

Immunopharmacology and inflammation

Toll-like receptor (TLR) 7 decreases and TLR9 increases the airway responses in mice with established allergic inflammation



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ABSTRACT

Toll-like receptor (TLR) 7 and TLR9 recognise microbial products of viral descent. Since viruses are a common trigger of asthma exacerbations these TLRs have emerged as interesting therapeutic targets. Even though their effects on allergic inflammation have been evaluated in several models their effects on established allergic airway inflammation remains to be described. Therefore, mice with an on-going ovalbumin (OVA)-induced allergic airway inflammation were given R848 or CpG (TLR7 and TLR9 agonists, respectively) intranasally during four consecutive days. At day five, the R848 treatment had reduced OVA-induced airway hyperresponsiveness (measured as the increased resistance to methacholine), counteracted the accompanying influx of eosinophils and macrophages, and decreased the OVA-enhanced release of interleukin (IL)-5 and leukotriene (LT) B₄ in bronchoalveolar lavage fluid. CpG, which by itself caused airway hyperresponsiveness, did not influence the OVA-induced airway hyperresponsiveness, and release of IL-5 and LTB₄, but decreased the OVA-induced influx of cells in bronchoalveolar lavage fluid, and increased the amount of pro-inflammatory mediators like IL-12, CXCL1 and CXCL9. To conclude, TLR7 dampens the allergic airway reactivity and local inflammation, whereas TLR9 that causes airway hyperresponsiveness and increased cellular response per se, do generally not interfere with the effects induced by allergic inflammation.

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1. Introduction

Viral infections are a common cause for exacerbation in asthmatic patients (Contoli et al., 2005; Gern and Busse, 2002; Micillo et al., 2000). Like other microbes they are upon intrusion recognised by the pattern recognition receptors. Among these receptors Toll-like receptors (TLRs) (Holt and Sly, 2012; Kawai and Akira, 2006) play a key role for virus detection and four out of the ten TLRs found in humans are devoted to this task. TLR3 via viral dsRNA, TLR7 and TLR8 via viral ssRNA, and TLR9 via unmethylated DNA containing CpG motifs (CpG) (Akira and Hemmi, 2003; Fransson et al., 2007; Iwasaki and Medzhitov, 2004). In humans TLR7 and TLR8 seems to be closely related but because TLR8 lacks a functional correlate in mice it will not be object for further analysis in the present paper. (Jurk et al., 2002). TLR7 is also structurally related to TLR9. They are both strictly localised in the

endosomal compartment of cells and they are both acting through a common Myd88-related pathway. Within the airways activation of TLR3 causes airway hyperresponsiveness (Starkhammar et al., 2012; Stowell et al., 2009). However, TLR7 and TLR9 are not known to induce such a direct response. Instead, they both appears to have role in influencing the subsequent adaptive response (Guiducci et al., 2009; Holt and Sly, 2012; Sun et al., 2007) and be more important than TLR3 in antiviral immunity (Bowie and Unterholzner, 2008). Thus, the role of TLR7 and TLR9 in asthma is of specific interest.

Despite structural similarities, TLR7 and TLR9 are reported to promote inflammatory responses via different pathways (Chen et al., 2006; Schoenemeyer et al., 2005; Uematsu et al., 2005) and they sometimes end up causing opposing effects. In example, TLR7 seems to aggravate most autoimmune diseases, whereas activation of TLR9 appears protect against damage improving the disorder, thereby revealing a complex interplay between the receptors (Fukui and Miyake, 2012). The TLR7/8 gene is thought to be associated with allergic and hyperreactive airway disease (Moller-Larsen et al., 2008) and the activity of TLR7 in peripheral mono-nuclear cells is reduced in young patients suffering from asthma. The TLR7 agonist S-28463 has been found to prevent

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allergen-induced airway hyperresponsiveness and eosinophilia in a murine model of allergic asthma (Moisan et al., 2006) and to inhibit airway remodelling in a rat model of chronic asthma (Camateros et al., 2007). Intranasal administration of R-848, a TLR7/8 ligand, mediates acute and long-lasting suppression of experimental asthma in mice by inducing production of type I interferons and inhibiting Th2-type responses (Xirakia et al., 2010). Similarly, activation of TLR9 by CpG has been shown to decrease airway hyperresponsiveness and eosinophilia in mouse asthma models (Hayashi et al., 2004; Kline et al., 1998). In addition, vaccination of allergic patients with CpG conjugated ragweed has been shown to suppress symptoms of rhinitis (Creticos et al., 2006). However, both the role of TLR7 and TLR9 in an established asthmatic inflammation remains to be defined.

Hence, the present study was designed to investigate how activation of TLR7 and TLR9, respectively, affects a persistent, on-going allergic inflammation, using an ovalbumin (OVA) mice model of allergic asthma. In order to simulate a viral infection, TLR7 or TLR9 agonists were given during four consecutive days (Starkhammar et al., 2012) after the completion of the OVA challenge. Methacholine was subsequently used to assess the airway hyperactivity and changes in cell composition and mediators were used for characterisation of the on-going inflammation.

2. Methods

2.1. Animals

Female BALB/c mice (approximately 20 g, 8–12 weeks old) were obtained from Charles River (Sulzfeld, Germany). The mice were housed in a conventional animal house with 12 h dark/light cycles and were housed in plastic cages with adsorbent bedding material. Water and pelleted food were provided ad libitum. All animal procedures were approved by the regional committee of animal experimentation ethics.

2.2. Treatment protocol

Mice were sensitised with 10 µg ovalbumin (OVA) (grade II; Sigma-Aldrich, St Louis, MO, USA) and 1 mg Al(OH)₃ (Sigma-Aldrich) suspended in 200 µl PBS, given as an intraperitoneal injection on days 0 and 7. On days 15, 16 and 17 the animals were anaesthetized by isoflurane inhalation and 20 µl OVA (50 µg) or PBS were administered intranasally (Swedin et al., 2010). After another 4 days (18–21) the animals were again anaesthetized by isoflurane inhalation and 20 µl of the single stranded RNA mimetic TLR7 agonist R848 (Invivogen, San Diego, CA, USA; 20 µg), or the TLR9 agonist phosphorothioate-modified CpG oligodeoxynucleotide 2006 (CpG; 5′-tcgtcgttttgcgttttgcgtt-3′, from DNA Technology A/S, Aarhus, Denmark; 10 µg) were administered intranasally. The doses were chosen according to the report from Duechs and colleagues (Duechs et al., 2011). R848 and CpG were dissolved in PBS which also was used as vehicle control. Lung mechanics together with collection of tissues and bronchoalveolar lavage fluid were assessed 24 hours after the last treatment (day 22; Fig. 1).

2.3. Lung mechanics

The mice were anaesthetised (pentobarbital sodium, 90 mg/kg bw given intraperitoneally), put on a heating pad (body temperature, 37 °C), tracheotomised (18-gauge cannula) and connected to the flexivent animal ventilator (Scireq, Montreal, Canada). After ventilation was started, bilateral thoracotomies were performed to equalise pleural pressure to atmospheric and to exclude chest wall

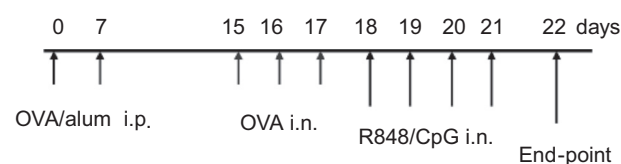


Fig. 1. Scheme over the protocol. An established allergic inflammation was assessed of sensitization with ovalbumin (OVA) adsorbed to aluminium hydroxide (alum) by intraperitoneal (i.p.) injection and thereafter challenged with OVA intranasally (i.n.). Thereafter the R848 or CpG (TLR7 and TLR9 agonists, respectively) were given i.n. during four consecutive days. All measurements were performed after the end-point.

contribution to the mechanics. The mice were ventilated at a frequency of 2.5 Hz in a quasi-sinusoidal fashion and in this manner, the pressure waveform was sinusoidal during inflation. The tidal volume was 12 ml/kg body weight and the positive end-expiratory pressure (PEEP) was 3 cm H₂O. Four sigh manoeuvres to three times the tidal volume were performed in order to stabilise the baseline lung resistance. After a five minutes resting period, methacholine (Sigma-Aldrich) was injected through the tail vein in increasing doses (0.01, 0.03, 0.1, 0.3, 1, 3 and 10 mg·kg⁻¹·body weight), in order to induce airway hyperresponsiveness. Lung mechanics were measured using forced oscillation technique (Swedin et al., 2010). In brief, the ventilation piston delivers 19 superimposed sinusoidal frequencies during the forced oscillatory manoeuvre, at the airway cannula. The frequency ranges from 1 to 20.5 Hz over 3 s (quick prime-3). The respiratory mechanics data were fitted to a constant-phase model and could be separated into central and peripheral compartments (Hantos et al., 1992). The parameters obtained were the Newtonian resistance (R_N), a close approximation of resistance in the conducting airways; tissue resistance (G), which reflects energy dissipation in the lung tissue consisting of airway closure and heterogeneity of airway distribution; and tissue elastance (H), which characterises tissue stiffness and reflects the energy storage within the tissue.

2.4. Bronchoalveolar lavage

Bronchoalveolar lavage was performed directly after lung function measurements. 1 ml ice cold PBS containing 0.6 mM EDTA was lavaged three times in the lung. The fluid was centrifuged at +4 °C, 1200 rpm, for 10 min and the supernatant was stored at –80 °C until use. Lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2), was used for 2 min, to lyse the red blood cells, followed by washing in PBS. Total cell number was counted and expressed as cells/ml bronchoalveolar lavage fluid. Differential cell counts were performed on May-Grünwald/Giemsa stained cytopins, counting a minimum of 300 cells, in a blinded manner.

2.5. Lung histology

Lungs were removed and immersed with 4% buffered formaldehyde. They were then embedded in paraffin, sectioned and stained in haematoxylin and eosin. Histological findings, such as peribronchial, parenchymal and perivascular cell infiltration, were semi-quantitatively graded in a blinded manner as 0 to 5 (zero to abundant cell infiltration) with a focus on inflammatory cells. Cells were identified according to their morphology in 1000x magnification.

2.6. Measurement of inflammatory mediators in bronchoalveolar lavage fluid

IL-1 α , IL-5, IL-12(p40), IL-17, CXCL1 (KC), CXCL9 (MIG), CCL2 (MCP-1), CCL3 (MIP-1 α), CXCL10 (IP-10) and TNF α were measured in bronchoalveolar lavage fluid using the Bio-Plex Pro. (BIO-RAD Laboratories, Hercules CA, USA) and run on the Luminex[®] 200[™] system. The immunoassays were carried out according to instructions of the manufacturer. In brief, samples were diluted with assay diluent and samples or standards were applied to the spectrally encoded magnetic beads. After incubation, beads were washed and mixed with specific biotinylated detector antibodies. Streptavidin-R-phycoerythrin was added to conjugate and the spectral properties of the beads were monitored by the Bioplex[™] System (Bio-Rad Laboratories) giving the amounts of cytokines.

Leukotriene B₄ (LTB₄) were measured in bronchoalveolar lavage fluid using the Luminex[®] Leukotriene B4 kit (Cayman Chemical Company, U.S.A) that were run on the Luminex[®] 200[™] system. The immunoassay was carried out according to manufacturer's instructions. In brief, samples or standards were applied on a 96-well plate, after which LTB₄ phycoerythrin tracer and the spectrally encoded beads were added. Following incubation, the spectral properties of the beads were monitored by the Bioplex[™] System (Bio-Rad Laboratories).

2.7. Statistical analysis

Data were analysed using Graph Pad Prism[®], version 5.01, software (GraphPad Software Inc., San Diego, CS, USA.). Results are presented as mean $\pm$ standard error of mean (S.E.M.) and *n* equals' number of subjects. For comparison of airway reactivity and compliance data, two-way analysis of variance was followed by Bonferroni's Multiple Comparison Test. Log ED₅₀ values were estimated due to their normal distribution. bronchoalveolar lavage fluid cell data and lung histology data were analysed with Kruskal–Wallis analysis of variance, followed by Dunn's test for between group comparisons. A *P* value of less than 0.05 was considered significant.

3. Results

3.1. Airway function

In order to investigate how specific TLR7 and TLR9 stimulation affect airway functions during an established, on-going allergic airway inflammation, the effect of TLR7 and TLR9 ligands on non-inflamed airway was investigated for comparison. The methacholine dose-response curve was not altered by R848 treatment in any of functional parameters compared to the non-treated mice (Fig. 2). On the other hand, CpG caused a marked increase in *R_N*, the resistance in the central airways, but did not affect *G*, the tissue resistance, or *H*, the tissue elastance. The existence of an on-going allergic inflammation five days after the final OVA challenge was established using the assessment of three lung function parameters, *R_N*, *G* and *H*, all reflecting the presence of airway hyperresponsiveness. Treatment with R848 during the 4 days period after the final OVA-challenge clearly reduced the OVA induced response for all parameters (Fig. 2). In contrast, similar treatment regime with CpG did not have any significant effect on the OVA-induced airway hyperresponsiveness showing unaffected increases in *R_N*, *G* and *H* (Fig. 2).

3.2. Cells in BAL fluid and lung tissue

To further depict the local inflammatory response, the infiltration of cells in lung tissue along with their subsequent accumulation in

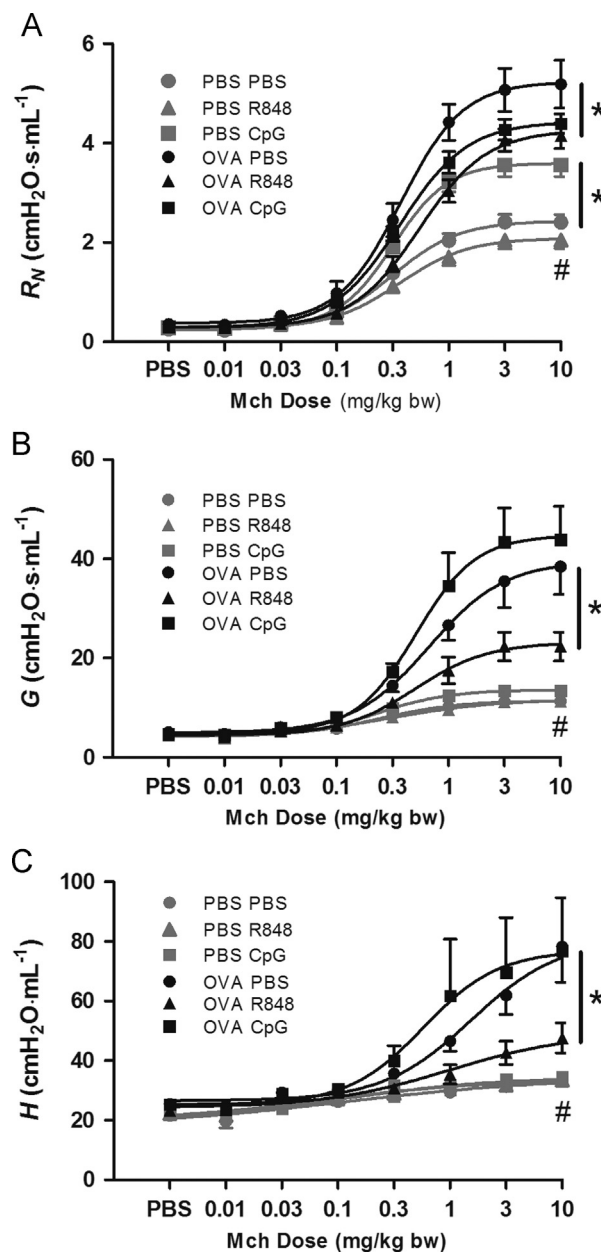


Fig. 2. Methacholine induced changes in (A) central lung resistance (*R_N*), tissue resistance (*G*), and tissue elastance (*H*) after 4 days of treatment with R848, CpG or vehicle (PBS) in either PBS- or OVA-challenged mice. Results are shown as mean $\pm$ S.E.M. (**P* < 0.05 TLR-agonist vs. PBS treatment, #*P* < 0.05 OVA vs. PBS treatment between all groups; *n* = 11–12).

bronchoalveolar lavage fluid was investigated for all treatment regimens given. The cell influx in bronchoalveolar lavage fluid in non-treated mice was similar after R848 treatment whereas CpG treatment caused an increase of neutrophils and lymphocytes (Fig. 3). OVA-treatment augmented the total amount of cells. This enhancement was mainly comprised of eosinophils, but an increase of macrophages and lymphocytes was also seen. Treatment with R848 and CpG after the OVA-challenge decreased the total number of cells by reducing the number of macrophages and eosinophils. In addition, R848 also reduced the influx of lymphocytes. In order to study TLR-induced cell infiltration in the lung, HE staining on lung sections was performed. In control mice, R848 caused no cell influx in the lung whereas CpG induced a strong influx of both neutrophils and lymphocytes (Fig. 4). OVA challenge caused a pulmonary invasion of

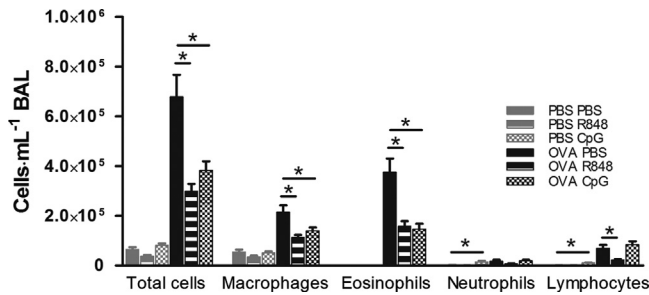


Fig. 3. Total cell count and cellular composition in bronchoalveolar lavage fluid after 4 days of treatment with R848, CpG or vehicle (PBS) in either PBS- or OVA-challenged mice. Results are shown as mean $\pm$ S.E.M. (* $P < 0.05$; $n = 10$ –15).

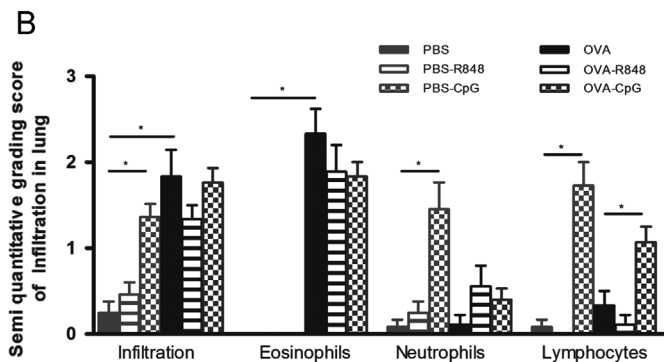
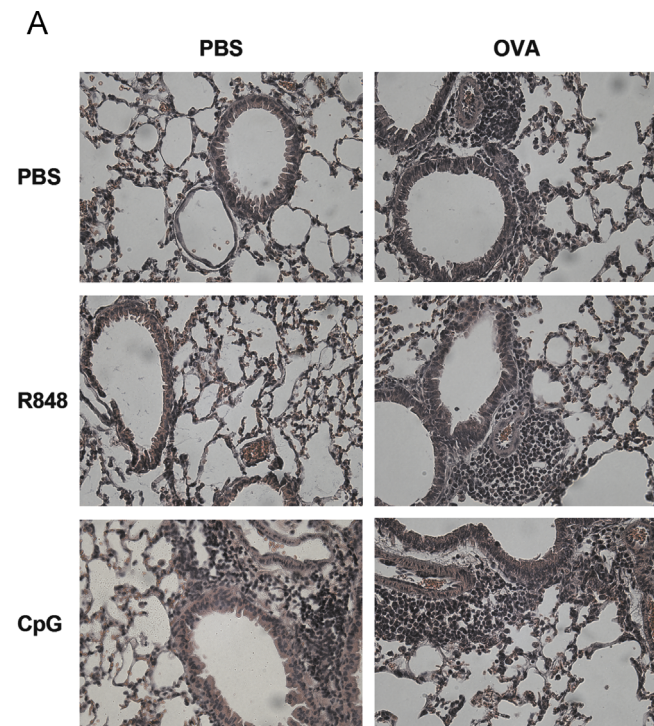


Fig. 4. Cellular infiltrations in the lung after 4 days of treatment with R848, CpG or vehicle (PBS) in either PBS- or OVA-challenged mice. (A) Typical pictures of histological sections stained in haematoxylin and eosin (200 $\times$ magnification). (B) Compiled data of semi-quantitatively graded cellular infiltration. Results are shown as mean $\pm$ S.E.M. (* $P < 0.05$; $n = 9$ –15).

eosinophils. This enhancement was not affected by the TLR treatment, whereas the neutrophil augmentation caused by CpG, was lower in the OVA-challenged mice.

3.3. Inflammatory mediators in BAL fluid

In order to find out how the stimulation of the innate immune receptors might affect the subsequent adaptive response, pro-inflammatory cytokines (IL-1 α , IL-12, IL-17 and TNF α) and chemokines (CCL2, CXCL9 and CXCL10 recruiting lymphocytes, and CCL3 and CXCL1 recruiting neutrophils) were measured. IL-5 and LTB₄ were also assessed since they both are well-known to increase during allergic airway inflammation (Henderson et al., 1996). The mediators measured in bronchoalveolar lavage fluid from non-treated mice were not affected by R848 treatment whereas CpG increased levels of IL-12, CCL2, CCL3 CXCL1 and CXCL9, (Fig. 5). In the OVA challenged group the levels of IL-5 and LTB₄ were enhanced, an increase that was reduced by the R848-treatment, but not by CpG (Figs. 5B and 6). The CpG-induced increase of IL-12 and CXCL9 was not altered by the OVA challenge whereas CXCL1, CCL2 and CCL3 became lower without losing their initial trend to increase. IL-1 α and IL-17 were not altered (not shown), regardless of treatment and TNF α and CXCL10 were found to be below the detection limit of the assays used.

4. Discussion

Four days of continuous TLR7 activation with R848 neither affected the airway smooth muscle function nor the inflammatory parameters measured. However, when the challenge was repeated during allergic inflammatory conditions caused by repeated OVA challenges, the OVA induced airway hyperresponsiveness was counteracted and the subsequent influx of eosinophils and macrophages were reduced along with a depletion of the OVA enhanced release of IL-5 and LTB₄ in bronchoalveolar lavage fluid. In contrast, 4 days of TLR9 activation with CpG induced a marked airway hyperresponsiveness together with a distinct Th1 response of cells and mediators. When given after the OVA challenge, CpG did not affect the airway hyperresponsiveness and although a decreased the OVA-induced influx of cells in bronchoalveolar lavage fluid was seen the general Th1 response was maintained.

To establish two levels of control the effects of R848 and CpG per se were investigated in mouse model of asthma as well as in unchallenged animals. R848 did not alter the dose-response curves to methacholine in the PBS-challenged controls, whereas CpG induced a marked airway hyperresponsiveness of the central resistance. This latter is a novel finding and interesting since the airway hyperresponsiveness caused by TLR9 stimulation is similar to that reported after stimulation of TLR3 (Starkhammar et al., 2012; Stowell et al., 2009). TLR3 is known to be triggered by viruses but its mechanistic pathways does not, like TLR7/9, involve Myd88 (Sato et al., 2002). Further, CpG also increased the number of neutrophils and lymphocytes seen in bronchoalveolar lavage fluid and lung tissue whereas R848 did not affect the cell influx. The CpG-induced neutrophil increase is, due to the nature of these cells, probably short acting. This assumption is supported by previous findings of increases in the amount of natural killer and dendritic cells along with unaltered neutrophil numbers 4 days after a single tracheal CpG installation in mice (Pesce et al., 2010). The present R848 data stands in contrast to a previous study, reporting increased amounts of cells in bronchoalveolar lavage fluid four hours after activation (Xirakia et al., 2010). Evaluation related time differences might account for part of this discrepancy.

The new approach in this study was that R848 and CpG were given during four consecutive days after the OVA-challenges were completed. This was done in order to mimic the effect of an acute viral infection on top of an on-going allergic inflammation. The long duration of the OVA induced inflammation is reflected as a sustained airway hyperresponsiveness, a continuous influx of cells

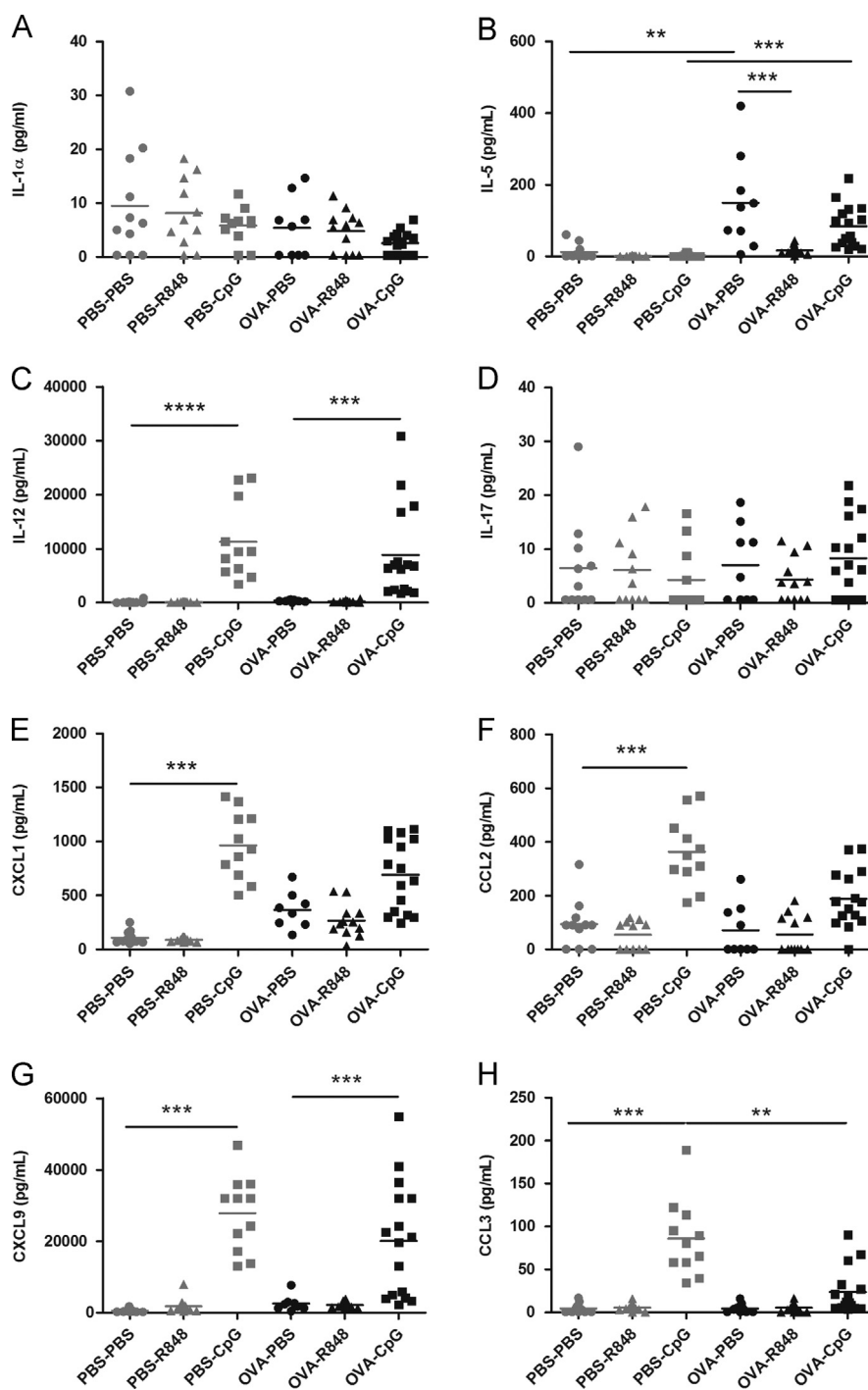


Fig. 5. Cytokines in bronchoalveolar lavage fluid after 4 days of treatment with R848, CpG or vehicle (PBS) in either PBS- or OVA-challenged mice. Results are shown as mean $\pm$ S.E.M. (* $P < 0.05$; $n = 8-14$).

in lung tissue and bronchoalveolar lavage fluid, and reaching high levels of IL-5 and LTB₄ in bronchoalveolar lavage fluid. The sensitization protocol presently used is known to induce a robust airway hyperresponsiveness with an increase of eosinophils that remains stable during at least two days after the last OVA-challenge (Swedin et al., 2010). The response is also characterised by increased levels of the IgE and IgG1, the Th2 cytokines IL-4, IL-5 and IL-13 (Swedin et al., 2009) and LTB₄ (Henderson et al., 1996). Taken together the presented data supports the ability to use the presented model for studies of viral interference during at least a five day period after the final OVA challenge is given.

Previous studies of TLR7/9 have been focused on the action seen when the TLRs were given before the OVA-challenges. Some studies have investigated the effect of TLR activation during the sensitization period (Sel et al., 2007), before the challenge period (Duechs et al., 2011; Hayashi et al., 2004; Kline et al., 1998; Xirakia et al., 2010), during the main challenge period (Du et al., 2009; Moisan et al., 2006) or in the late phase of the challenge period (Duechs et al., 2011; Sel et al., 2007; Van et al., 2011). According to these protocols the last allergen challenges were performed subsequent to the TLR agonist administration (Sel et al., 2007; Xirakia et al., 2010). Such studies have shown that TLR7 activation

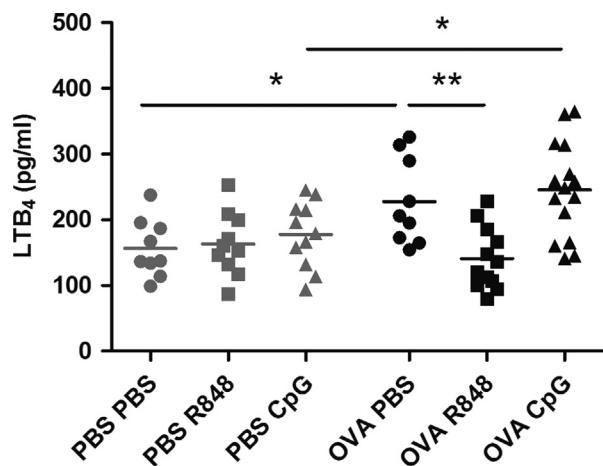


Fig. 6. LTB₄ in bronchoalveolar lavage fluid after 4 days of treatment with R848, CpG or vehicle (PBS) in either PBS- or OVA-challenged mice. Results are shown as mean $\pm$ S.E.M. (* P < 0.05; n = 9–15).

decreased airway hyperresponsiveness (Du et al., 2009; Duechs et al., 2011; Moisan et al., 2006; Sel et al., 2007; Xirakia et al., 2010) and that the corresponding TLR9 activation either decreased airway hyperresponsiveness (Hayashi et al., 2004; Kline et al., 1998) or were without effect (Duechs et al., 2011). In the present study we deliberately used a model for interference during an on-going inflammation, clearly differing from the other studies by the approach to give a boost of the TLR agonists during 4 days without any influence of further allergen challenge. In the present set up R848 decreased airway hyperresponsiveness. In addition to what has been previously reported, we could show that this take place at all levels of the bronchial tree, from the central to the most distal parts of the lung. This is clearly mirrored in a TLR7-induced response that affects smooth muscle contractility (R_N), tissue impedance (G) and tissue elastance (H) (Hantos et al., 1992). Mechanistically this could be a reflection of a direct activation of TLR7 located on the airway smooth muscle resulting in airway dilation (Ekman et al., 2011; Kaufman et al., 2011). A direct smooth muscle effect is probably best reflected in the response of the large conductive airways (R_N), whereas the more peripheral response also involves an inflammatory component (discussed below). TLR9 did not affect the allergen-induced airway hyperresponsiveness in the present model something that both stands in contrast (Hayashi et al., 2004; Kline et al., 1998) and is similar (Duechs et al., 2011) to when TLR9 has been given before the inflammatory (Hayashi et al., 2004; Kline et al., 1998). The reason for these differences is most likely dependent to be related to variations in the experimental design of the protocols. With the present straight-forward approach using two levels of controls, TLR7, but not TLR9, reduce allergen-induced airway hyperresponsiveness.

The OVA challenge caused an augmentation of the bronchoalveolar lavage fluid cell content, dominated by eosinophils and macrophages with a smaller neutrophils and lymphocytes component. R848 and CpG both decreased the influx of eosinophils and macrophages. Lymphocytes were reduced only with R848 and neutrophils remained unaltered by both TLR agonists. The attenuating effect of eosinophils caused by TLR7 and TLR9 stimulation has also found in previous studies using TLR agonist treatment before, during or at the end of the challenge period (Du et al., 2009; Duechs et al., 2011; Hayashi et al., 2004; Kline et al., 1998; Moisan et al., 2006; Sel et al., 2007; Van et al., 2011; Xirakia et al., 2010). In some of these studies, R848 also decreased the lymphocyte numbers (Moisan et al., 2006; Sel et al., 2007) and CpG reduced the amount of macrophages (Hayashi et al., 2004). Although, the mechanisms behind these reductions is not are

clear, the more pronounced effect seen in the present study may be related to the interruption of the challenge procedure. Further, it should be recognised that CpG increases lymphocytes in both non-challenged and challenged mice, suggesting a CpG effect independent of the allergic inflammation. In the lung tissue, OVA caused a strong eosinophil-dominated cell infiltration, which, in contrast to bronchoalveolar lavage fluid, was not affected by the TLR agonists. This suggests that pulmonary cell influx may take longer to reduce. Thus, a longer period might be necessary in order to show the same effect in the tissue. In the pulmonary tissue CpG per se increased the amount of neutrophils and lymphocytes. The infiltration of neutrophils, but not lymphocytes was then affected by the OVA-induced allergic response, suggesting that some but not all mediator pathways involving CpG also is related to the process behind an on-going allergic reaction. Taken together the outcome of our experiments confirms our previously reported notion about a separation between the mechanisms behind the development of airway hyperresponsiveness and the local recruitment of inflammatory cells, (Wardlaw et al., 2002) confirming the involvement of separate mediator pathways (Swedin et al., 2009).

Pulmonary viral infections trigger the release of Th1 cytokines with an ability to interact with the Th2 cytokines known to dominate during an allergic inflammation (Abbas et al., 1996). In analogy, four hours after an intratracheal administration of R848 the levels of IL-6, IL-12 and CXCL1 have been reported to increase (Garcia et al., 2010). In a similar manner, CpG induced the release of pro-inflammatory mediators like TNF- α and IL-1 β , peaking 2 h after a similar installation (Knuefermann et al., 2007). In contrast to these data measured hours after the last stimulation, the present study measured the levels one day after final challenge. This may explain why Th1 cytokines like IL-1 α and TNF α remained unaltered by the given treatments. Neither could it be excluded that the consecutive treatment with TLR agonists during four days might have caused tachyphylactic effects affecting the outcome. Van and colleagues have suggested that the attenuating effect induced by TLR7 stimulation is due to an activation of T-regulatory cells and a release of TGF β (Van et al., 2011). This idea was not tested in the present study but it certainly merits further evaluation in future studies. However, CpG induced a release of other mediators like IL-12, CCL2 and CXCL9. They all are known to be involved in recruitment and differentiation of T-cells which matches with the observed lymphocyte increase. The levels of CXCL1 and CCL3 involved in neutrophil recruitment, also increased and it likely that they are involved in the same process in the present experiments.

Five days after the final OVA challenge IL-5 was still present, indicating a sustained Th2 inflammation. The lipid mediator LTB₄ was also increased. This mediator has been shown to increase in mouse models of asthma (Henderson et al., 1996) and it is believed to be important for leucocyte migration in asthma (Ohnishi et al., 2008). Following the same pattern in response to R848 as the eosinophils and macrophages, IL-5 and LTB₄ were markedly reduced. No such reduction was seen following CpG treatment. These R848 data are in line with results from experiments where R848 was given during the OVA challenge (Du et al., 2009; Duechs et al., 2011; Moisan et al., 2006; Sel et al., 2007). It is likely that the decrease in IL-5 and LTB₄ is a direct effect of the reduction of the number of eosinophils and macrophages seen in bronchoalveolar lavage fluid (Henderson et al., 1996), but a more general effect on the progression of the allergic reaction cannot be excluded. CpG-treatment increased the levels of IL-12, CXCL1, CCL2, CXCL9 and CCL3 in both control and OVA-challenged mice although the increase of CXCL1, CCL2 and CCL3 did not reach statistical significance in the OVA group. This mediator increase, with special emphasis on IL-12 and CXCL9, is probably directly related to the enhancement of especially lymphocytes in lung tissue and

bronchoalveolar lavage fluid. It is also tempting to speculate in a role for a reciprocal interactions between Th1 and Th2 responses, cutting both ways (Abbas et al., 1996). Along these lines it is also possible that the massive Th1 response seen is involved in the eosinophil and macrophage decrease in bronchoalveolar lavage fluid. However, it must be stressed that both Th1 and Th2 signalling with appurtenant inflammatory reactions were present in all CpG- and OVA-treated animals

5. Conclusion

TLR7 and TLR9 are situated in the same intracellular compartment sharing adaptor proteins, but when stimulated locally during an on-going allergic airway inflammation the resulting response differs significantly. TLR7 dampens both the hyperactive airways and the inflammatory component, whereas TLR9 mainly appears to further activate the cellular component. Hence, the TLR9 stimulation is in the present set up actually worsening the inflammatory situation. It is worth noticing that in contrast to what has been reported for TLR3 (Torres et al., 2010) neither stimulation of TLR7 nor TLR9 appear to increase the airway hyperresponsiveness caused by OVA. What this means in terms of the role viruses during allergic asthmatic exacerbations remains to be established, but it underscores the complexity of an on-going inflammatory situation. The basic message of our study, stresses the positive effects of TLR7 stimulation, supporting the use of TLR7 agonists as a component in future asthma treatment (Drake et al., 2012) and calls some cautiousness when using TLR9 agonists in treatment attempts, acknowledging the potential negative effects of this receptor (Fukui and Miyake, 2012).

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