods section. **A**, Cells were stained for expression of CD11b and CD11c, and staining for intracellular cytokines was performed with antibodies against IL-10–phycoerythrin or IL-12–phycoerythrin. **B**, Upregulation of costimulatory molecules assessed by staining of lung leukocytes with antibodies against CD11c, CD11b, CD40, CD80, and CD86. Shown are uninfected control animals *(gray)*, mice infected with high-dose *C pneumoniae (dotted line)* or low-dose *C pneumoniae (bold line)* 5 days before analysis, and mice infected with low-dose *C pneumoniae* 10 days before analysis *(thin line)*. Shown are representative results from 1 of 3 experiments performed with 5 mice. **C**, Total numbers of MHC class II[high] DCs in mediastinal lymph nodes *(LN)* 5 days after infection. Shown are representative dot blots of cells gated for CD11b, as well as combined results of 3 separate experiments (n = 4 per group). **D**, Percentages of DCs in infected mice undergoing apoptosis. DCs were identified by using CD11c, CD11b, and side-scatter. Apoptotic cells were identified with the Vybrant Apoptosis Assay Kit (Invitrogen).
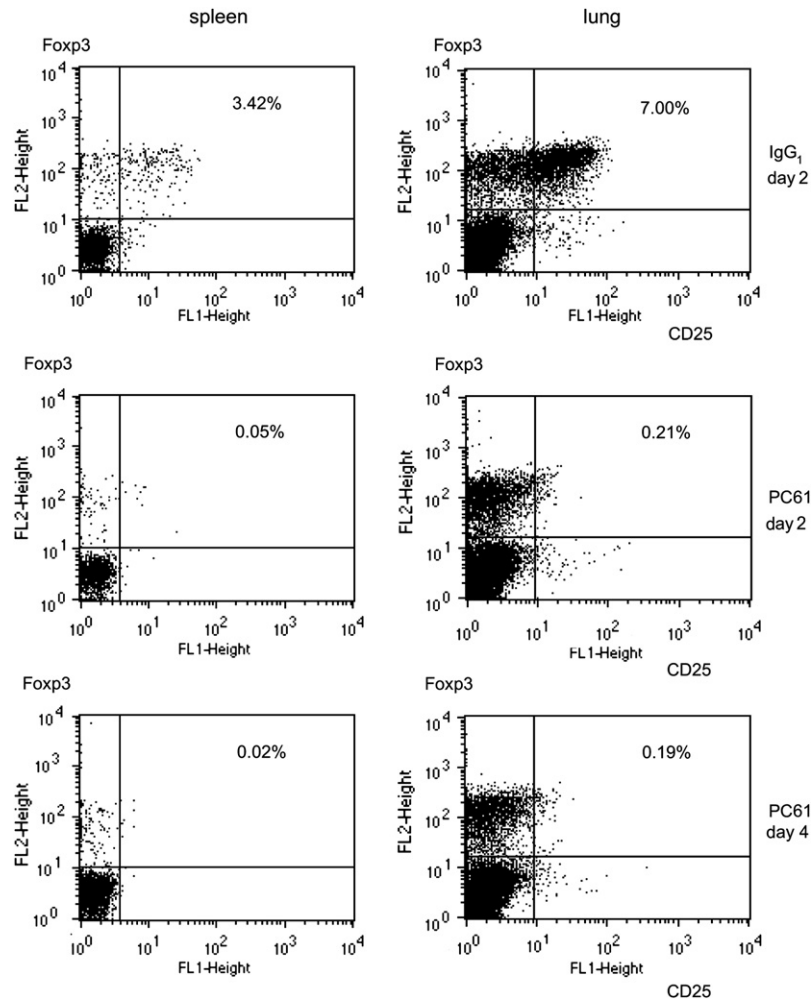
FIG E3. Depletion of CD4$^+$CD25$^+$ Treg cells in the lungs after injection of anti-CD25 antibody. Mice received intraperitoneal injections of 100 μg of either anti-CD25 antibody (clone PC61) or rat IgG1 isotype control on day 0. Mice were killed 2 or 4 days after injection. Splenocytes and lung leukocytes were prepared as described in the Methods section and were then analyzed for Foxp3$^+$ Treg cells by means of flow cytometry with antibodies against CD4, CD25 (clone 7D4), and Foxp3. Percentages refer to total CD4$^+$ lymphocytes. Shown are representative results from 1 of 3 experiments performed with 3 mice.
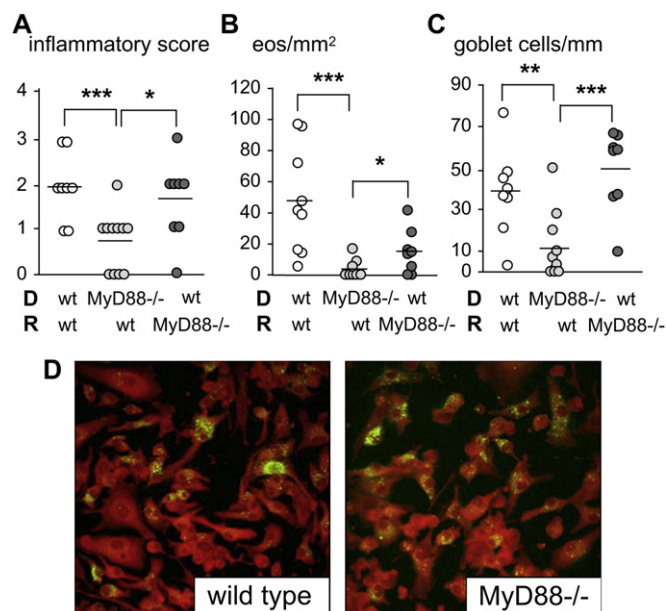
FIG E4. MyD88-dependent signaling in DCs is required for airway sensitization. BMDCs from wild-type *(wt)* and MyD88$^{-/-}$ mice were exposed to *C pneumoniae* (multiplicity of infection = 2.5) and HSA (50 μg/mL) in the presence of gentamicin for 16 hours, followed by intratracheal transfer into recipient mice. Wild-type mice were injected with wild-type DCs (n = 8) or MyD88$^{-/-}$ DCs (n = 11). One group of MyD88$^{-/-}$ mice received wild-type DCs (n = 10). **A,** Inflammatory scores determined by using hematoxylin and eosin–stained lung sections. **B** and **C,** Eosinophil *(eos)* numbers and goblet cell numbers in lung sections. *$P \leq$ .05, **$P \leq$ .01, ***$P \leq$ .001. **D,** BMDCs generated from wild-type and MyD88$^{-/-}$ mice were infected with *C pneumoniae* (multiplicity of infection = 2.5), and content of *C pneumoniae* was determined after 24 hours by means of immunofluorescence with the Pathfinder staining kit (Sigma). Shown are representative results from 1 of 3 separate experiments.