Exacerbation or resolution of asthmatic reaction by microbial environmental agents: involvement of innate immune cells

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Objectives

Airway inflammation and hyperresponsiveness, increased Th2 cytokine production in bronchoalveolar lavage and enhanced IgE secretion in serum are the hallmarks of allergic asthma [1].

The improvement of the hygienic conditions, which leads to a decrease of the microbial infections in particular during the early childhood, is considered as a triggering factor for the allergic diseases by decreasing the tolerogenic response [2]. On the contrary, some respiratory infections, as those caused by Influenza virus, favor the development of the asthmatic disease [3]. Pathogen-associated molecular patterns on microbes are sensed by the innate immune system through a variety of specific receptors. Among them, Toll-like receptors (TLRs), which are expressed in a variety of both structural and immune cells [4, 5], play an important role.

Our objective was to better understand mechanisms involved during an inhibition or an exacerbation of the allergic asthma by TLR agonists (FSL-1 as TLR2 agonist and poly(I:C) as TLR3 agonist, respectively), mimicking some microbial components. More particularly, the importance of innate immune cells was defined by using mice sensitized to ovalbumine (OVA).

Materials and Methods

Sensitization, airway challenges and treatment

All procedures were approved by regional ethical committee. 7-10 weeks old female C57BL/6 mice were sensitized by intraperitoneal injection of 20 µg OVA emulsified in 100 µl of aluminium hydroxide on days 0 and 7 (OVA mice). For inhibition protocol, mice were anesthetized and challenged by nasal instillation of 100 µg OVA in 40µl Phosphate Buffer Saline (PBS), on days 14, 15, 16, 28, 29 and 30 (figure 1A). Control group (afterwards called PBS mice) received intraperitoneal injection of PBS emulsified in 100 µl of aluminium hydroxide and was challenged by nasal instillation of PBS. TLR2/TLR1 or TLR2/TLR6 agonist treatment (synthetic tripalmitoylated lipopeptide Pam3CSK4 and Synthetic diacylated lipoprotein FSL-1, respectively) was administrated on days 21, 22 and 23 by nasal instillation (0.5µg/40µl) on anesthetized mice. For exacerbation protocol, endotoxin-free poly(I:C) (50 µg of double-stranded RNA, dsRNA) was administered intranasally with OVA challenges on D14-15-16 (figure 1B). dsRNA preparations were devoid of endotoxin as determined by the Limulus amebocyte test and did not activate a cell line transfected with TLR4 (data not shown).

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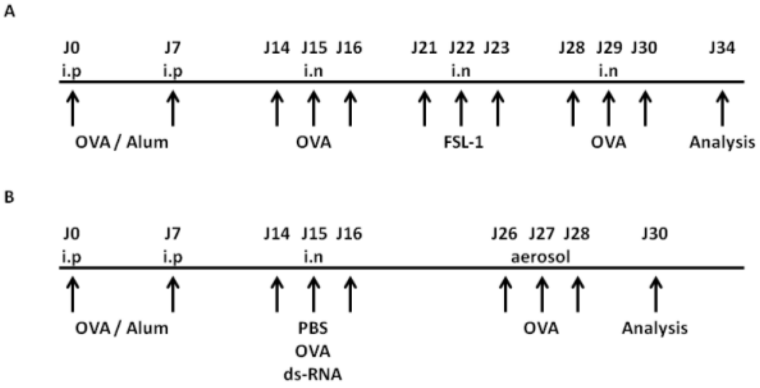

Figure 1: Protocols for inhibition (A) or exacerbation (B) of the allergic reaction induced by FSL-1 or dsRNA administration in C57BL/6 mice, respectively.

Measurement of Airway Hyperresponsiveness

Responsiveness to β-metacholine was assessed either in conscious mice using the measurement of enhanced pause (PenH) by chamber whole-body plethysmography, or in anesthetized and ventilated mice using lung resistance measurement by Flexivent® (Scireq®, Montreal, Canada). Briefly, mice were anesthetized (5 ml/kg body weight of 10% medetomidine and 10% ketamine), paralyzed (5 ml/kg pancuronium bromide 1%) and immediately intubated, followed by mechanical ventilation. Mice were exposed to nebulized PBS followed by increasing concentrations of nebulized metacholine (3-50mg/ml in PBS) using an ultrasonic nebulizer. Baseline lung resistance (R) was restored before administration of the subsequent doses of methacholine. Only resistance values corresponding to COD values > 0.95 were kept. For each dose, the maximum resistance value measured was taken and was expressed as the percentage of maximum resistance value measured after PBS exposure (% increase above PBS).

Generation of myeloid dendritic cells (mDC) and adoptive transfer of OVA-pulsed DC

In brief, bone marrow-derived DCs (BMDCs) were differentiated in the presence of GM-CSF (20 ng/ml). After 10 days, a pro-Th2 environment was constituted by addition of IL-4 (10 ng/ml) into some cultures. At day 13, both types of BMDCs were stimulated with OVA (100 ng/ml) alone or in association with dsRNA (10 µg/ml). Control cells were incubated in medium alone. After 24 h of incubation, BMDC phenotype and cytokine production were analyzed by flow cytometry. To address the role of DC in asthma exacerbation, C57BL/6 mice were sensitized as described above. At day 14, 10^6 BMDC were injected intratracheally and then challenged with OVA aerosol (D26–28) before evaluation of the allergic reaction.

Assessment of allergic airway inflammation in BAL, lung tissue, and draining lymph nodes

Lungs were lavaged via the tracheal cannula with 1ml of PBS immediately after the measurement of lung resistance (i.e. 96 or 48 hours after the last OVA challenge for the inhibition or exacerbation protocols, respectively). Total leucocyte numbers were counted, cytocentrifuged and stained with May Grünwald Giemsa. Cells were identified as macrophages, eosinophils, neutrophils and lymphocytes by standard hematological procedures and at least 200 cells were counted under x400 magnification.

Lung homogenates were prepared after dispersion of the middle right lobe in 1 ml lysis buffer for cytokine measurements by ELISA. After dissociation of the left lobe, lung cells were counted, and the cell phenotype was analyzed by flow cytometry. For lung histology and immunohistochemistry, caudal right lobe of the lung from each mouse was fixed in Immunohistofix and embedded in resin using the Immunohistowax processing method. Inflammatory infiltrate was analyzed on lung sections stained with Hematoxylin and eosin. CCL11, CCL17, and CCL20, as well as MHC class II (MHC II)-positive cells, were also studied by immunohistochemistry on lung sections.

Mediastinal lymph node cells ($3x10^5$/well) were plated and restimulated with 20 µg/ml OVA and 5 µg/ml fixed anti-mouse CD3 mAb.

Measurement of OVA-specific IgE and IgG1 in serum

Serum levels of OVA-specific IgG1 and IgE were measured by ELISA. Briefly, 96-well plates were coated with either purified anti-IgE (2µg/ml, clone R35-72) for the specific IgE quantification or OVA for the specific IgG1 quantification. After overnight incubation (+4°C) with serum dilutions, binding of specific antibodies was detected by addition of biotinylated anti-mouse IgG1 or home-made biotinylated-ovalbumin for specific IgE quantification. Binding of biotinylated proteins was revealed by addition of avidin-horseradish peroxidase and TMB substrate solution. The OVA-specific antibody titers of the samples were related to pooled standards generated in the laboratory. Results are expressed as the inverse of the dilution corresponding to 50% of the maximal OD.

Murine airway epithelial cells (AEC)

After sacrifice of WT mice and TLR3$^{-/-}$ mice, AEC were prepared by enzymatic digestion within the airways. After cell culture, >95% of AEC stained for cytokeratin and were negative for vimentin (data not shown). After cell starvation, AEC were activated by dsRNA (10 mg/ml) for 24 h before cell supernatant collection.

Chemotaxis assays

Chemotaxis assays were performed using 24-well Transwells (5 µm-pore polycarbonate filter). After 2 h at 37°C, the number of migrated BMDC (CD11c^{+}MHC II^{+}) was numbered and identified by flow cytometry. The implication of CXCL10, CCL5, CCL11, and CCL20 in the chemotactic activity of AEC supernatant was determined by using specific neutralizing Abs.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical significance of the differences between experimental groups was calculated by ANOVA with a Bonferroni, Mann-Whitney, Dunn's or unpaired Student t posttest (GraphPad Prism 4 Software). Results with a value of $p < 0.05$ were considered as significant.

Scientific Results

1. Pulmonary administration of TLR2/6 agonist reverses established experimental asthma [4]

OVA-sensitized mice receiving PBS nasal instillation treatment (OVA-PBS mice) exhibited significant increased BAL eosinophilia (figure 2) and airway resistance (figure 3) compared to control PBS-PBS mice. FSL-1 treatment once experimental asthma has established (OVA-FSL-1 mice) significantly decreased inflammation and more particularly eosinophil numbers in mice, as well as airway hyperresponsiveness (AHR). FSL-1 treatment did not modify airway resistance in PBS mice. In contrast, whilst OVA-sensitization significantly increased OVA-specific IgE, FSL-1 treatment did not modify OVA-specific serum IgE (data not shown).

FSL-1 effect was checked to be TLR2 dependent. Indeed, FSL-1 treatment did not decrease airway resistance and bronchoalveolar eosinophilia in TLR2 deficient mice (data not shown).

In contrast, Pam3CSK4 treatment was found to inhibit airway inflammation but not AHR (data not shown), and was therefore excluded from further analysis.

In conclusion, the TLR2/TLR6 agonist FSL-1, administered in the lungs by nasal instillation after the experimental asthma has established, significantly decreased airway hyperresponsiveness and eosinophilia. The effect was local and not systemic as OVA-specific IgE was unchanged by FSL-1 treatment.

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[4] Barrier M, et al (in preparation)

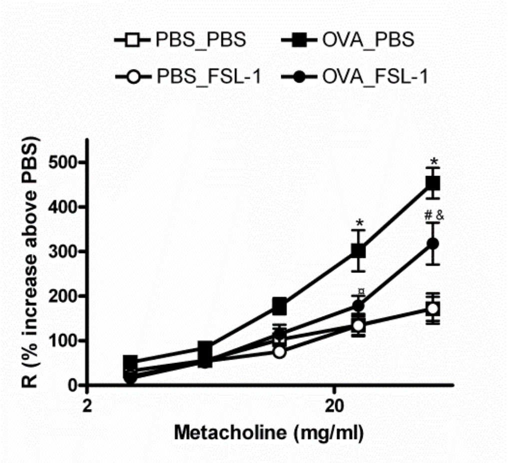

Figure 2: Administration of FSL-1 after OVA-sensitization inhibits airway hyperresponsiveness

Mice sensitized to OVA were treated with FSL-1 (OVA-FSL-1, n=8) or PBS as a control (OVA-PBS, n=10) administered locally to the lungs by nasal instillation. Control mice were sensitized with PBS and treated with FSL-1 (PBS-FSL-1, n=6) or PBS (PBS-PBS, n=10). All mice underwent a second set of OVA or PBS challenge before analysis. Lung resistance (R) was measured in response to increasing doses of metacholine. Results are expressed as mean ± sem of R percentage increase above PBS inhalation. *, p<0.001 for PBS-PBS compared to OVA-PBS. $^{\#}$, p<0.01 for PBS-FSL-1 compared to OVA-FSL-1. $^{\&}$, p<0.001 for OVA-PBS compared to OVA-FSL-1. $^{¤}$, p<0.01 for OVA-PBS compared to OVA-FSL-1.

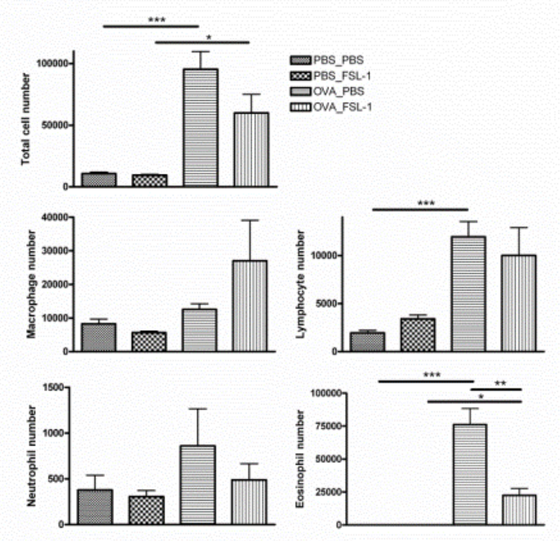

Figure 3 Administration of FSL-1 after OVA-sensitization inhibits BAL eosinophilia

Mice sensitized to OVA were treated with FSL-1 (OVA-FSL-1, n=8) or PBS as a control (OVA-PBS, n=9) administered locally to the lungs by nasal instillation. Control mice were sensitized with PBS and treated with FSL-1 (PBS-FSL-1, n=6) or PBS (PBS-PBS, n=9). All mice underwent a second set of OVA or PBS challenge before analysis. Total cell, macrophage, lymphocyte, neutrophil and eosinophil numbers in BAL were microscopically identified and counted after May Grünwald staining. ***: p<0.001, **: p<0.01, *: p<0.05.

Administration of TLR2 agonists in animal models was shown to have contradictory effects: it can shift Th2 towards Th1, aggravate Th2 or induce tolerance depending on agonist and its sequence of administration [6-9]. Concerning TLR2/6 stimulation, Fuchs et al performed treatment during the course of allergen sensitization. They showed that, in a model of chronic allergic airway inflammation induced by intranasal administration of Timothy grass pollen allergen extract, early TLR2/6 agonism (by bisacyloxypropylcysteine polyethylene glycol conjugate, a pegylated derivative of MALP-2) reduced airway eosinophilic inflammation and IL-5 and IL-10 production from lymph node. Neither neutrophil, nor lymphocyte numbers were modified. TLR2/6 agonist did not affect CD11c^{+} antigen presenting cells nor CD4^{+}foxp3^{+} regulatory T cell numbers in draining lymph nodes [6].

Our study showed that local TLR2/6 agonist treatment once the asthma like reaction established is still able to inhibit airway responses in a different model. The mechanisms involved in FSL-1-induced inhibition of allergic airway responses are currently under investigation. Although the repartition of Natural Killer cell subsets (for phenotype, see [10]) in mediastinal lymph nodes is affected by FSL-1 treatment, we did not evidence other phenotypic differences. In contrast, percentage of CD25^{+} T cells was increased in the lungs of FSL-1-treated mice, suggesting that regulatory T cells may be involved.

2. Double-Stranded RNA Exacerbates Pulmonary Allergic Reaction through TLR3: Implication of Airway Epithelium and Dendritic Cells [11]

Administration of dsRNA in OVA-sensitized mice induces exacerbation of the pulmonary allergic reaction

Allergic asthma is a complex, multifactorial inflammatory disease that may be exacerbated by viral infection. To reproduce virus induced exacerbation of asthma, OVA-sensitized mice were challenged intranasally with OVA in the presence of dsRNA, a viral mimetic. We showed that dsRNA/OVA administration induced a significant increase of AHR, lung inflammation (increased BAL eosinophilia, peribronchial leucocyte infiltration, $CD4^+$ and $CD8^+$ cell infiltration and lung DC (MHC $II^+CD11c^+CD11b^{+/-}$) numbers) (figure 4). dsRNA treatment also heightened serum titers of OVA-specific IgG1 and IgE but not IgG2a (data not shown). Mice treated with dsRNA exhibited increased Th2-associated cytokine (IL-5, IL-13) and chemokine (CCL11, CCL17) concentrations in lung tissues, whereas no significant difference in Th1-associated cytokine (IFN-γ) and chemokine (CXCL10) was observed compared to mice sensitized to OVA alone (data not shown).

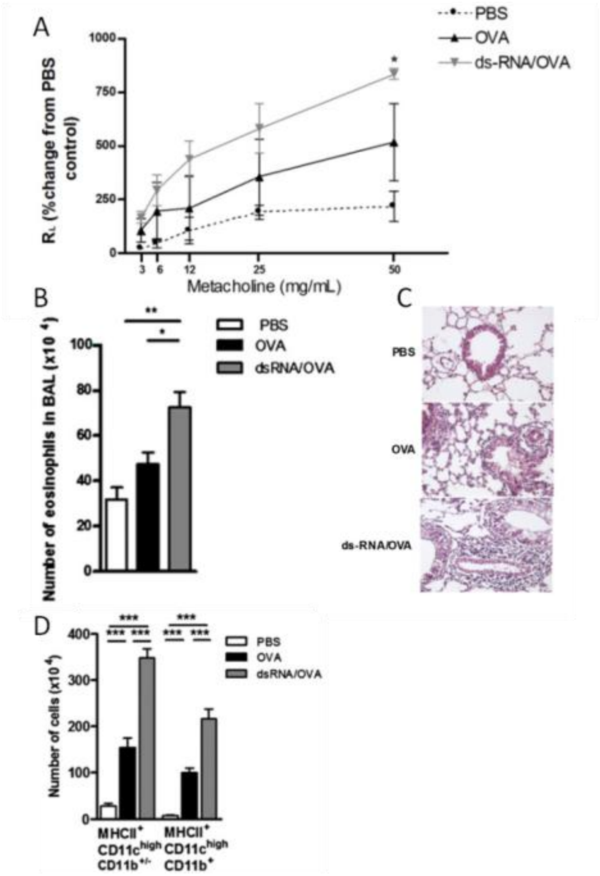

Figure 4: Local administration of dsRNA exacerbates the lung allergic reaction in WTC57/BL6 mice.

(A) OVA sensitized mice received dsRNA or PBS concomitantly with OVA challenge (ds-RNA/OVA and OVA mice, respectively). Lung resistance (RL) was measured after a second set of OVA challenges. Control mice received PBS only. * $p < 0.05$ versus mice treated with OVA. Experiments were performed two times with at least five mice per group. One representative experiment out of these replicates is shown.

Lung inflammation was analyzed measuring eosinophil numbers in BAL (B) and after Hematoxylin and eosin staining of lung sections (original magnification x300) (C). (D) Number of DC in lung tissue was determined by flow cytometry. DC were defined as non autofluorescent $MHCII^+CD11c^+CD11b^{+/-}$ and $MHCII^+CD11c^+CD11b^+$. Data are expressed from one representative experiment out of three as the mean ± SD (n = 5 mice). * $p <0.05$; ** $p < 0.01$; *** $p < 0.001$ versus mice treated either with OVA or PBS.

Activation of TLR3, through TRIF signaling, plays an essential role in the inflammatory response induced by dsRNA exposure [12]. To determine the role of this signaling pathway in our experimental model, OVA-sensitized WT mice and $TRIF^{-/-}$ mice were challenged with or without dsRNA. In contrast to WT animals, treatment with dsRNA did not enhance AHR, BAL eosinophilia nor peribronchial infiltrate in $TRIF^{-/-}$ mice (data not shown). These results indicate that TRIF signaling is essential in the dsRNA-induced exacerbation of the pulmonary allergic reaction.

Transfer of dsRNA-stimulated IL-4/BMDCs exacerbates the pulmonary allergic reaction

To study the involvement of mDC in dsRNA induced exacerbation of experimental asthma, in vivo transfer experiments were performed. mDC were differentiated in vitro from bone marrow progenitors in the absence (BMDC) or the presence (IL-4/BMDC) of IL-4. The transfer of OVA-pulsed BMDC did not modulate AHR, whereas mice receiving dsRNA-stimulated IL-4/BMDC or BMDC significantly increased AHR and promoted strong recruitment of eosinophils and mononuclear cells (including CD4+ and CD8+ T lymphocytes) in the lungs and in the BAL (figure 5A, B, C). Higher levels of IL-5, IL-13 but not IFN-γ (Figure 5D) were found in the lungs of mice that received dsRNA/OVA-pulsed IL-4 BMDCs, compared with the animals receiving OVA-pulsed IL-4 BMDCs. CCL11 and CCL17 were also increased (data not shown). Finally, exposure of IL-4 BMDCs with dsRNA also enhanced the production of OVA-specific IgG1 and IgE but did not affect IgG2a synthesis (data not shown).

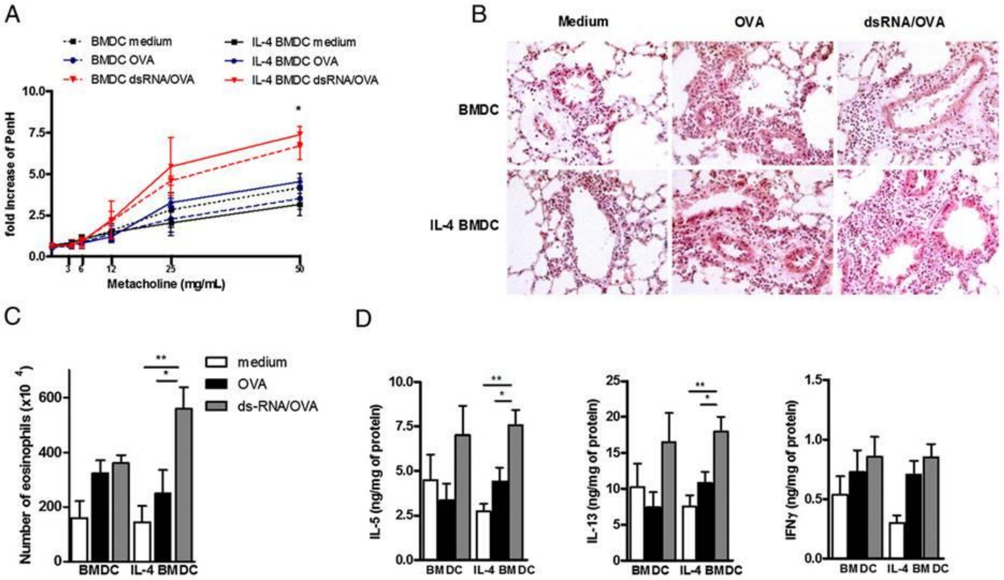

Figure 5: Intratracheal administration of BMDCs primed with dsRNA/OVA exacerbates the lung allergic reaction in sensitized mice. (A) Lung function determined by measurement of PenH of mice receiving intratracheal administration of BMDCs differentiated with GM-CSF (BMDCs) or GM-CSF plus IL-4 (IL-4 BMDCs) and activated with OVA, with dsRNA/OVA or unstimulated (medium) (*$p < 0.05$ versus mice treated with IL-4 BMDCs plus OVA); (B) H&E staining of lung sections (original magnification x300); (C) eosinophil numbers in BAL; (D) cytokine production as determined by ELISA in the protein lung extract. Data are expressed as the mean ± SD (n = 5 mice) and are from one representative experiment out of three. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus mice receiving either BMDCs or IL-4 BMDCs primed with medium or OVA.

dsRNA-activated Airway Epithelial cells (AEC) participate to mDC recruitment

Our data suggest that dsRNA exposure induced DC recruitment into the lungs through a TRIF-dependent mechanism. Because AEC are one of the first targets of dsRNA and are involved in DC migration [13, 14], we hypothesized that dsRNA-activated AEC might participate in mDC recruitment through chemokine synthesis. Immunohistochemistry showed increased percentage of CCL20-expressing AEC associated with an augmented number of peribronchial MHC II+ cells, after dsRNA treatment (figure 6A). In vitro, isolated TLR3-competent AEC activated with dsRNA significantly enhanced their secretion of CCL5, CCL20, CXCL10, CCL11, and CCL17 (figure 6B), in contrast to TLR3-deficient AEC. The supernatant from dsRNA-activated TLR3-competent AEC strongly increased the migration of BMDC in chemotaxis assays (figure 6C). CXCL10 and CCL20 were involved in this migration as shown by neutralizing antibodies (figure 6D).

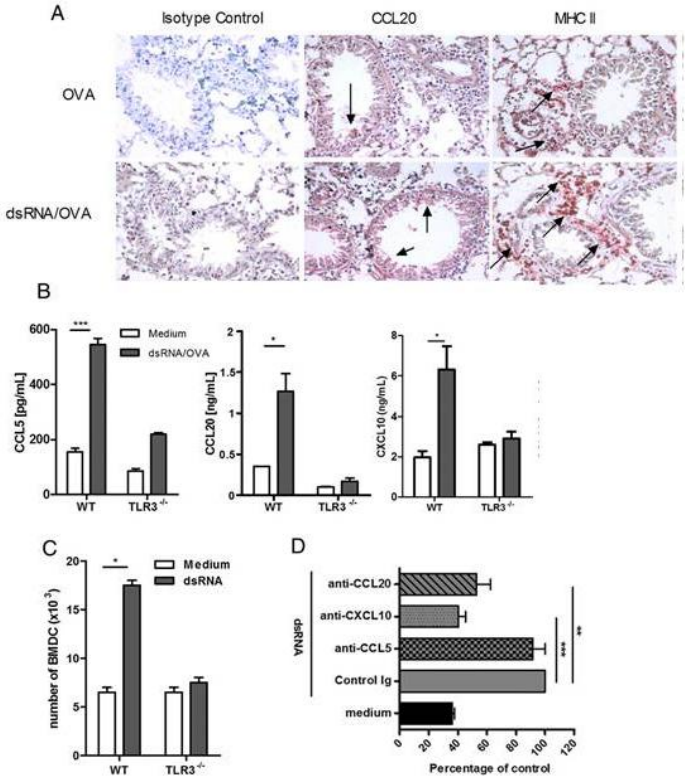

Figure 6: dsRNA-activated AEC participate to mDC recruitment through chemokine secretion. (A) Immunohistochemistry with mAb anti-CCL20 and anti-MHC II, or with a control isotype on lung sections from sensitized mice exposed to OVA and dsRNA/OVA (lungs are collected at day 17) (original magnification x300). Black arrows indicate positive AEC stained in red. (B) dsRNA-induced secretion of CCL5, CCL20 and CXCL10 by AEC is dependent on TLR3 expression. In vitro, AEC from WT and $TLR3^{-/-}$ mice are activated or not with dsRNA; (C) chemotactic activity for BMDC of supernatants from WT and $TLR3^{-/-}$ AEC stimulated with dsRNA; (D) effect of neutralizing Ab against CCL5, CCL20, and CXCL10 on the BMDC migration induced by dsRNA-stimulated AECs. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ versus the indicated condition.

Therefore, we showed in this study for the first time that dsRNA instillation in sensitized mice has a longterm effect by inducing exacerbation of the allergic reaction after a secondary allergen challenge. The route and the timing of dsRNA administration are critical for the development of the allergic reaction. In our experimental setting, dsRNA acts as a pro-Th2 adjuvant in sensitized animals and amplifies the allergic reaction through an enhanced Th2-mediated allergen-specific immune response. In vitro and in vivo dsRNA administrations were shown to reproduce most of the proinflammatory effect of pulmonary viruses [15], suggesting that our model may mimic the clinical situation associated with virus-induced asthma exacerbation. We showed that mDC and airway epithelial cells (AEC) cooperate to induce dsRNA effect.

Conclusion

Therefore, our study shows that microbial agents may inhibit or exacerbate experimental allergic asthma, depending on the innate receptor involved. Innate cells like dendritic cells and airway epithelial cells are involved in the exacerbation of the allergic asthma.

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