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Intranasal administration of TLR agonists induces a discriminated local innate response along murine respiratory tract

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ABSTRACT

Adjuvants are relevant for mucosal immunization in order to induce long lasting protective immunity. It has been shown that targeting to different regions of the airway results in different capacity to trigger adaptive/protective immunity. Nevertheless there is scarce knowledge regarding topological responsiveness along airways to TLR agonists. We analyzed the effects of intranasal administration of lipopolysaccharide (LPS), poly I:C and flagellin on the expression of a panel of innate response markers along murine airways by laser microdissection and RTqPCR. In all cases treatment induced recruitment of inflammatory cells to airways. However, regional gene expression indicated that whereas deeper airways (mainly alveoli) respond with high expression of IL6, CXCL1 and CXCL10, the response in conductive airways (bronchi and bronchioles) is dominated by expression of CCL20. On the other hand, triggering TLR3 elicits a response dominated by CXCL10, showing higher expression at 6 h compared to 2 h, whereas LPS and flagellin induce a response peaking at 2 h and dominated by IL6 and CXCL1. The results presented here showed difference in topological response triggered by different TLR agonist. These results make the targeting of different sites of airways a variable to evaluate when selecting the appropriate combinations of TLR and vaccinal antigens for intranasal delivery.

1. Introduction

Respiratory mucosa is one of the body surfaces that are heavily exposed to external environment interacting with thousands of liters of air inhaled daily. This poses a permanent challenge to local immune system which has to deal with potentially harmful particles and pathogenic microorganisms that should be kept under control to preserve the primary function of gas exchange at alveolar surfaces [1]. In this context functionality of innate immunity results essential, with pattern recognition receptors (PRR) as key players in the initial induction of host-defenses. Between PRR, toll like receptors (TLRs) are the best characterized family so far and their participation in protection against a diverse array of respiratory pathogens including virus, fungi and bacteria has been well documented [2]. Recognition of ligand by these transmembrane proteins leads to activation of MyD88 and/or TRIF dependent signaling pathways which promote differential activation of NFkB, the mitogen-activated protein kinase (MAPK) pathways and some members of the interferon responsive factor (IRF) family [3]. These events result in transcription of inflammatory and immunoregulatory genes such as cytokines, chemokines and co-stimulatory molecules allowing the establishment of initial containment mechanisms and instruction of dendritic cells for the development of specific adaptative response [4]. This ability has prompted the use of TLR agonist for development of therapeutic and prophylactic approaches against different pathologies [5,6]. Among them, strategies involving intranasal delivery of TLR agonists have been proposed to improve local and systemic immune response to peptide vaccines, achieving protection in several experimental models of respiratory infections [7–10]. These studies have established the proof of concept that TLR ligands can be combined with the appropriate antigens and delivered by intranasal route as vaccination strategy.

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However different aspects regarding TLR dependent orchestration of innate immune response at respiratory mucosa are not completely understood. Airways present a particular histological landscape, with several cell types, including epithelial cells, alveolar macrophages and different subsets of dendritic cells (DCs), which can respond in a different way to TLR activation [1]. Another particular aspect of the intranasal route, which has been relatively well established in humans [11], is that by changing the size of the delivered particle differential depths of the respiratory tract can be reached. In addition, studies in mouse models, as well as in human reports, have shown that targeting to different depths of the respiratory tract may also influence the outcome of the elicited response [12,13]. In this context, there is a lack of information related to the responsiveness of the different sectors of the airways to TLR ligand stimulation *in vivo*. In a previous work we have characterized the cellular specificity of flagellin-dependent innate response profile of the airways [14]. In the present work we used three TLR agonists to study the *in vivo* innate response at different regions of lower respiratory tract after intranasal treatment of mice. We focused on the response promoted by intranasal delivery of Poly I:C, lipopolysaccharide (LPS) and flagellin (FliC) whose performance as inducers of lung immune response have been clearly stated [14–16]. Through the use of these agonists we also contemplated diversity of TLR signaling pathways. Thus, while Poly I:C is sensed through TLR-3 leading to activation of TRIF dependent pathways and TLR-5, which recognize FliC, involves MyD88 dependent pathways, TLR-4 detection of LPS is able to trigger both signaling routes. By comparing gene expression patterns of a group of cytokines and chemokines we showed that although the three agonists had an effect on functional response of cells belonging to different anatomical compartment, these responses were not exactly equal having different kinetics of activation. Understanding this aspect of TLR functionality at respiratory mucosa would help not only to discern how recognition of different type of pathogens occurs along respiratory tract but also to improve design of immune-intervention strategies based on use of TLR agonist by intranasal route.

2. Materials and methods

2.1. Mice and reagents

Six-week-old Balb/C female mice were purchased from the School of Animal Science at the National University of La Plata (La Plata, Argentina). Mice were housed in appropriate conventional animal care facilities and handled according to the guidelines set by the National Institutes of Health (NIH publication vol. 25, No. 28 revised, 1996).

Endotoxin-free flagellin (FliC) was prepared and detoxified from *Salmonella enterica serovar Typhimurium* ATCC 14.028 as described previously [17]. *Escherichia coli* LPS O111:B4 and Poly I:C were purchased from Sigma.

2.2. Intranasal stimulation and tissue collection for gene expression

Mice were intranasally stimulated by administration of 25 μl of sterile saline solution containing 1 μg FliC, 1 μg LPS or 50 μg Poly I:C into the nostrils under light anesthesia. These dose were selected since they were already described to promote functional effects by intranasal administration [19]. Administration of vehicle alone was employed as control treatment. Volume of delivery was selected upon optimization experiments as the highest volume showing no significant induction of the selected markers when compared vehicle treated mice with non-treated mice.

At different times post-administration mice were euthanized by cervical dislocation. Trachea and inferior lobe of right lungs were collected in lysis buffer for RNA isolation. Left lung lobe were immediately placed in CRYOPLAST® medium (Biopack, Argentina) and flash frozen with liquid nitrogen. Experiments were performed at least twice using 3 mice per condition.

2.3. Laser capture microdissection (LCM)

Laser microdissection was performed as previously described [18]. Briefly, frozen lung sections of 15 μm were obtained with Shandon Cryotome from Thermo Scientific (Waltham, MA, USA) at −20 °C and placed on membrane Slides PEN-Membrane 2.0 μm (Leica, Weslar, Germany). Tissue sections were fixed in 70% ethanol for 1 min, stained with hematoxylin for 30 s and putted in 100% ethanol for 1 min. According to histology cells fractions from bronchi, bronchioles and lung parenchyma was obtained with microscope LMD6000 from Leica and collected in lysis buffer for RNA isolation. As average 30 microdissected pieces were collected in a single tube.

2.4. Gene expression analysis: RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was isolated using the Illustra RNAspin Kit (GE, CA, USA) following the manufacturer's instructions. Quantification of RNA was performed using a Heλios β Thermo spectrophotometer (Sirio S SAECS, Rome, Italy) at 260 nm. Measurements of A260/280 were used to determine the purity of the RNA.cDNA was obtained using 500 ng of total RNA of each sample as previously described [19]. All reagents used were from Invitrogen (Carlsbad, CA, USA). Briefly, reverse transcription was performed using a Mastercycle gradient thermal cycler (Eppendorf, Hamburg, Germany) using 10 min at 25 °C for annealing, 90 min at 42 °C for extension and 15 min at 95 °C for inactivation of enzymes.

SYBR green master mix 2× (Bio-Rad, USA) and 1:20 diluted cDNA were used to determine gene expression on the iCycler (BioRad) real-time PCR detection system (BioRad). All samples and non-template controls were run in duplicates as previously described [19]. The specificity of the PCR reaction was confirmed by melting curves. Data were analyzed using ΔΔCt method using β-actin expression levels as normalizer [19]. Results were expressed as fold increase over values from PBS-treated mice. The specific primers pairs used were:

IL6: Fwd: 5′GTT CTC TGG GAA ATC GTGGAAA 3′; Rev: 5′AAGTGCATCATCG TTG TTCATACA 3′
CXCL1: Fwd: 5′ CTT GGTTCA GAA AAT TGT CCA AAA 3′; Rev: 5′ CAG GTG CCATCAGAGCAGTCT 3′
CXCL10: Fwd: 5′ GGA GTG AAG CCA CGC ACA C 3′; Rev: 5′ TGA TGG AGA GAG GCT CTC TGC 3′
CCL20: Fwd: 5′ TTTTGGGATGGAATTGGACAC 3′; Rev: 5′ TGCAGGT-GAAGCCTTCAACC 3′
Ccsp Fwd: 5′ AAGCCTCCAACCTCTACCATG 3′; Ccsp Rev: 5′ ATGTC-CGAAGAAGCTGAGCTG 3′
Spc Fwd: 5′ CACTGGCATCGTTGTGTATGACT 3′; Spc Rev: 5′AGGTTCCTGGAGCTGGCTTATA 3′

2.5. Bronchoalveolar lavage (BAL) and leukocytes recruitment

After 24 h of intranasal administration of 25 μl of sterile saline solution containing 1 μg FliC, 1 μg LPS or 50 μg Poly I:C mice were euthanized by cervical dislocation. Vehicle alone treated mice were used as control. To perform BAL, trachea was partially cut and 1 ml sterile PBS 0.1% BSA was flushed into the lungs and then withdrawn. This procedure was repeated 3 times. To eliminate contaminating

red blood cells, BAL fluid was treated with potassium acetate Lysis Buffer (BD Biosciences, CA, USA) during 3 min at room temperature, then cells were washed and counted in Neubauer's chamber.

Cells from BAL were stained with fluorocrome conjugated antibodies for 1 h at 4 °C, followed by flow cytometry analysis using a FACSCalibur from Becton Dickinson. The FITC-, PE-, APC- and PerCP-conjugated monoclonal specific antibodies for CD11c (clone N418, hamster IgG: eBioscience, San Diego, CA, USA), CD11b (clone M1/70.15, Rat IgG2b: Caltag Laboratories, CA, USA), Ly-6G (clone 1A8, Rat IgG2a: BD Pharmingen) and Ly-6C (AL-21, Rat IgM: BD Pharmingen) were used to label the cells. Experiments were performed at least two times using 3 mice per condition.

2.6. Statistical analysis

Differences among means were assessed by one-way and two-way ANOVA with significance accepted at the $p < 0.05$ level. Differences between groups were analyzed using Bonferroni Test (GraphPad Prims®).

3. Results

3.1. Poly I:C, LPS and FliC treatment induce a robust innate immune response in lungs and airways

To analyze the ability of TLR agonist to promote response of respiratory tract, we assessed the recruitment of leukocytes to bronchoalveolar space after intranasal administration of 50 μg of Poly I:C or 1 μg of LPS or FliC. A significant increase in total cellularity of BAL samples was detected after 24 h of treatment with LPS and flagellin compared with PBS treated mice (Fig. 1A). This increase was equivalent for all tested agonist and corresponded to neutrophils recruitment, characterized as CD11b+, CD11cLy6G+, Ly6C+ (Fig. 1B), which were not found in airways of control mice (Fig. 1C). Poly I:C showed a slight increase in leukocytes also associated to neutrophils recruitment but unlike LPS and FliC it did not reach statistical significance.

In order to understand differences in the capacity of these TLR agonists to promote lung innate immunity we analyzed gene expression of classical proinflamatory molecules including chemokines responsible for neutrophil recruitment, such as CXCL1. As preliminary experiment we performed a time course expression of different innate activation markers along the first 24 h post-treatment. According to the transient response described for TLR stimulation [20], we found that highest response was found in the first hours post-stimulation, coming back almost to basal expression upon 24 h post-treatment (Supplementary Fig. 1). Consequently, we focused our analysis in the first 6 h post stimulation. Intranasal delivery of Poly I:C, LPS and FliC promoted robust expression of IL-6, CCL20, CXCL10 and CXCL1 as early as 2 h post-stimulation (Fig. 1D). However differences in lung response to each agonist could be found. Response elicited by FliC was the strongest, with average induction levels around 800 fold for IL-6 and CXCL1 (***$p < 0.001$ *vs* CTR) which represents almost 7 times higher expression than those induced by Poly I:C or LPS ($p < 0.001$ LPS/Poly I:C *vs* FliC for both markers). A similar profile was found for the chemokine CCL20, responsible for lymphocyte and immature dendritic cells recruitment (Fig. 1D) and for the neutrophil chemoattractant CXCL2 whose levels were about 4.5 times higher for FliC treatment ($p < 0.001$ FliC *vs* LPS/Poly I:C, not shown). On the other hand, the LPS that induced leukocyte recruitment similar to FliC, evoked an equivalent lung gene expression to those generated by the TLR-3 agonist, even for CXCL1.

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Gene expression was also evaluated at 6 h post-intranasal administration (Fig. 1D). Response to FliC was lower compared to 2 h but still significant at 6 h for all markers evaluated ($p < 0.05$). FliC was also the only treatment inducing expression of CCL2 (66.8 ± 10.7 *vs* control treatment $p < 0.05$, not shown) responsible for monocyte recruitment [21]. A Poly I:C dependent response was also detected at 6 h as shown by up-regulation of IL-6 and CXCL10 ($p < 0.05$ and $p < 0.01$ respectively). Nevertheless, no induction of CXCL1 was assessed while IL-6 expression was almost 6 times lower than for FliC treatment. Oppositely, CXCL10 up-regulation was similar for both agonists. In the case of LPS treatment no marker was significantly induced at 6 h.

Altogether our data showed that even when TLR activation gives a common prototypic response, there are slight differences in lung response with TLR-5 agonist promoting the stronger response after intranasal delivery.

Upon analysis of Poly I:C, LPS and FliC dependent lung's response we wonder if central conducting airways also contribute differentially to lower respiratory tract immune response induced by these TLR agonists. To this aim, we evaluated transcriptional expression of IL6 and CXCL1, which were the markers with highest induction levels in the previous analysis (Fig. 1E). We observed that at 2 h post-stimulation all treatments produced a significant tracheal induction of both markers ($p < 0.001$). However, tracheal expression levels were around 4–60 times lower compared to those from lung, depending on marker and agonist considered (Fig. 1E). At 6 h post-stimulation, FliC and Poly I:C induced both markers while LPS showed only up-regulation of IL-6 (Fig. 1E). Moreover, Poly I:C showed a differential capacity to trigger tracheal induction of IL6 and CXCL1, since it was similar at 2 and 6 h post-treatment, being higher than lung's response to Poly I:C at 6 h post-treatment (Fig. 1E). Thus, while in lung FliC was the agonist promoting the most pronounced and long-lasting effect, in tracheal tissue Poly I:C treatment induced the more pronounced response. Altogether our results indicate that central conducting airways are responsive to Poly I:C, LPS and FliC but the profile of response differs among them and from lung.

3.2. Poly I:C, LPS and FliC promoted differential activation of diverse lung regions

In order to assess whether the observed differences in the immune responses to the 3 tested agonists are maintained in different anatomical regions of the lungs, we evaluated the expression levels of IL6, CXCL1, CXCL10 and CCL20 at different cellular fractions obtained by laser capture microdissection. By histological analysis we defined 3 anatomical regions: central intrapulmonary conducting airways (bronchi), smaller airways encompassing diameters lower than 300 μm (bronchioles) and parenchymal lung tissue. To sample this last compartment, the presence of blood vessels or airways were excluded from the microdissected tissue (Fig. 2A). To assess proficiency of microdissection, we used expression of known markers of type II alveolar cells (surfactant C protein) and conductive airways (clara cell secreted protein) [22,23] to verify enrichment of these markers in the corresponding microdissected samples (Supplementary Fig. 2). Transcriptional response of all fractions was evaluated at 2 h and 6 h after intranasal delivery of each agonist. For the three agonists, significant induction of all markers was detected at bronchi, bronchioles and lung parenchyma as soon as 2 h after intranasal administration (Fig. 2B). However, differences according agonist and the region considered were found. A global analysis indicated a highly inducible functionality of airways compartments in response to all agonist, with increments in gene

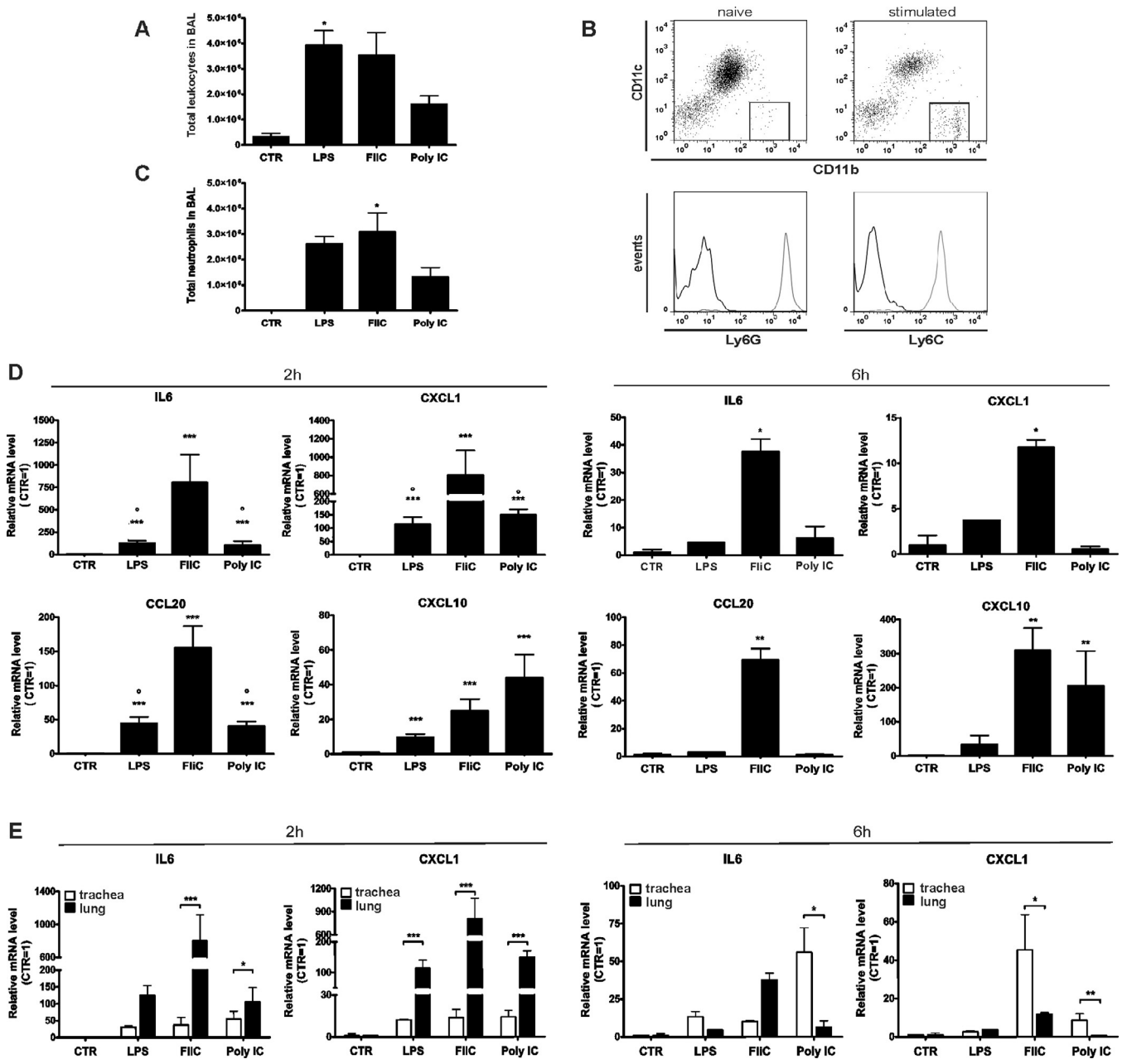

Fig. 1. Lungs and central airways inflammatory response induced by intranasal treatment with different TLR agonists. Mice received 50 μg Poly I:C, 1 μg LPS or 1 μg FliC by i.n. route. (A–C) Cellular populations in airways were sampled by broncho-alveolar lavage (BAL) and analyzed by flow cytometry at 24 h after treatment ($n = 3$). (A) Quantitative analysis of BAL leukocytes. (B) Upper panel: representative dot plot of BAL showing leukocyte populations in control and agonist treated mice. Neutrophils are depicted as CD11c− CD11b+. Lower panel: Ly6G and Ly6C expression on neutrophils (gray line). Isotype controls are indicated in black. (C) Total neutrophils in BAL. Results are given as mean ± SEM and are representative of two experiments. (D–E) Induction of pro-inflammatory markers was determined by RT-qPCR at 2 and 6 h after treatment. (D) IL-6, CXCL1, CXCL10 and CCL20 expression in lung. (E) Comparative expression of IL-6 and CXCL1 in lungs and central conducting airways. Data represents means of folds increase over PBS treatment ± SEM. Results are representative of two similar independent experiments. (A–D) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ statistically different from control treatment. $^{\circ}p < 0.05$ statistically different from FliC treatment. (E) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ statistical differences between trachea and lung for each treatment.

expression higher than 1000-fold over control for IL6 and CCL20 expression at 2 h upon FliC or LPS stimulation. In addition, comparisons of expression levels suggest that bronchiolar and bronchi fractions have a differential response dominated by CCL20 among the markers tested (Fig. 2B) whereas in lung parenchyma all agonists induced comparable levels of expression of CXCL1, CXCL10, CCL20 and IL6. In the case of LPS, all markers – with exception of CXCL1 – presented higher induction levels in airways compared to lung parenchyma, dominated by IL-6 and CXCL10 in bronchioles and CCL20 induction in bronchus ($p < 0.05$). Response promoted by FliC was also stronger in airways (Fig. 2B) and dominated by CCL20 expression, being even higher than that promoted by LPS ($p < 0.001$). Furthermore, FliC triggered a high induction of all analyzed markers in parenchymal tissue.

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In the case of Poly I:C stimulation, levels of CXCL1 and CXCL10 induced in parenchymal fraction was higher than in bronchi (Fig. 2B). The same pattern was found for CXCL2 expression with 300-fold induction in parenchymal tissue compared with no significant increase in bronchi ($p < 0.001$). Moreover, comparison of

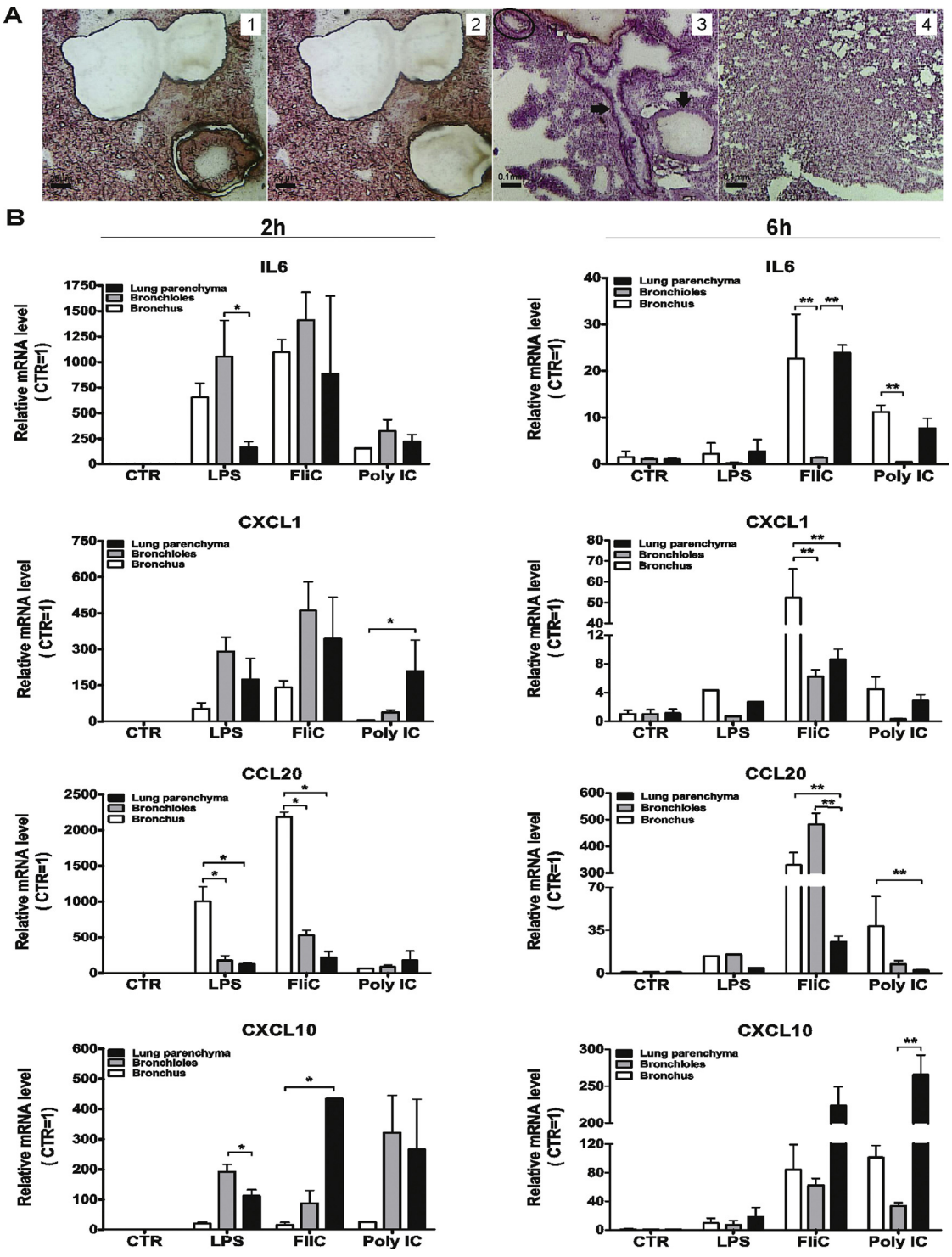

Fig. 2. Expression of inflammatory molecules at different anatomical intra-lung compartments induced by intranasal treatment with different TLR agonists. (A) Definition of lung anatomical regions for laser microdissection analysis (LDM). (1 and 2) Microdissection process. (1) Partial sectioning of bronchiolar tissue. (2) Complete sectioned tissue. (3 and 4) Region definition for microdissection. (3) Lung section showing bronchiole (circle) and bronchus (black arrow) selected for microdissection. Big black arrow indicates presence of blood vessel excluded of all previously defined regions. (4) Lung region selected as lung parenchyma fraction where airways and blood vessel were excluded. (B) Mice received 50 μg Poly I:C, 1 μg LPS or 1 μg FliC. IL-6, CXCL1, CXCL10 and CCL20 were determined by RT-qPCR in cellular fractions obtained by LDM at 2 h and 6 h after treatment. Material from three mice per condition was processed in each experiment. Data represents means of folds increase over PBS induced response at each compartment ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ statistical differences between response of anatomical regions to the same TLR agonist.

airways response to different agonist suggested that Poly I:C was the less powerful treatment after 2 h of intranasal delivery. Bronchiolar expression of CXCL1, CCL20 and IL6 induced by the TLR-3 agonist were around 3–12 times lower than for FliC or LPS ($p < 0.05$).

Microdissection study at 6 h also highlighted differences between agonist dependent responses along pulmonary compartments. Analysis of gene expression elicited by each tested agonist showed that the response to LPS was lower than the response elicited at 2 h post treatment. In this case, significant level of expression of CXCL10 in bronchi and parenchymal tissue and CCL20 in bronchi and bronchioles was observed (Fig. 2B). Absence of gene induction for some compartments, together with

increments lower than 20-folds over the control could explain the absence of response seen in whole lung 6 h after the TLR agonist administration. Moreover, no significant induction was detected for IL6 or CXCL1 even when both were highly increased in all compartments at 2 h post-stimulation (Fig. 2B). FliC treatment induced the up-regulation of all markers analyzed although with lower levels than observed at 2 h post-stimulation. As mentioned above, CCL20 was also the marker with the highest level of induction upon FliC stimulation at 6 h post-treatment with average increments as high as 300 and 450 fold for bronchi and bronchioles respectively. CXCL10 was also highly up-regulated by FliC in both airways and lung parenchyma where the detected levels of expression were the highest. Contrary to response seen at 2 h, bronchial levels of these two markers were significantly higher than those of bronchioles ($p < 0.01$). All together, this data showed that in the case of FliC, airways still contribute significantly to whole lung response at 6 h while parenchymal lung tissue evidence stronger diminutions in the intensity of the response.

Intranasal administration of Poly I:C promoted an innate response along all compartments analyzed which was detectable 6 h after stimulation. For this agonist CXCL10 and CCL20 were also the most up-regulated genes and increment's magnitude was conserved or increased compared with response observed at 2 h post-stimulation. Bronchial gene expression was induced for all markers analyzed.

Globally our results indicate that even when intranasal delivery of Poly I:C, LPS and FliC leads to activation of diverse lungs regions their contribution is not exactly equivalent for the 3 of them. Moreover, participation of each region to global response promoted by each agonist could vary along the first 6 h post-stimulation.

4. Discussion

Airways responsiveness to TLR agonist intranasal administration has been extensively documented [24,25]. It has been established that response to intranasally delivered LPS has an important contribution from structural cells [26], although alveolar macrophages are also responsive to this agonist [27]. Similar picture was described for flagellin [14,28]. Expression of several TLR receptors in the apical side of epithelial cells along airways has been described, explaining the high responsiveness to TLR ligand intranasally administered [24]. Nevertheless, although multiple cell types are TLR responsive along airways, there is a lack of knowledge regarding *in vivo* response elicited at different anatomical compartments of respiratory tract. In our study we employed 3 TLR agonists, FliC, LPS and Poly I:C, at doses already described to promote functional effects by intranasal administration [19]. Although it is difficult to establish an equivalence of dosing among different TLR ligands, we could confirmed that in all cases analyzed, a functional response was established by assessment of neutrophil recruitment to airways (Fig. 1). To analyze innate response induction by the effects of triggering different TLRs along airways we selected a set of genes typically induced by TLR activation in airways [29,30]. Regional characterization of response was performed at the transcriptional level since nucleic acid amplification techniques provides highest sensitivity for the small amount of material recovered by LCM techniques. Concordantly to previous reports [12], we detected a non-homogeneous distribution of stimuli along airways using intranasal administration of methylene blue in preliminary experiments (not shown). Thus, while proximal airways were highly exposed, small amounts of the stimuli reach deep airways (data not shown). However, innate response patterns observed were complex and did not correlate with stimuli exposure levels. Whereas deeper airways (mainly alveoli) are producing high response of IL6, CXCL1 and CXCL10, conductive airways (bronchi and bronchioles) response is dominated by expression of CCL20 (Fig. 2). The higher CCL20 response observed in bronchus and bronchioles compared to alveolar regions and compared to other innate activation markers has not been described so far. CCL20 is the single ligand of CCR6, that is expressed in immature DCs and activated T and B cells [31]. A dense network of DCs is formed around conductive airways and functional differences have been described for different subsets of DCs present in separate lung microcompartments [32,33]. Upon activation of innate response in airways, the higher expression of CCL20 may contribute to differential DC migration to the airways epithelial compartment. Recruitment of DCs upon proinflammatory activation of airways has been described [34]. Local activation of DCs and increase in sampling and antigen processing has been associated to indirect activation depending on TLR-triggered epithelial response [14,35]. Although CCR6 has been involved in constitutive recruitment of DCs to mucosal sites [36], it has also been shown that it also plays a role in proinflammatory settings [37,38]. The increase of CCL20 expression mainly in conductive airways described in our study could be related to this epithelial-dependent process of recruitment and activation of DCs upon intranasal delivery of TLR agonists. In all cases, we use histopathological analysis to rule out the possibility of including of iBALT-like lymphoid structures that are known to express CCL20, in microdissected sample [39].

On the other hand, we have also shown that triggering TLR3 elicits a response dominated by CXCL10, which has higher expression at 6 h compared to 2 h, whereas flagellin and LPS induce a response peaking at 2 h and dominated by IL6 and CXCL1 (Figs. 1 and 2). This observation is consistent with the capacity of triggering IRF-type I interferon pathways by TLR3 activation, that results in critical activation of CXCL10 chemokine expression [40,41]. We have previously shown that CXCL10 exhibits a different kinetics of induction upon innate response activation when compared to other proinflammatory markers such as IL6 or CXCL2 showing an initial rise but reaching highest levels upon 24 h post stimulation [29].

Although there are several differences at the histological and physiological level between the airways of mice and human [42], there are evidences in both species that vaccinal efficacy varies if different regions of airway are targeted. Minne et al. [12] have shown that targeting the alveolar compartment in mice using a split influenza virus vaccine induces virus neutralizing antibody titers as high as obtained with intramuscular vaccination whereas it produces a much higher secretory antibody response than i.m. route. Furthermore, by careful evaluation of antigen deposition studies, they showed that when antigen was delivered to the alveolar compartment, elicited immune response was higher than when antigen remained in the nasal cavity or in the conductive airways. Similar results were reported by Menzel et al. [43] and Meyer et al. [13] working on healthy human volunteers that received intranasal delivery of pneumococcal antigens. In this case, antibody responses were higher when antigen was delivered to the deeper airways. In both studies the antigens used were different, however in both cases the superior capacity of deeper airways was documented and it was speculated that differences in the local microenvironment originates in differences in cellular composition or in the type of response elicited by local populations may skew the resulting adaptive response. In this scenario, our proof of concept study indicates that even when using a single TLR agonist, regional differences in response along the airways are established. This draws the necessity to improve our understanding on the type of local response elicited by TLR activation and how this may condition the outcome of the resulting adaptive response. Furthermore, taking into account that different regions of the respiratory tree can be targeted by changing the particle size or using different administration methods [11,44,45] it could be envisaged a rational formulation of

intranasal vaccines by combining different TLR agonists with different targeting strategies in order to shape the generated adaptive response according to the type of pathogen confronted.

We have shown here that intranasal treatment with different TLR agonists elicit a local response that may be dependent on the agonist used; on the other hand, general differences in the response were observed between the alveolar region and the conductive airways, which may contribute to the different microenvironments generated upon innate response activation. Although a deeper characterization is needed, this rise the possibility of considering the targeting site of the airways as other variable to take into consideration when selecting the appropriate combinations of TLRs and vaccinal antigens for intranasal delivery.

Conflict of interest statement

Authors have no conflicts of interest to declare.

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References

[1] Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL. Regulation of immunological homeostasis in the respiratory tract. Nat Rev Immunol 2008;8:142–52.
[2] Kovach MA, Standiford TJ. Toll like receptors in diseases of the lung. Int Immunopharmacol 2011;11:1399–406.
[3] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010;11:373–84.
[4] Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. Adv Exp Med Biol 2005;560:11–8.
[5] Lahiri A, Das P, Chakravortty D. Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond. Vaccine 2008;26:6777–83.
[6] Dunne A, Marshall NA, Mills KH. TLR based therapeutics. Curr Opin Pharmacol 2011;11:404–11.
[7] Honko AN, Sriranganathan N, Lees CJ, Mizel SB. Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with Yersinia pestis. Infect Immun 2006;74:1113–20.
[8] Buffa V, Klein K, Fischetti L, Shattock RJ. Evaluation of TLR agonists as potential mucosal adjuvants for HIV gp140 and tetanus toxoid in mice. PLoS ONE 2012;7:e50529.
[9] Oma K, Zhao J, Ezoe H, Akeda Y, Koyama S, Ishii KJ, et al. Intranasal immunization with a mixture of PspA and a Toll-like receptor agonist induces specific antibodies and enhances bacterial clearance in the airways of mice. Vaccine 2009;27:3181–8.
[10] Nguyen CT, Hong SH, Ung TT, Verma V, Kim SY, Rhee JH, et al. Intranasal immunization with a flagellin-adjuvanted peptide anticancer vaccine prevents tumor development by enhancing specific cytotoxic T lymphocyte response in a mouse model. Clin Exp Vaccine Res 2013;2:128–34.
[11] Carvalho TC, Peters JI, Williams 3rd RO. Influence of particle size on regional lung deposition – what evidence is there? Int J Pharm 2011;406:1–10.
[12] Minne A, Louahed J, Mehauden S, Baras B, Renauld JC, Vanbever R. The delivery site of a monovalent influenza vaccine within the respiratory tract impacts on the immune response. Immunology 2007;122:316–25.
[13] Meyer P, Menzel M, Muellinger B, Weber N, Haeussinger K, Ziegler-Heitbrock L. Inhalative vaccination with pneumococcal polysaccharide in patients with chronic obstructive pulmonary disease. Vaccine 2006;24:5832–8.
[14] Van Maele L, Fougeron D, Janot L, Didierlaurent A, Cayet D, Tabareau J, et al. Airway structural cells regulate TLR5-mediated mucosal adjuvant activity. Mucosal Immunol 2013;2013:66.
[15] Wikstrom ME, Batanero E, Smith M, Thomas JA, von Garnier C, Holt PG, et al. Influence of mucosal adjuvants on antigen passage and $CD4^+$ T cell activation during the primary response to airborne allergen. J Immunol 2006;177:913–24.
[16] Ainai A, Tashiro M, Hasegawa H. Cross-protective immunity against influenza virus infections induced by intranasal vaccination together with a TLR3-mucosal adjuvant. Hum Vaccin 2011;7:174–82.
[17] Hiriart Y, Errea A, Gonzalez Maciel D, Lopez JC, Rumbo M. A method for the purification of bacterial flagellin that allows simple upscaling. World J Microbiol Biotechnol 2012;28:15–21.
[18] Rumbo M, Sierro F, Debard N, Kraehenbuhl JP, Finke D. Lymphotoxin beta receptor signaling induces the chemokine CCL20 in intestinal epithelium. Gastroenterology 2004;127:213–23.
[19] Errea A, Moreno G, Sisti F, Fernandez J, Rumbo M, Hozbor DF. Mucosal innate response stimulation induced by lipopolysaccharide protects against *Bordetella pertussis* colonization. Med Microbiol Immunol 2010;2010:103–8.
[20] Alexopoulou L, Kontoyiannis D. Contribution of microbial-associated molecules in innate mucosal responses. Cell Mol Life Sci 2005;62:1349–58.
[21] Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 1999;398:718–23.
[22] Glasser SW, Senft AP, Maxfield MD, Ruetschilling TL, Baatz JE, Page K, et al. Genetic replacement of surfactant protein-C reduces respiratory syncytial virus induced lung injury. Respir Res 2013;14:19.
[23] Stripp BR, Lund J, Mango GW, Doyen KC, Johnston C, Hultenby K, et al. Clara cell secretory protein: a determinant of PCB bioaccumulation in mammals. Am J Physiol 1996;271:L656–64.
[24] Ioannidis I, Ye F, McNally B, Willette M, Flano E. Toll-like receptor expression and induction of type I and type III interferons in primary airway epithelial cells. J Virol 2013;87:3261–70.
[25] Iwamura C, Nakayama T. Toll-like receptors in the respiratory system: their roles in inflammation. Curr Allergy Asthma Rep 2008;8:7–13.
[26] Perros F, Lambrecht BN, Hammad H. TLR4 signalling in pulmonary stromal cells is critical for inflammation and immunity in the airways. Respir Res 2011;12:125.
[27] Hoppstadter J, Diesel B, Zarbock R, Breinig T, Monz D, Koch M, et al. Differential cell reaction upon Toll-like receptor 4 and 9 activation in human alveolar and lung interstitial macrophages. Respir Res 2010;11:124.
[28] Raoust E, Balloy V, Garcia-Verdugo I, Touqui L, Ramphal R, Chignard M. Pseudomonas aeruginosa LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells. PLoS ONE 2009;4:e7259.
[29] Moreno G, Errea A, Van Maele L, Roberts R, Leger H, Sirard JC, et al. Toll-like receptor 4 orchestrates neutrophil recruitment into airways during the first hours of *Bordetella pertussis* infection. Microbes Infect 2013;15:708–18.
[30] Jenner RG, Young RA. Insights into host responses against pathogens from transcriptional profiling. Nat Rev Microbiol 2005;3:281–94.
[31] Williams IR. CCR6 and CCL20: partners in intestinal immunity and lymphorganogenesis. Ann N Y Acad Sci 2006;1072:52–61.
[32] Huh JC, Strickland DH, Jahnsen FL, Turner DJ, Thomas JA, Napoli S, et al. Bidirectional interactions between antigen-bearing respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC activation occurs in the airway mucosa but not in the lung parenchyma. J Exp Med 2003;198:19–30 [Epub 2003 Jun 2030].
[33] Lambrecht BN, Hammad H. Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. Annu Rev Immunol 2012;30:243–70.
[34] Lommatzsch M, Bratke K, Bier A, Julius P, Kuepper M, Luttmann W, et al. Airway dendritic cell phenotypes in inflammatory diseases of the human lung. Eur Respir J 2007;30:878–86.
[35] Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med 2009;15:410–6.
[36] Cook DN, Prosser DM, Forster R, Zhang J, Kuklin NA, Abbondanzo SJ, et al. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. Immunity 2000;12:495–503.
[37] Vanbervliet A, Homey B, Durand I, Massacrier C, Ait-Yahia S, de Bouteiller O, et al. Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. Eur J Immunol 2002;32:231–42.
[38] Osterholzer JJ, Ames T, Polak T, Sonstein J, Moore BB, Chensue SW, et al. CCR2 and CCR6, but not endothelial selectins, mediate the accumulation of immature dendritic cells within the lungs of mice in response to particulate antigen. J Immunol 2005;175:874–83.
[39] Botelho FM, Rangel-Moreno J, Fritz D, Randall TD, Xing Z, Richards CD. Pulmonary expression of oncostatin M (OSM) promotes inducible BALT formation independently of IL-6, despite a role for IL-6 in OSM-driven pulmonary inflammation. J Immunol 2013;191:1453–64.
[40] McNally B, Willette M, Ye F, Partida-Sanchez S, Flano E. Intranasal administration of dsRNA analog poly(I:C) induces interferon-alpha receptor-dependent accumulation of antigen experienced T cells in the airways. PLoS ONE 2012;7:e51351.
[41] Starkhammar M, Kumlien Georen S, Swedin L, Dahlen SE, Adner M, Cardell LO. Intranasal administration of poly(I:C) and LPS in BALB/c mice induces airway hyperresponsiveness and inflammation via different pathways. PLoS ONE 2012;7:e32110.
[42] Hyde DM, Hamid Q, Irvin CG. Anatomy, pathology, and physiology of the tracheobronchial tree: emphasis on the distal airways. J Allergy Clin Immunol 2009;124:S72–7.
[43] Menzel M, Muellinger B, Weber N, Haeussinger K, Ziegler-Heitbrock L. Inhalative vaccination with pneumococcal polysaccharide in healthy volunteers. Vaccine 2005;23:5113–9.
[44] Foo MY, Cheng YS, Su WC, Donovan MD. The influence of spray properties on intranasal deposition. J Aerosol Med 2007;20:495–508.
[45] Nadithe V, Rahamatalla M, Finlay WH, Mercer JR, Samuel J. Evaluation of nose-only aerosol inhalation chamber and comparison of experimental results with mathematical simulation of aerosol deposition in mouse lungs. J Pharm Sci 2003;92:1066–76.