Toll-like receptor 4 ligation enforces tolerogenic properties of oral mucosal Langerhans cells

Jean-Pierre Allam, MD,[a] Wen-Ming Peng, MSc,[a] Torsten Appel, MD,[b] Matthias Wenghoefer, MD,[b] Bernd Niederhagen, MD,[b] Thomas Bieber, MD, PhD,[a] Stefaan Bergé, MD,[c] and Natalija Novak, MD[a]

Bonn, Germany, and Nijmegen, The Netherlands

Background: Despite high bacterial colonization, acute infections are rare in the oral mucosa, implicating tolerogenic predominance. Bacterial antigens like LPSs are recognized by innate immunity receptors such as Toll-like receptor 4 (TLR4), associated with LPS receptor (CD14).

Objectives: Toll-like receptor 4 agonist monosphoryl lipid A has been successfully used as adjuvant in subcutaneous immunotherapy, suggesting reinforcement of allergen-specific tolerance. Recently sublingual immunotherapy (SLIT) has been shown to be an effective alternative to subcutaneous immunotherapy. We observed CD14 expression on human oral Langerhans cells (oLCs), representing a major target of SLIT. However, not much is known about TLR4 expression and its effect on oLCs.

Methods: Cell suspensions were obtained by trypsinization of human oral mucosa and analyzed by flow cytometry, RT-PCR, cytometric bead arrays, ELISA, and mixed lymphocyte reactions.

Results: We could show that oLCs express TLR4, and its ligation by monosphoryl lipid A upregulated expression of coinhibitory molecules B7-H1 and B7-H3 while surface expression of costimulatory molecule CD86 was concomitantly decreased. Furthermore, TLR4 ligation on oLCs increased their release of the anti-inflammatory cytokine IL-10 and decreased their stimulatory capacity toward T cells. Moreover, TLR4-ligation on oLCs induced IL-10, TGF-β1, Forkhead box protein 3, IFN-γ, and IL-2 production in T cells.

Conclusion: In view of these data, TLR4-ligation on oLCs might not only play a role in pathogen recognition for efficient immunity but also contribute to the tolerogenic state predominating in the oral cavity. (J Allergy Clin Immunol 2008;121:368-74.)

Key words: *Human, Toll-like receptors, Langerhans cells, immunotherapy, tolerance, mucosa*

Bacterial infections are rarely seen within the oral mucosa, although it is frequently exposed to a great number of gram-positive and gram-negative bacterial components that originate from the oral microflora.[1] This leads to the assumption that the oral mucosa represents a highly tolerogenic environment. Approximately 500 different bacterial species reside within the oral cavity, including commensals and pathogens[1] that contain, among other components, LPS.[2] Bacterial antigens are recognized by structures of the innate immune system, so-called *pathogen-specific pattern recognition receptors*, to which the Toll-like receptor (TLR) family belongs.[3] Bacterial ligands identified by TLRs include bacterial lipopeptides (TLR2), LPS (TLR4), and CpG DNA (TLR9).[3] However, LPS recognition is not restricted to TLR4 alone but requires an innate immunoreceptor complex formed by TLR4 combined with LPS receptor/CD14 (CD14) and the connecting molecule MD2.[4,5] It has been shown that TLR ligation activates a complex signal transduction cascade leading to transcription of proinflammatory cytokines such as IL-1, IL-6, and TNF-α.[3] Nevertheless, a recent report demonstrated that TLR recognition within the commensal microflora is required to sustain intestinal homeostasis.[6] It is more than likely that this is an active process, in which antigen-presenting cells such as Langerhans cells (LCs) are involved. As part of the adaptive immune system, LCs are capable to mount an immune response by activating antigen-specific T cells.[7] Nevertheless, LCs are not only able to drive antigen-specific T-cell inflammatory immune responses but are also capable of operating protective immune reactions, which are characterized by tolerance toward these pathogens.[7] This concept, to "turn off" inflammatory immune responses despite constant antigen exposure and instead "turn on" tolerance, is clinically used in immunotherapeutic strategies for allergic disorders.[8] Because increasing doses of allergen the individual is sensitized to are injected subcutaneously during allergen-specific immunotherapy, there is a relatively high risk of serious allergic side effects. In search for immunotherapeutic strategies with fewer side effects, sublingual immunotherapy (SLIT) has been shown to represent a relatively safe and effective alternative.[9,10] However, not much is known about the mechanisms underlying SLIT. It is likely that in regard to SLIT, especially oral mucosal Langerhans LCs (oLCs) as antigen-presenting cells serve as primary cellular targets. In this context, we showed that human oral oLCs express the high-affinity receptor for IgE (FcεRI),[11] which enables them to take up and process allergens applied during SLIT. Further on, we observed that oLCs, in contrast with epidermal LCs, express the LPS receptor CD14, which makes them susceptible to LPS.[12] Recently an impaired expression of TLR2, TLR4, and TLR5 of epidermal LCs resulting in

From the Departments of [a]Dermatology and Allergy and [b]Oral and Maxillofacial Surgery, University of Bonn; and [c]the Department of Oral and Maxillofacial Surgery, Radboud University Nijmegen.

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Reprint requests: Natalija Novak, MD, Department of Dermatology, University Bonn, Sigmund-Freud-Str 25, 53105 Bonn, Germany. E-mail: Natalija.Novak@ukb.uni-bonn.de.

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Abbreviations used
APC: Antigen-presenting cell
DC: Dendritic cell
LC: Langerhans cell
MFI: Mean fluorescence intensity
MPL: Monophosphoryl lipid A
oLC: Oral mucosal Langerhans cell
rFI: Relative fluorescence index
rSI: Relative stimulation index
SLIT: Sublingual immunotherapy
TLR: Toll-like receptor

a low responsiveness to bacterial stimuli has been shown.[13] The LPS derivative and TLR4 ligand monosphoryl lipid A (MPL) has been used as an adjuvant in subcutaneous immunotherapy in the clinical practice already.[14-16] Therefore, approaches of the near future will most likely use MPL as an adjuvant for SLIT to improve its efficacy. However, it is yet unknown in which way MPL might act on oLCs as target cells of SLIT. To address this question, we analyzed the expression of the whole LPS recognition receptor complex on oLCs and investigated in which way its ligation by MPL might alter the phenotype and functional properties of oLCs.

METHODS

Reagents

For discussion of reagents, see this article's Online Repository at www.jacionline.org.[17]

Oral mucosa specimen

Specimens of oral mucosa from the vestibular region were obtained from patients undergoing intraoral surgery for molar extraction or revision of mandibular fracture at the Department of Oral and Maxillofacial Surgery. Tumor patients were excluded. Only clinically noninflamed tissue was collected. All specimens were obtained with the approval of the local ethics committee and the medical board ethics committee and only after informed consent from patients had been obtained.

Amplification of mRNA and analysis of transcripts

For discussion of amplification of mRNA and analysis of transcripts, see this article's Online Repository at www.jacionline.org.

Preparation of cell suspensions

Crude epithelial cell suspensions of oral mucosa were prepared by trypsinization in a 0.5% trypsin buffer without Ca^{2+} for 1 hour at 37°C as described elsewhere.[11] For PCR experiments, oLCs were enriched by CD1a MicroBeads and AutoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany) as described in the user manual. Purity of enriched CD1a cells was above 90%.

TLR4 ligation of oLCs

Toll-like receptor 4 agonist MPL in injectable distilled water was kindly provided by Dr Drachenberg (Bencard, Munich, Germany). Crude cell suspension was incubated in RPMI1640 (Invitrogen, Carlsbad, Calif) containing 10% heat-inactivated FCS (Sigma-Aldrich, Seeze, Germany), 1% antibiotics/antimycotics (Invitrogen). Cells were either stimulated with 50 μg MPL or as control condition with a respective volume of injectable distilled water for 12 hours or 18 hours before analysis.

Immunolabeling of cell suspensions

An indirect extracellular or intracellular staining for a number of 0.5 to 2 × 10^5 unfixed or fixed cells was performed as described elsewhere.[18] Finally, the cells were acquired on a FACS-Canto (BD Biosciences, Heidelberg, Germany) as described in detail before[19] and analyzed by FACSDiva (BD Biosciences) and FlowJo (TreeStar Inc, Ashland, Ore) software. For quantitative evaluation, dead cells were excluded by 7-Amino-Actinomycin D staining. CD1a population was gated out manually and expressed either as a percentage of positive cells or by the relative fluorescence index (rFI) determined as follows:

$$rFI = (MFI\ [receptor] - MFI\ [isotype\ control])/MFI\ (isotype\ control)$$

where MFI is the mean fluorescence intensity.

Cytokine analysis

Oral mucosal LC IL-10 cytokine production was detected in supernatants by ELISA from R&D Systems (Wiesbaden, Germany) as described in the instruction manual. TGF-β1 was detected in unactivated supernatants of unstimulated and TLR4-activated oLCs cultures as well as in oLC/T-cell cocultures by ELISA from R&D Systems as described in the instruction manual and by intracellular cytokine staining. T-cell production of IL-2, IL-4, IL-5, IL-10, and INF-γ was detected in supernatants by using the T_H1/T_H2 cytokine bead array detecting kit from BD Biosciences and the BDArray (BD Biosciences) as described in the instruction manual. Briefly, different cytokines can be detected in 1 sample where each cytokine is restricted to a bead population of defined fluorescence intensity in the near infrared channel. Cytokine concentration level is calculated by fluorescence intensity in the yellow channel in correlation to fluorescence intensity of recombinant standards of each cytokine. Cytokine concentration was calculated by using FCAP software (BD Biosciences), and zebra plots and histograms were created by using FlowJo Version 8.4.5 (TreeStar Inc).

T-cell proliferation assays

Allogeneic naive $CD4^+$ T cells were isolated from PBMCs from healthy donors by magnetic microbead labeled antibodies (Miltenyi Biotech) and the AUTOMACs techniques (Miltenyi Biotech) as described in the manufacturer's instructions. Triplicates of oLCs containing 200 viable $CD1a^+$ oLCs/well were incubated with 100,000 viable allogeneic T cells at 37°C for 3 days. Proliferative response was then measured by addition of 1 μCi ^3H-thymidine incorporation for 12 hours. The incorporated radioactivity was measured in counts per minute (cpm) with a Wallac Microbeta Jet 1450 Microplate Scintillation/Luminescence Counter (Long Island Scientific, East Setauket, NY). Relative stimulation indices (rSIs) were calculated as follows: rSI = (cpm oLC – cpm T cells)/cpm T cells.

Statistical analysis

For statistical evaluation of significances, the Mann-Whitney *U* test or Wilcoxon was performed by using the SPSS 12.0 for Windows software (Microsoft, Redmond, Wash). Results are shown as arithmetic means ± SEMs ($P < .05$; $P < .01$; no indication = not significant unless otherwise indicated).

RESULTS

oLCs express TLR4

The LPS receptor CD14 forms a complex with TLR4 and MyD88 after activation with LPS.[5] In semiquantitative PCR experiments, we could detect transcripts for TLR4 in complete oral mucosal tissue (Fig 1, *A*) and in oLCs enriched from oral mucosal tissue (Fig 1, *B*). Flow-cytometric analyses revealed the presence of 3 different CD14 bearing oLC populations expressing Langerin/CD207. Besides a $CD14^{neg}/CD207^{high}$ oLC population, we could detect a $CD14^{low}/CD207^{high}$ and a $CD14^{high}/CD207^{high}$ population, of which the latter displayed the highest TLR4 expression (Fig 1, *C*). Therefore, the LPS derivative and TLR4 ligand MPL were used for stimulation. Because some recent studies

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