

Imprinted DC mediate the immune-educating effect of early-life microbial exposure

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It has been long proposed that exposure to environmental factors early in life may have an educating effect on the development of immune regulatory functions. However, experimental studies on this issue are limited and the related molecular and cellular basis remains unclear. Here we report that neonatal exposure to killed bacteria (*Chlamydia muridarum*, originally called *Chlamydia trachomatis* mouse pneumonitis (MoPn)) changed the pattern of the hosts' immune responses to a model allergen (OVA) in adulthood. This was associated with altered phenotype and function of DC. We found that DC from adult mice treated neonatally with UV-killed MoPn exhibited distinct patterns of surface marker and TLR expression and cytokine production from control mice (DC from adult mice neonatally treated with vehicle, (Sham-DC)). More importantly, DC from adult mice treated neonatally with UV-killed MoPn induced significantly lower type-2 antigen-specific T-cell responses than Sham-DC shown in DC:T co-culture experiments *in vitro* and in adoptive transfer experiments *in vivo*. In addition, depletion of T cells *in vivo* largely abolished the phenotypic and functional alterations of DC caused by bacterial exposure, suggesting the involvement of T cell in this process. Our study demonstrates a central role of DC in linking the early-life exposure to microbial products and the balanced development of immune regulatory functions and the involvement of T cells in imprinting of the DC function.

Key words: Allergy · DC · Hygiene hypothesis · IL-10 · TLR

Introduction

The immune system of a host experiences multiple influences from the outside world during development from birth to adult life. Numerous studies have demonstrated that environmental factors encountered during the developmental stage have considerable effect in shaping the pattern of immune responses in adult life. Therefore, the exposure to “proper” environmental antigens in early life might be important for the formation and maintenance of a balanced immune regulatory system in the host, thus preventing autoimmunity and allergic diseases [1, 2].

Over the past decades, the general burden of infections in the developed areas of the world has been reduced due to a better hygiene, and the “hygiene hypothesis” has been raised to explain the significant increase of allergic diseases [3, 4]. Specifically, it has been hypothesized that exposure to microbial products during early childhood may have an inhibitory effect on the development of allergic diseases [4–6]. Notably, in a series of well-performed epidemiological studies on children of European farmers (Allergy and Endotoxin), it was demonstrated that children who grew up in a farming environment and had close contact with farm animals exhibited significantly low levels of allergy and asthma in later life [7–9]. However, the proposed educating effect of exposure to various microbial products during early life on the development of immune regulatory function has not been tested in detail using experimental approaches.

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More importantly, how early life environmental factors influence adulthood immune regulatory function remains largely unknown.

DC, as the most efficient professional APC, play a crucial role in the linkage between innate and adaptive immune responses [10]. DC can direct the activation and differentiation of primary T cells through sensing the microenvironment via expressed receptors (including pattern-recognition receptors), and producing various immune regulatory cytokines. Recent studies have shown that DC play a critical role in the initiation as well as maintenance of allergic immune responses [11, 12].

Chlamydiae are a group of obligate intracellular bacteria that can cause infections and various diseases in all age groups of humans including neonates and young children [13]. *Chlamydia muridarum*, originally called *Chlamydia trachomatis* mouse pneumonitis (MoPn) is a chlamydial strain that causes respiratory tract infection in mice [13]. We therefore chose MoPn as a model bacterium for this study. We experimentally examined the linkage between the exposure to bacterial products in early life and the pattern of immune responses to unrelated antigen in adulthood by examining the effect of neonatal exposure to killed MoPn on allergic responses induced by allergen in adult mice. Our results showed that neonatal exposure to UV-killed MoPn (UV-MoPn) significantly inhibited eosinophilic airway inflammation, mucus over-production and Th2 cytokine production induced by OVA sensitization and challenge in adulthood. More importantly, we found that DC from adult mice treated neonatally with UV-killed MoPn (UV-MoPn-DC) differ in phenotype and function from those isolated from mice exposed to buffer solution only (DC from adult mice neonatally treated with sucrose-phosphate-glutamic acid (SPG), Sham-DC). Furthermore, adoptive transfer of UV-MoPn-DC significantly inhibited the development of allergic inflammation in the recipient mice. The results indicate that early microbe exposure can modulate immune responses to unrelated antigen in adult life and, more importantly, this educating effect is largely mediated through alterations in DC function.

Results

Neonatal exposure to UV-MoPn-inhibited allergic responses in adult life

To investigate the effect of neonatal exposure to microbial products on the development of allergic responses in adulthood, newborn mice (5–6 days after birth) were initially injected (i.p.) with UV-MoPn or buffer only. When these mice were 35 days old (adult), they were sensitized and challenged with OVA according to a standard protocol [14, 15]. At 7 days after challenge, the mice were killed and analyzed for allergic reaction and immune responses. Both the total number of inflammatory cells (3.7×10^5 versus 6.6×10^5 , $p < 0.05$), and the absolute number (6.0×10^4 versus 3.9×10^5 , $p < 0.01$) and percentage (16.2% versus 61.6%, $p < 0.001$) of eosinophils in bronchoalveolar lavage (BAL) were dramatically reduced in the mice with neonatal UV-MoPn treatment, compared with the buffer-treated control mice

(Fig. 1A and B). As a baseline, the total number of cells in the BAL of the naive mice and UV-MoPn-treated mice without OVA treatment was less than 10^4 , and these mostly comprised epithelial cells without notable eosinophils. The histological mucus index, a quantitation of mucus-positive epithelium in the airway, was 15–20% in UV-MoPn-treated mice, compared with 40% in control mice (Fig. 1C, $p < 0.01$). The histological analysis showed significantly less inflammation and fewer infiltrating eosinophils in the peribronchial and perivascular areas of the lung tissues in the UV-MoPn-treated mice (Fig. 1D(i) and ii). Moreover, mucus-producing goblet cell hyperplasia within the airway was also significantly reduced in the UV-MoPn-treated mice (Fig. 1Diii). In airway blood vessels, the expression of VCAM-1, which plays a pivotal role in eosinophil recruitment into the lung, was significantly reduced in the mice with neonatal exposure to UV-MoPn (Fig. 1D(iv)). The results demonstrate that early-life exposure to chlamydial components can inhibit allergic airway inflammation in adult mice.

Neonatal exposure to UV-MoPn changed allergen-driven cytokine profiles in adulthood

Since Th2 cytokines play a critical role in airway eosinophilic inflammation in allergy, we examined the effect of neonatal UV-MoPn treatment on cytokine secretion profiles of T lymphocytes in response to allergen in adulthood. We cultured the splenocytes of the OVA-sensitized/challenged adult mice that had been treated neonatally with UV-MoPn or buffer only with further OVA-specific stimulation. Analysis of the cytokine profiles in the culture supernatants showed that the cells from mice with neonatal UV-MoPn treatment secreted significantly lower levels of IL-4 ($p < 0.01$), IL-5 ($p < 0.01$) and IL-13 ($p < 0.01$) than those from sham-treated mice (Fig. 2A–C). No significant difference was observed in IFN- γ production between the two groups (Fig. 2D). Intracellular cytokine staining also showed reduced IL-4 production by CD3⁺CD4⁺ T cells from the mice treated neonatally with UV-MoPn (Fig. 2E and F). The results demonstrate that neonatal exposure to UV-MoPn can inhibit type 2 T-cell responses induced by allergen in adult life.

Neonatal exposure to UV-MoPn changed the phenotype and cytokine production of DC in adulthood

Since DC are critical in determining T-cell differentiation and cytokine production, we then examined the effect of neonatal exposure to UV-MoPn on DC in adult mice. As showed in Fig. 3A and Table 1, UV-MoPn-DC expressed significantly higher levels of CD86 (both in percentage and MFI) and CD80 (MFI) than those from sham-treated mice (Sham-DC). Moreover, a larger proportion of CD8 α ⁺ DC was observed in the mice with neonatal exposure to UV-MoPn (Fig. 3A, Table 1). In addition, neonatal exposure to UV-MoPn also altered cytokine patterns of DC in adulthood. UV-MoPn-DC exhibited significantly higher

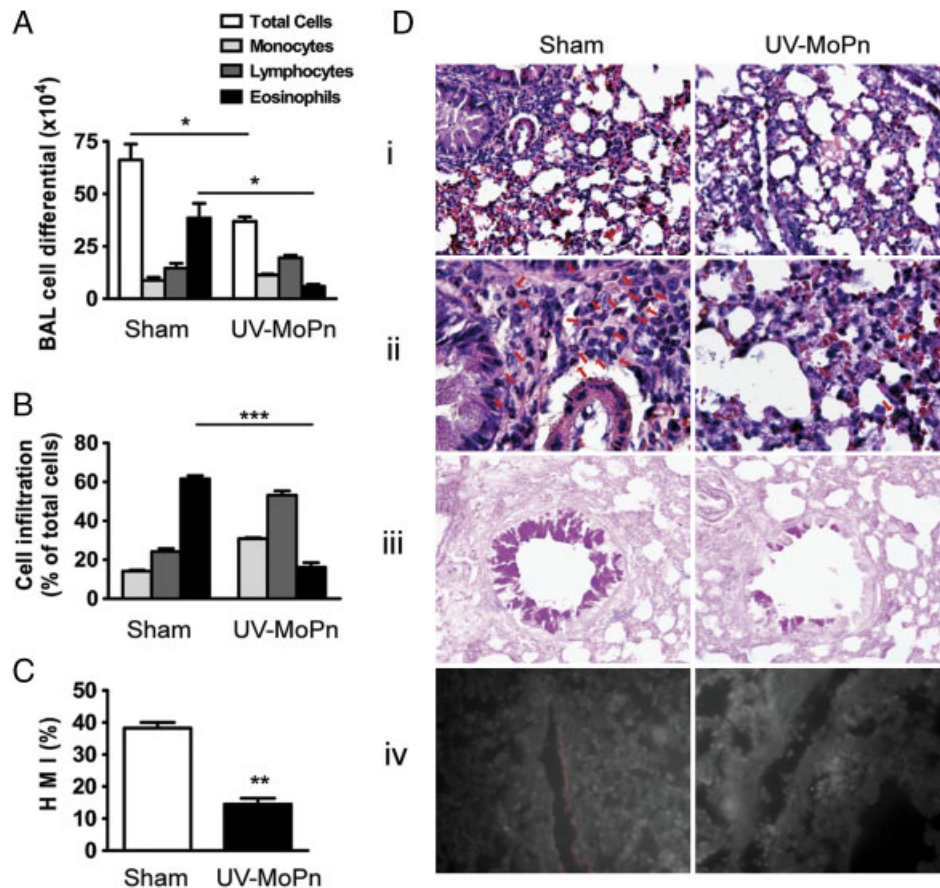


Figure 1. Neonatal exposure to UV-killed *C. muridarum* (UV-MoPn) inhibits airway allergic reactions in adult mice. Newborn C57BL/6 mice (four mice/group) were immunized i.p. with UV-MoPn (2×10^6 original IFU in 20 μ L SPG buffer) at 5–6 days after birth. The control group (Sham) received SPG buffer only. At 35 days of age, all mice were sensitized with OVA (2 μ g in alum) by i.p. immunization. At 14 days after sensitization, mice were challenged i.n. with OVA (50 μ g in 40 μ L PBS). The mice were killed at day 7 after i.n. challenge and the cell differential in BAL fluid was counted using Fisher Leukostat Stain kit. (A) The absolute number of each type of infiltrated cell population. (B) The proportion of each type of infiltrating cells. (C) Mucus-producing epithelium was quantified as histological mucus index (HMI) as described previously [16]. (D) The lung sections were stained by hematoxylin and eosin for inflammation (i) $\times 400$; (ii) $\times 1000$; eosinophils are pointed by red arrows and by PAS staining for mucus production; (iii) VCAM-1 expression and (iv), red) was analyzed as described in *Materials and methods*. One representative experiment of five independent experiments (four mice in each group in each experiment) is shown. Data are shown as the mean $\pm$ SD of each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

production of both IL-12 (Fig. 3B, $p < 0.001$) and IL-10 (Fig. 3C, $p < 0.01$) than Sham-DC. Furthermore, UV-MoPn-DC expressed significantly lower level of TLR9 than Sham-DC, while the levels of TLR2 and TLR4 were comparable between the two groups (Fig. 3D and E). Taken together, the results indicate that neonatal exposure to microbes can modulate the co-stimulatory molecule expression and cytokine production of DC at adulthood.

UV-MoPn-DC inhibited the differentiation of naive allergen-specific CD4⁺ T cells in a Th2 direction

To determine whether the alteration in DC observed in the mice with neonatal exposure to UV-MoPn can translate into differences in their ability to polarize antigen-specific CD4⁺ T cells, we co-cultured UV-MoPn-DC or Sham-DC with naive OVA-specific CD4⁺ T cells isolated from DO11.10 mice in the presence of OVA stimulation. As shown in Fig. 4, CD4⁺ T cells co-cultured with

UV-MoPn-DC showed significantly lower IL-4, IL-5 and IL-13 production than those co-cultured with Sham-DC, while IFN- γ production was comparable between the two groups (Fig. 4A). Moreover, intracellular cytokine staining of CD4⁺ T cells co-cultured with UV-MoPn-DC for 72 h also showed reduced IL-4 production but comparable IFN- γ production (Fig. 4C) compared with those co-cultured with Sham-DC. To further test the role played by IL-12 and IL-10 produced by UV-MoPn-DC in altering the pattern of cytokine production by antigen-specific CD4⁺ T cells, we used anti-IL-12 and anti-IL-10 antibodies to neutralize the corresponding cytokines in the UV-MoPn-DC:CD4⁺ T cell co-culture. The data showed that blockade of IL-12 significantly increased IL-4, IL-5 and IL-13 production, but decreased IFN- γ (Fig. 4B) production, by CD4⁺ T cells in the co-culture system. However, blockade of IL-10 dramatically increased Th2 cytokines without significant effect on IFN- γ production (Fig. 4B). The results suggest that the higher production of both IL-12 and IL-10 by UV-MoPn-DC influences

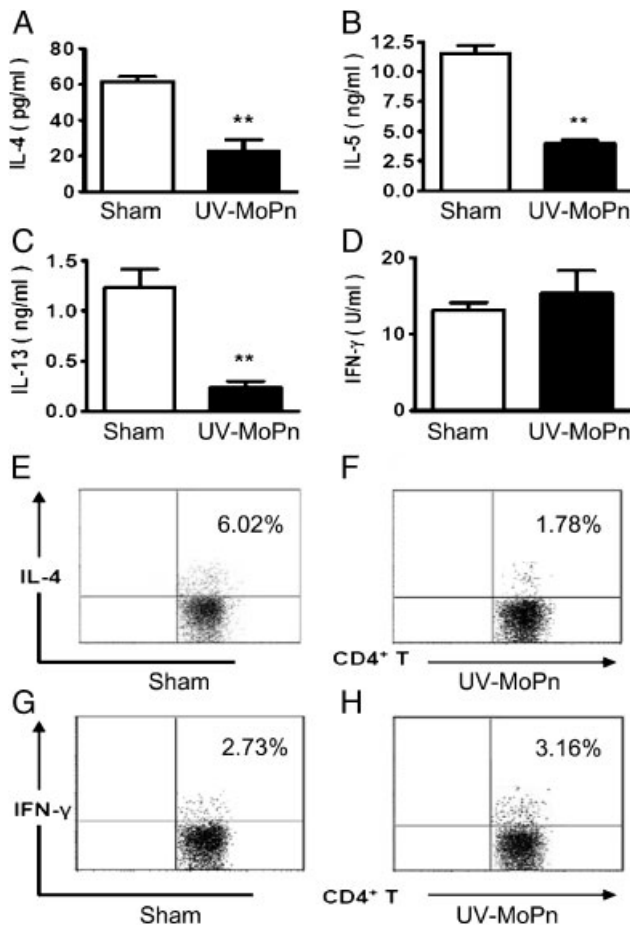


Figure 2. Neonatal exposure to UV-MoPn inhibits Th2 cytokine production in adult mice. Mice were treated neonatally with UV-MoPn or SPG (Sham) and sensitized and challenged with OVA in adulthood as described in the legend to Fig. 1. At 7 days after i.n. OVA challenge, mice were killed and the splenocytes from individual mice were cultured with OVA-specific stimulation. The concentrations of cytokines (A–D) in culture supernatants were measured using ELISA. Levels for cytokine production by splenic cells from mice without *in vivo* OVA treatment were virtually undetectable by ELISA. (E–H) Cytokine production by CD3⁺CD4⁺ T cells was analyzed by intracellular cytokine staining using labeled antibodies and flow cytometry as described in *Materials and methods*. Cells were gated on CD3⁺ T cells and the percentages of the respective cytokine-producing CD4⁺ T cells are indicated. (E and G) Adult OVA sensitized/challenged mice exposed neonatally to SPG; (F and H), adult OVA sensitized/challenged mice exposed neonatally to UV-MoPn. Data are presented as mean ± SD of each group. One representative experiment of three independent experiments (four mice in each group in each experiment) is shown. ***p* < 0.01.

the polarization of antigen-specific T cells, while IL-10 may be particularly important for inhibiting Th2-like cell development.

Adoptive transfer of UV-MoPn-DC, but not Sham-DC, inhibited airway allergic reactions in recipient mice

We then examined the function of UV-MoPn-DC in modulating allergen-specific T cells and airway allergic reactions *in vivo* using an adoptive transfer approach. Syngeneic naive adult mice were

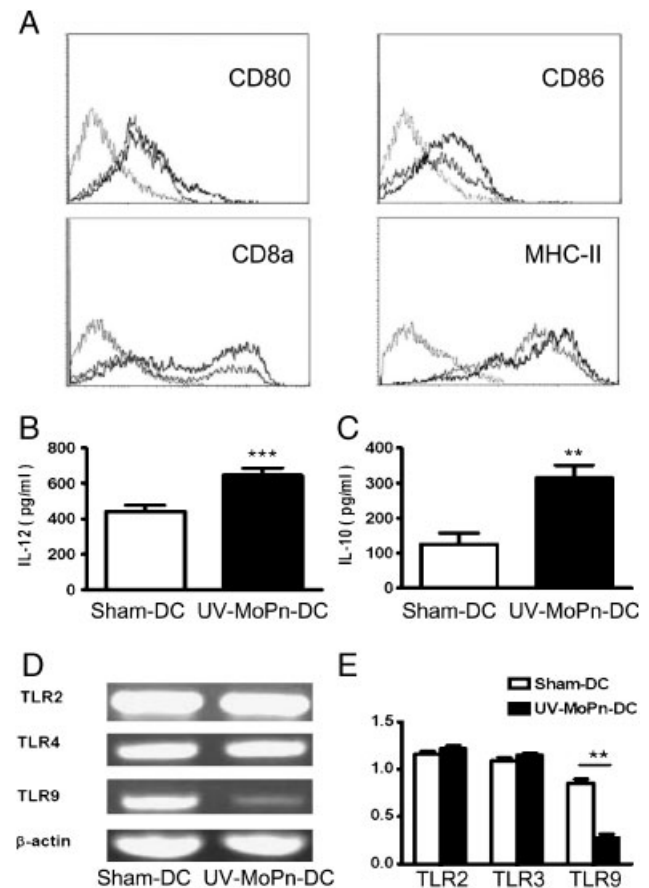


Figure 3. UV-MoPn-DC show different patterns of surface marker expression and cytokine production from Sham-DC. DC were purified from the spleens of 35-day-old adult mice exposed neonatally to UV-MoPn (UV-MoPn-DC) or SPG (Sham-DC) using MACS CD11c⁺ cell isolation column. (A) The expression of surface markers on the freshly isolated DC was assessed by flow cytometry. The dotted line indicates isotype control; the thinner solid line represents Sham-DC; the thicker line indicates UV-MoPn-DC. (B and C) Freshly isolated DC were cultured at 5×10^5 cells/well in complete culture medium for 72 h. The culture supernatants were measured for IL-12 and IL-10 levels by ELISA. One representative experiment of three independent experiments (four mice in each group in each experiment) is shown. Data are shown as mean ± SD. ***p* < 0.01; ****p* < 0.001. Comparison between Sham-DC and UV-MoPn-DC. (D) Total cellular RNA from the freshly isolated DC was extracted and RT-PCR was performed for measuring mRNA of TLR2, TLR4 and TLR9. (E) β-Actin-normalized quantification of the PCR products from Sham-DC and UV-MoPn-DC. Pooled data from three independent experiments (four mice in each group in each experiment) on RT-PCR analysis are shown. Data are presented as mean ± SEM. ***p* < 0.01. Comparison between TLR9 mRNA expressions by Sham-DC and UV-MoPn-DC.

adoptively transferred with UV-MoPn-DC or Sham-DC followed by OVA sensitization and challenge. The recipients of UV-MoPn-DC showed significantly fewer eosinophils in BAL fluids (Fig. 5A and B) and peribronchial tissues (data not shown) than those receiving Sham-DC. The goblet cell hyperplasia and mucus production was also significantly lower in the recipients of UV-MoPn-DC than in those receiving Sham-DC (Fig. 5C). Consistent with the reduction in allergic inflammation, Th2 cytokine (IL-5 and IL-13) production in the UV-MoPn-DC recipients was significantly lower than in the Sham-DC recipients

Table 1. Summary of flow cytometric analysis of surface markers expressed on DC (mean $\pm$ SEM)

		Sham-DC (n = 23)	UV-MoPn-DC (n = 28)	p value
CD80	Percentage (%)	41.7 $\pm$ 2.7	46.9 $\pm$ 4.8	0.107
	MFI	23.7 $\pm$ 3.9	42.7 $\pm$ 6.1	0.024*
CD86	Percentage (%)	25.4 $\pm$ 4.2	36.8 $\pm$ 2.1	0.031*
	MFI	19.2 $\pm$ 3.1	30.3 $\pm$ 6.4	0.047*
MHCII	Percentage (%)	69.7 $\pm$ 6.1	73.2 $\pm$ 5.0	0.542
	MFI	490.0 $\pm$ 49.1	766.4 $\pm$ 41.7	0.002**
CD8 α	Percentage (%)	27.7 $\pm$ 3.1	36.8 $\pm$ 2.9	0.003**
	MFI	233.1 $\pm$ 41.7	373.6 $\pm$ 57.4	0.008**

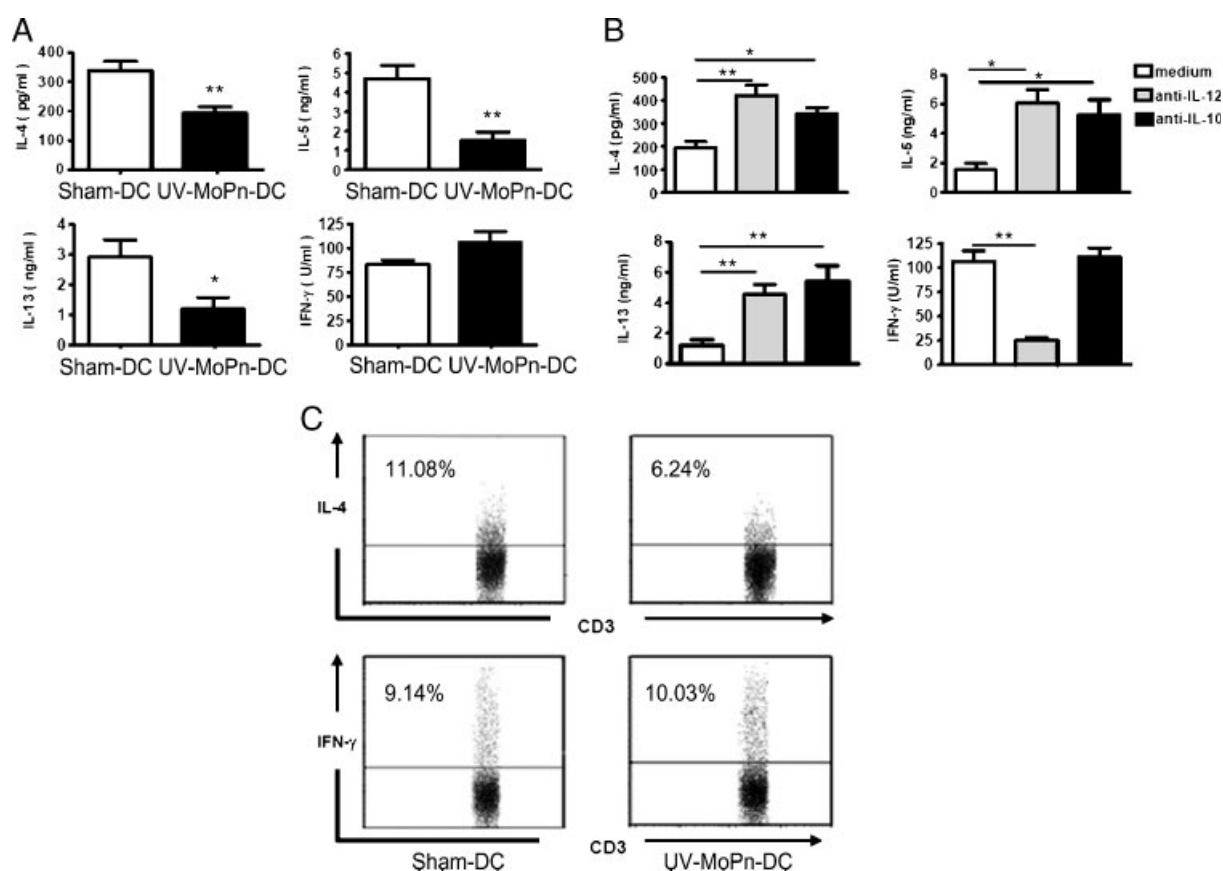
* $p < 0.05$; ** $p < 0.01$.

Figure 4. Co-culture with UV-MoPn-DC significantly reduces Th2 cytokine production by antigen-specific CD4⁺ T cells. Freshly purified splenic Sham-DC or UV-MoPn-DC (5×10^4 cells/well) from 35-day-old individual BALB/c mice (four mice/group) were co-cultured with naive CD4⁺ T cells (5×10^5 /well) isolated from DO11.10 OVA peptide-specific TCR- $\alpha\beta$ transgenic mice in 96-well culture plate at a 1:10 DC:T ratio with OVA (0.1 mg/mL) stimulation for 72 h. (A) The concentrations of IL-4, IL-5, IL-13 and IFN- γ in the culture supernatants were measured by ELISA. One representative experiment of three independent experiments is shown. Data are presented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$. (B) The concentrations of Th2 cytokines and IFN- γ in the supernatants of UV-MoPn-DC and CD4⁺ T cell co-culture with OVA stimulation in the presence or absence of anti-IL-12 mAb (5 μ g/mL) or anti-IL-10 mAb (5 μ g/mL) were measured by ELISA. One representative experiment of three independent experiments is shown. Data are presented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$, compared with the co-culture wells with the same OVA stimulation but without blocking antibodies (medium). The baseline cytokine production in the co-culture wells without OVA stimulation showed marginal levels, 16 pg/mL for IL-4, 0.2 ng/mL for IL-5, 0.1 ng/mL for IL-13 and 17 U/mL for IFN- γ . (C) Cytokine production by CD3⁺CD4⁺ T cells after 72 h co-culture with different DC was analyzed by intracellular cytokine staining. Left panel, T cells co-cultured with Sham-DC; right panel, T cells co-cultured with UV-MoPn-DC. One representative experiment out of three with a total number of nine mice examined per group is shown.

(Fig. 5D and E). The levels of allergic reaction were similar in Sham-DC recipients and the mice without cell transfer but having the same allergen treatment (data not shown). The data suggest that UV-MoPn-DC, but not Sham-DC, are capable of inhibiting allergen-induced Th2 responses *in vivo*. Collectively, the results indicate a significant modulating effect of early-life exposure to microbial components on DC function in adult life.

Depletion of T cells in UV-MoPn-treated mice reversed the alteration of DC

Since DC undergo a regular turnover, the DC that have original contact with the microbial products are unlikely to be present in

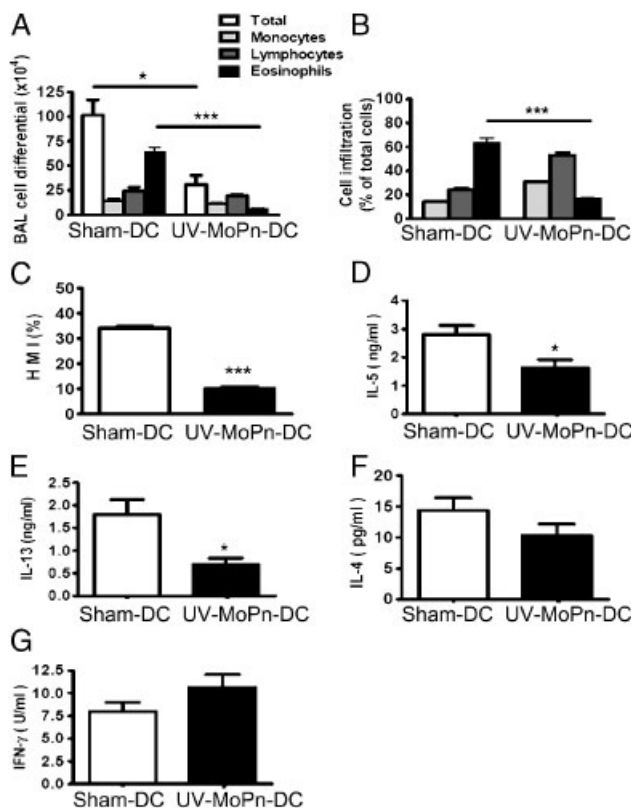


Figure 5. Recipients of adoptively transferred UV-MoPn-DC show significantly reduced airway allergic reaction. Naive 6–8-wk-old C57BL/6 mice (four mice per group) received i.v. adoptive transfer of Sham-DC or UV-MoPn-DC (1×10^6 cells/mouse) isolated from 35-day-old mice. At 1–2 h after cell transfer, the recipients were sensitized and then challenged with OVA using the standard protocol described in the legend to Fig. 1. Mice were killed on day 7 following OVA challenge. The lungs were washed with PBS and the cells in BAL fluid were stained using a Fisher Leukostat Stain kit. The absolute number (A) of total and each type of infiltrating cells and the proportion (B) of each cell types in the BAL fluid were calculated. Airway mucus-producing epithelium was stained by PAS and was quantified as HMI (C). Spleen cells were cultured in complete medium at 7.5×10^6 cells/mL in 48-well plates for 72 h with the stimulation of OVA, and the concentrations of cytokines (D–G) were measured by ELISA. Data are presented as mean $\pm$ SD. One representative experiment out of three with a total number of 12 mice examined per group is shown. * $p < 0.05$; *** $p < 0.001$, comparison between mice receiving Sham-DC and UV-MoPn-DC.

adult life. Why, then, do the UV-MoPn-DC in the adult mice show altered phenotype and function and have increased ability to reduce Th2 responses? We hypothesized that T cells in the MoPn-treated mice play a role in altering/maintaining DC function. To test this hypothesis, we depleted CD4 and CD8 T cells from the mice treated neonatally with UV-MoPn using mAb specific for CD4 and CD8 2 wk before the mice were killed at day 35. Flow cytometry analysis showed that this antibody treatment resulted in >95% depletion of CD4 and CD8 T cells without influencing the DC population (data not shown). As shown in Fig. 6, the depletion of T cells virtually abolished the modulating effect of neonatal microbial exposure on adult DC. Specifically, the UV-MoPn-DC from T-cell-depleted mice (UV-MoPn/T(–)-DC) showed a dramatic reduction in IL-10 and IL-12 production compared with UV-MoPn-DC from mice without T-cell depletion (Fig. 6A). Similarly, the alterations of surface markers in UV-MoPn-DC were reversed by *in vivo* T-cell depletion (data not shown). More importantly, UV-MoPn/T(–)-DC, unlike UV-MoPn-DC, failed to inhibit antigen-specific Th2 cytokine responses in the DC:T co-culture system (Fig. 6B). The results suggest that T cell plays a critical role in neonatal microbial product exposure-mediated alteration of adult DC function.

Discussion

In this study, we evaluated the educating effects of neonatal exposure to bacterial components on host immune responses to allergen in adulthood and the cellular and molecular mechanisms underlying this modulating effect. There are three major findings from this experimental study: (i) neonatal exposure to microbial components is inhibitory for the development of allergic reactions in adulthood; (ii) neonatal exposure to microbial components leads to altered phenotype and function of DC in adult life; and (iii) T cells are involved in imprinting of the DC function. To our knowledge this is the first report showing that neonatal exposure to bacterial components has a modulating effect on DC phenotype and function in adult life. Our data shed light on the educating effect of early-life environment factors on the balanced development of the immune regulatory function and emphasize the central role of DC imprinting and DC:T interaction for explaining “hygiene hypothesis”.

The inhibitory effect of early exposure to microbial components on the development of airway allergic reactions appears to be mediated by at least two mechanisms: reducing allergen-driven Th2 cytokine production (Fig. 2) and decreasing adhesion molecule, VCAM-1, expression (Fig. 1Dd). Since both Th2 cytokine production and VCAM-1 expression play important roles in allergic airway eosinophilic inflammation, these decreases may naturally lead to the inhibition of asthma. Notably, allergen-driven IFN- γ expression by T cells was not significantly different between UV-MoPn exposed and sham-exposed mice. Therefore, the inhibitory effect of early exposure to microbial components on allergic reactions was likely mainly through

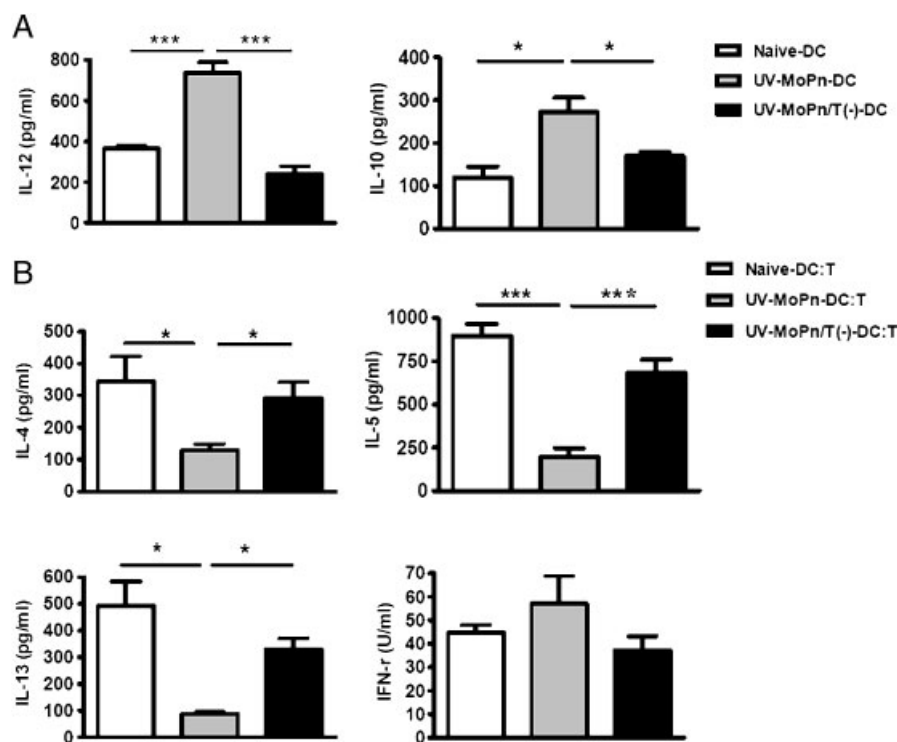


Figure 6. Depletion of T cells *in vivo* reversed the alteration of UV-MoPn-DC in cytokine production and T-cell-priming function. Newborn mice (four mice/group) were immunized i.p. with SPG buffer only or UV-killed *Chlamydia* (UV-MoPn, 2×10^6 original IFU in 20 μ L SPG buffer) at 6 days after birth. At day 21, one group of UV-MoPn-treated mice were injected (i.p.) with 0.5 mg purified anti-CD4 (GK1.5) and anti-CD8 (2.43) mAb to deplete T cells. At day 35, mice were killed and DC were purified. The DC from the T-cell-depleted group were designated as UV-MoPn/T(-)-DC. T-cell depletion *in vivo* was confirmed by flow cytometry. (A) Cytokine production by DC. Freshly isolated DC were cultured at 5×10^5 cells/well in complete culture medium for 72 h and cytokine levels in the culture supernatants were measured by ELISA. Data are shown as mean $\pm$ SD. * $p < 0.05$; *** $p < 0.001$. (B) Levels of cytokines in the 72 h culture supernatants of DC:T co-culture. Fresh CD4⁺ T cells (5×10^5 /well) from naive DO11.10 OVA peptide-specific TCR- $\alpha\beta$ transgenic mice were co-cultured with naive-DC, UV-MoPn-DC or UV-MoPn/T(-)-DC (5×10^4 cells/well) in the presence of OVA (0.1 mg/mL) stimulation for 72 h. The concentrations of IL-4, IL-5, IL-13 and IFN- γ in the culture supernatants were measured by ELISA. * $p < 0.05$; *** $p < 0.001$.

reducing allergen-specific Th2 responses rather than through immune deviation, *i.e.* enhancing Th1 response (IFN- γ production), which consequently inhibits Th2 responses. This result appears to differ from our previous finding in adult mice, in which mice exposed to MoPn showed higher IFN- γ and lower Th2 cytokine production [16, 17]. However, the previous studies using adult mice were performed with live MoPn, and the discrepancy in results more likely reflects the variable effect of timing in exposure to microbes. Since the imbalanced immune responses to normally non-harmful antigens characterized by dominant Th2 cytokine responses are the major mechanism for the development of allergic reactions, the reduced allergen-driven Th2 responses in the adult mice that were exposed to microbes in early life are a strong indication of a more balanced immune regulatory function.

The most important finding in the present study is the central role of DC in the linkage between early-life exposure to microbes and the balanced development of immune regulatory function, particularly in the prevention of allergic responses. Although numerous reported epidemiological and experimental studies have demonstrated the inhibitory role of live or killed microbes or microbial components on allergic responses [18–25], the basis for

this inhibition remains unclear. In particular, little experimental study has been performed on the effect and mechanism of neonatal microbial exposure on airway allergic inflammation. To examine the influence of early MoPn exposure on DC development in adulthood, we examined the phenotype and function of the DC from adult mice with or without neonatal exposure to killed MoPn. We found that UV-MoPn-DC showed higher co-stimulatory marker (CD86 and CD80) expression than Sham-DC. We also found that UV-MoPn-DC, compared with Sham-DC, induced significantly lower Th2-cell development in DC:CD4⁺ T co-culture experiments (Figs. 4 and 5). More importantly, recipients of adoptively transferred UV-MoPn-DC showed significantly lower allergen-induced airway inflammation and mucus production than Sham-DC recipients *in vivo* (Fig. 6). The results indicate that modulating DC function is a key mechanism by which early-life exposure to bacterial components prevents adulthood allergic responses. In addition, the changes observed in the splenic DC of the adult mice that were i.p. exposed neonatally to MoPn components and the significantly inhibitory effect of i.v. adoptively transferred splenic UM-MoPn-DC on airway allergic inflammation suggest that i.p. exposure of MoPn components may change circulating DC. These can migrate to the

lung for handling allergens when the airway is exposed to such allergens. Indeed, it has been reported that in the presence of inflammatory signals in the lung, circulating DC can be recruited to the inflammation sites due to changes in local adhesion molecules and chemokines [26]. Therefore, the modulating effect of bacterial infection/products on allergic asthma does not necessarily only occur when the infection/bacterial exposure is restricted to the lung. Indeed, we have found that s.c. injection of killed MoPn in neonates also inhibited airway allergic inflammation in adult mice (data not shown). These results therefore may have implications for designing prophylactic/therapeutic approaches against allergy and asthma. Since DC have a rapid turnover *in vivo*, the observed DC from the mice killed at day 35 are unlikely to be the original DC influenced by the microbial products in neonates; the data shown in Fig. 6 suggest that the continuous interaction between DC and T cells *in vivo* is critical for the relatively long-term effect of microbial exposure on DC phenotype and function. Further study on the T cells that influence DC *in vivo* would be important for understanding the whole picture of cell interaction in immune system development and function.

An interesting finding is the higher production of both IL-10 and IL-12 by UV-MoPn-DC than by Sham-DC. Notably, the increases in the production of IL-12 and IL-10 by DC are considered to be related to the two separate mechanisms, respectively, in explaining hygiene hypothesis, *i.e.* immune deviation and immunosuppression, because extensive evidence has shown that IL-12-producing DC are most efficient in inducing Th1-cell responses [27], while IL-10-secreting DC have an immune regulatory capacity to protect against allergic responses [28–30]. Clinical studies have shown that DC from asthmatic patients have an impaired ability to secrete IL-10 [29, 30]. Whether the decrease in atopic allergy in Western countries is mainly because of lack of immune deviation (decreased Th1 response to allergen) or a general lack of immunosuppression (decreased Treg function) is still a matter of controversy [31–38]. On one hand, some of our *in vitro* data might be used to argue that both IL-12 and IL-10 contributed to the inhibition of allergy, thus pointing to a co-operation of these two mechanisms. Indeed, neutralization of either IL-10 or IL-12 activity increased Th2 cytokine production by allergen-specific CD4⁺ T cells in the DC:T co-culture system (Fig. 4B). On the other hand, most of the *in vitro* data and all the *in vivo* data appear more supportive for suppressed Th2 responses rather than an increased Th1 response. Indeed, in the *in vitro* co-culture experiment, co-culture with UV-MoPn-DC led to less Th2 cytokine production rather than higher Th1 (IFN- γ) production in comparison with Sham-DC (Figs. 4A and 5). Moreover, neonatal UV-MoPn-treated mice showed reduced allergen-driven Th2 cytokine production but comparable IFN- γ production compared with control mice (Fig. 2). Similarly, adoptive transfer of UV-MoPn-DC significantly inhibited Th2 cytokine production without significant influence on Th1 cytokine production (Fig. 6). Collectively, the data suggest that the major reason for the inhibitory effect of neonatal exposure of UV-MoPn on allergic reactions in adult life is not by shifting the polarization of allergen-specific T cells from Th2-like

to Th1-like, but more likely reflects an inhibited polarization and development of allergen-specific Th2-like T cells, possibly mediated by IL-10-producing tolerogenic DC and/or Treg. The closer association of the inhibitory effect on allergy with Th2 suppression than with Th1 increase by UV-MoPn-DC may be also explained by the fact that, although both IL-10 and IL-12 production were statistically significantly higher in these DC than in Sham-DC, the magnitude of differences in IL-10 (about threefold) was more dramatic than IL-12 (about 1.5-fold). It should be pointed out, however, that it is also possible that the IL-12- and IL-10-producing DC may differ in their capacity to be recruited to the lung when allergens are locally exposed. For example, it is possible that the circulating IL-10-producing DC (released from spleen) are more easily recruited to the lung than IL-12-producing DC for unknown reasons; thus, the former showed a dominant *in vivo* effect on pulmonary inflammation (inhibiting Th2).

In addition to cytokine production, UV-MoPn-DC exhibited lower level of TLR9 mRNA than Sham-DC. This was in contrast to our finding in experiments using adult mice [16], which showed an increased TLR9 mRNA expression in the DC from MoPn-exposed mice. Similarly, experiments performed using bone-marrow-derived DC showed that, unlike live MoPn, killed MoPn failed to increase TLR9 mRNA (data not shown). The reason for this discrepancy remains unclear. Since in both of these studies DC from bacteria-exposed mice showed inhibitory effect on allergic responses, it can be argued that the changes in TLR9 is only a confounding phenomenon. However, it is also possible that the alteration in TLR9 gene expression is involved in DC function through different pathways in these two experimental systems, leading to a common outcome of inhibited allergic responses. Indeed, many studies have shown that TLR9 activation can enhance Th1 responses, thus inhibiting Th2 responses [39–41]. On the other hand, a recent study has shown that TLR9 activation by non-CpG oligodeoxynucleotides is important for the development of Th2 responses [42]. Our data here appeared in line with the latter scenario. It should be noted, however, that whether the variable TLR expressions were causally related to DC function in modulating allergic responses was not directly examined in either of our studies. It is also worth mentioning that the alterations in mRNA levels do not always reflect the levels of protein expression. Further study on these aspects is required to determine the role of TLR9 in infection-mediated inhibition of allergy.

In summary, our data provide an explanation for the cellular and molecular basis of the educating effect of early-life microbial exposure on the development of adult immune regulation and, in particular, in inhibiting allergy. However, many questions need to be addressed in future studies. For example, although there is strong evidence supporting the inhibitory effect of early-life exposure to microbes on allergy, many children who suffered from many different infections are still susceptible to allergy and asthma. This may be related to the genetic background of the children, the type of infection and the combination of different types of infection. Notably, a recent study showed that a combination of three infections led to a greater significant inhibitory

effect on allergy than one infection [43]. Future study using genetically different inbred mouse strains and the combinations of different infections will be helpful in addressing this question. Moreover, although certain early-life microbial exposures appear beneficial to host in preventing allergy, its effect on other diseases is not necessarily always beneficial. For example, although airway mucus over-production is detrimental to asthmatics (leading cause of death), mucus production is a part of the host's natural defense mechanism against helminth infections, most of which traverse through the lungs. Therefore, the implications of the early-life bacterial exposure on adult worm infections need to be studied. Furthermore, our data showed an increase of CD8 α^+ DC in the mice neonatally treated with UV-MoPn. It is unclear whether the change in the DC subset is related to the change in DC function. It has been reported that CD8 α^+ and CD8 α^- DC subsets function differently in several model systems [10]. We also reported that CD8 α^+ and CD8 α^- DC subsets differed in generating protective immunity against chlamydial challenge infection [44]. Therefore, it is possible that the increased CD8 α^+ DC population contributes more to the inhibitory effect on allergy than CD8 α^- DC. In addition, although we have shown a significant effect of neonatal microbial exposure on DC function in adult mice, the length of time from the microbial exposure to analysis was still relatively short. Further studies on these issues, including the role of DC subsets, the longer-term effect of neonatal microbial exposure on allergy and the effect of neonatal bacterial exposure on the development of other diseases, are required.

Materials and methods

Mice

Female BALB/c and C57BL/6 mice were bred at the University of Manitoba breeding facility and housed under specific pathogen-free conditions. Breeding pairs of I-A^d-restricted DO11.10 TCR- $\alpha\beta$ -transgenic mice (TCR recognizes OVA323–339 peptide), purchased from the Jackson Laboratory (Bar Harbor, ME), were bred and maintained at the breeding facility of University of Manitoba. All experiments were performed in accordance with the guidelines issued by the Canadian Council on Animal Care. The animal experimental protocol was approved by the ethical committee of The University of Manitoba.

Organism

C. muridarum (MoPn) were grown in HeLa 229 cells and purified by discontinuous density gradient centrifugation as described previously [45]. The partially purified organisms were resuspended in SPG buffer, and frozen at -80°C until used. The original infectivity of the stock MoPn, as measured by inclusion-forming units (IFU), was determined by infection of HeLa 229 cells and enumeration of inclusions that were incubated with a

Chlamydia genus-specific murine mAb and stained with goat anti-mouse IgG conjugated to HRP and developed with substrate (4-chloro-1-naphthol; Sigma-Aldrich). The same seed stock of MoPn was used throughout the study. Because the use of live MoPn is life threatening to the newborn mice, we used UV-MoPn to treat newborn mice to deliver sufficient amount of microbial components. For UV killing, live MoPn was exposed to UV light at a distance of 5 cm for 2 h at room temperature, which led to complete killing of MoPn confirmed by viability testing.

Treatments of mice

Newborn C57BL/6 and BALB/c mice were injected i.p. with UV-MoPn (2×10^6 original IFU) in a volume of 20 μL SPG at day 5–6 after birth. Control newborn mice received an i.p. injection of 20 μL SPG only. On day 35, all the mice were sensitized with 2 μg OVA (ICN Biomedicals, Montreal, Canada) in 2 mg AL(OH)₃ (alum) by i.p. injection. At 2 wk after sensitization, mice were challenged i.n. with OVA (50 μg in 40 μL saline). All mice were killed for detection of pulmonary allergic inflammation and immune reactions 7 days after challenge as described in [14].

BAL

BAL was performed as described previously [14]. Briefly, at 5–7 days following OVA challenge, mice were killed, the mouse trachea was cannulated and lungs were washed two times by injection and aspiration of 1 mL sterile PBS. The total cell numbers in the BAL fluids were determined using a hemocytometer. The fluids were then centrifuged and cell pellets were resuspended in 100 μL PBS (pH 7.4) to prepare BAL smears [14]. The slides were air-dried, fixed with ethanol and then stained with Fisher Leukostat Stain Kit (Fisher Scientific, Ont., Canada). The numbers of monocytes, lymphocytes and eosinophils were determined based on their morphology and staining characteristics by two independent observers.

Histological analysis

Lung tissues were routinely fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Slides were examined for pathological changes by light microscopy [15]. Bronchial mucus and mucus-containing goblet cells within airway bronchial epithelium were stained by a periodic acid Schiff (PAS) staining kit (Sigma) as described [15]. The mucus-producing epithelium (Goblet cells) was quantified by histological mucus index, which represents the percentage of the area of mucus-positive epithelium in the total area of airway epithelium, using Image-Pro Plus software (Media Cybernetics) [15]. For VCAM-1 expression, slides of frozen lung tissues were incubated with a rat anti-mouse VCAM-1 antibody followed by a secondary rabbit anti-rat antibody conjugated to HRP, and

developed with 3-amino-9-ethylcarbazol chromogen as previously described [14].

Spleen cell culture

Mice were killed 7 days after OVA challenge and spleens were aseptically removed. To examine cytokine production, single-cell suspensions were prepared from spleens and cultured at 7.5×10^6 cells/mL (1 mL/well) with or without OVA stimulation (1 mg/mL) at 37°C in complete culture medium: RPMI 1640 containing 10% heat-inactivated FBS, 25 µg/mL gentamicin, 2 mM L-glutamine and 5×10^{-5} 2-ME (Kodak) for 72 h in a 5% CO₂ atmosphere. Culture supernatants were harvested for the measurement of cytokine levels by sandwich ELISA as described [14].

DC isolation and culture

Mice treated neonatally with UV-MoPn or SPG were killed at 35 days of age (adult). Spleens were aseptically collected and DC were isolated using magnetic CD11c columns from MACS (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions as described previously [16, 17]. The purity of the CD11c⁺ DC preparations was >95% based on flow cytometry. To test the spontaneous cytokine production by the isolated DC, the cells were incubated at 5×10^5 cells/well in 200 µL complete culture medium in 96-well culture plates for 72 h. Levels of IL-12 and IL-10 in the culture supernatants were measured by ELISA as described [16, 17].

RT-PCR analysis

To analyze the expression of TLR gene expression in isolated DC, RT-PCR was performed as described [16]. Briefly, total cellular RNA was extracted from DC using the phenol-guanidinium method (TRIzol reagents, Invitrogen, Burlington, Ont., Canada), followed by ethanol precipitation. The first-strand cDNA was synthesized from 1.2 µg RNA in a final volume of 15 µL using M-MLV reverse transcriptase (Invitrogen) and oligo(dT) primer; 1 µL cDNA was used for each PCR in a total volume of 20 µL. The reaction condition for PCR was as follows: 1 cycle at 95°C for 5 min; 32 cycles (for TLR2) or 35 cycles (for TLR4 and TLR9) at 95°C for 1 min, at 55°C for 1 min, and 72°C for 1 min. β-Actin was used as a loading control. PCR products were run on a 1% agarose gel containing 0.1 mg/mL ethidium bromide. Image analysis was performed using Gel Doc 2000 gel documentation system (Bio-Rad, Hercules, CA, USA) and quantified using Scion Image software (Scion, Frederick, MD, USA). The specific primers for TLR2, TLR4, TLR9 and β-actin were as follows: TLR2, 5'-GCTCCAGGTCTTTCACCTCTATTC-3' (sense) and 5'-TCCAGCAGGAAAGCAGACTCGCTTA-3' (anti-sense); TLR4, 5'-GGAAGCTTGAATCCCTGCATAGAG-3' (sense) and 5'-TCCACATGTAC-

TAGGTTCGTCAG-3' (anti-sense); TLR9, 5'-AACCTGCGGCAGCTGAACCTCAA-3' (sense) and 5'-GAGTTCAGTGTATGGAGAGAGCTG-3' (anti-sense); β-actin, 5'-GTGGGCCGCCCTAGGCACCA-3' (sense) and 5'-CTCTTTGATGTCACGCACGATTTC-3' (anti-sense).

Isolation of CD4⁺ T-cell and DC—T-cell co-culture

Naive CD4⁺ T cells were isolated from the spleens of DO11.10 OVA peptide-specific TCR-αβ transgenic mice (BALB/c background) using an MACS-positive selection column (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the CD4⁺ cells was more than 95%. The isolated naive CD4⁺ T cells (5×10^5 cells/well) were co-cultured with DC isolated from UV-MoPn- or SPG-treated BALB/c mice (5×10^4 cells/well) in the presence of OVA (0.1 mg/mL) in complete culture medium at 37°C with 5% CO₂ as described [16]. In designated experiments, anti-IL-12 or anti-IL-10 mAb (PharMingen, San Diego, CA, USA) was added at 5 µg/mL to the co-culture wells to block endogenous IL-12 or IL-10 activity. The culture supernatants were harvested at 72 h for cytokine analysis by ELISA.

Adoptive transfer of DC

DC were isolated from 35-day-old mice that were treated neonatally with UV-MoPn or SPG buffer. The DC (1×10^6 cell/mouse) in 200 µL sterile protein-free PBS were i.v. transferred to syngeneic naive mice. At 1–2 h after transfer of DC, the recipient mice were sensitized by OVA (2 µg in alum). At 14 days after OVA sensitization, mice were i.n. challenged with OVA (50 µg in PBS) and killed 7 days after challenge to examine pulmonary allergic inflammation.

Flow cytometry

Anti-CD11c-FITC, anti-MHCII-PE, anti-CD80-PE, anti-CD86-PE and anti-CD8α-PE antibodies and corresponding isotype controls were purchased from e-Bioscience (San Diego, CA, USA). To evaluate the expression of surface markers on DC, the freshly isolated DC were stained using these labeled antibodies as previously described [17]. Analyses were performed using an FACSCalibur flow cytometer and CellQuest program (BD Biosciences).

Intracellular cytokine staining

Freshly isolated splenocytes and cells collected from DC:T co-culture were analyzed for intracellular cytokines as described [45]. For splenocytes, erythrocytes were first depleted, and for cells from co-culture, cells were collected at 72 h of culture. All the cells were washed twice and then

stimulated with PMA (50 ng/mL, Sigma) and ionomycin (1 µg/mL, Sigma) and incubated for 6 h in complete RPMI 1640 medium at 37°C. For the last 4 h of incubation, Brefeldin A (Sigma) was added to accumulate cytokines intracellularly. After stimulation, cells (2×10^6) were washed twice and incubated with FcR block antibodies (anti-CD16/CD32; e-Biosciences) for 15 min at 4°C to block non-specific staining. Cell surface markers (CD3 and CD4) were first stained. The cells were then fixed and permeabilized with Cytofix/cytoperm (BD PharMingen) and stained intracellularly with anti-IFN-γ-allophycocyanin (XMG 1.2), and anti-IL-4-FITC (BVD6-24G2) mAb (e-Biosciences) or with corresponding isotope control Ab in permeabilization buffer (BD PharMingen). Finally, the cells were washed, resuspended in PBS containing 2% FCS and 2 mM EDTA and analyzed by flow cytometry for intracellular cytokines by gating on CD3⁺ CD4⁺ T cells.

Statistical analysis

Results are expressed as mean ± SEM. The unpaired Student's *t*-test was used for comparison of the data between two experiment groups. The ANOVA Newman–Keuls multiple comparison test was used to determine statistical significance among multiple groups (see Figs. 4B and 6). A *p* value of <0.05 was considered significant.

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Abbreviations: BAL: bronchoalveolar lavage · IFU: inclusion-forming units · MoPn: *Chlamydia muridarum*, originally called *Chlamydia trachomatis* mouse pneumonitis · PAS: periodic acid Schiff · SPG: sucrose-phosphate-glutamic acid buffer · UV-MoPn-DC: DC from adult mice treated neonatally with UV-killed MoPn · Sham-DC: DC from adult mice neonatally treated with sucrose-phosphate-glutamic acid · UV-MoPn: UV-killed MoPn · UV-MoPn/T(–)-DC: UV-MoPn-DC from T-cell-depleted mice

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