Immunology Letters 164 (2015) 33–39

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

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

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ARTICLE INFO

Article history:
Received 29 July 2014
Received in revised form
30 December 2014
Accepted 19 January 2015
Available online 28 January 2015

Keywords:
Toll like receptors
TLR agonist
Airways
Innate response
Topological response

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.

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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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http://dx.doi.org/10.1016/j.imlet.2015.01.004
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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

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