

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

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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 monophosphoryl 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 monophosphoryl 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- β 1, 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.

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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 pathogens¹ 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 (Fc ϵ RI),¹¹ which enables them to take up and process allergens applied during SLIT. Further on, we observed that oLCs,

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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

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 a low responsiveness to bacterial stimuli has been shown.¹³ The LPS derivative and TLR4 ligand monophosphoryl 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 Biotec, 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, Seize, 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 × 10⁵ 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 before¹⁹ 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:

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

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 (TreStar Inc).

T-cell proliferation assays

Allogeneic naive CD4⁺ T cells were isolated from PBMCs from healthy donors by magnetic microbead labeled antibodies (Miltenyi Biotec) and the AUTOMACS techniques (Miltenyi Biotec) 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

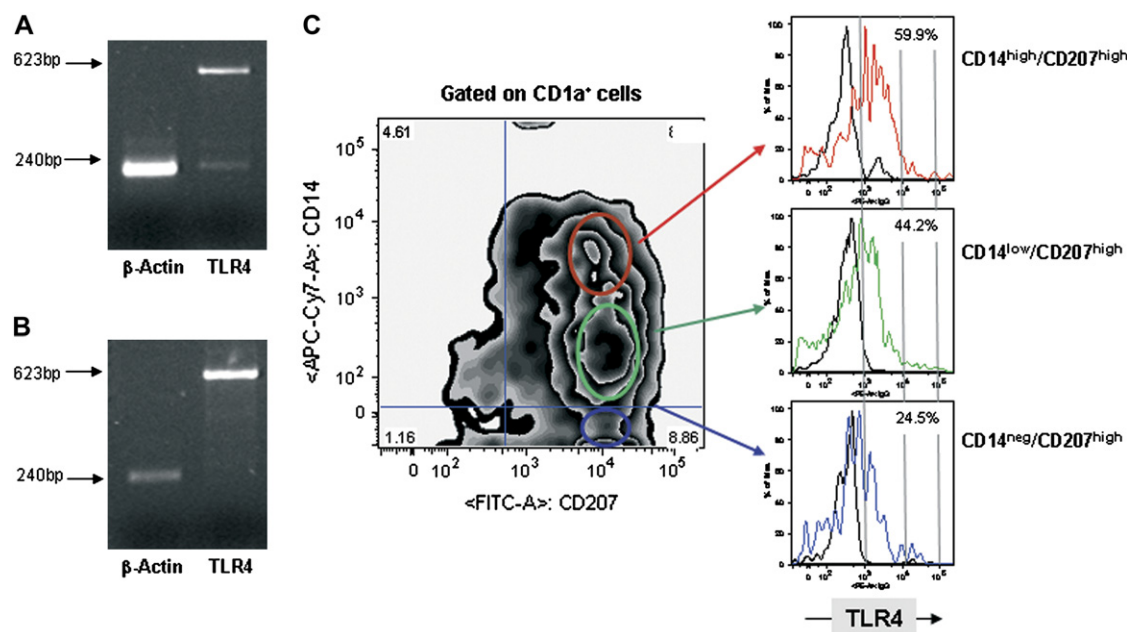


FIG 1. oLCs express TLR4. **A**, TLR4 transcripts were detected by RT-PCR in full oral mucosa tissue as well as **(B)** in highly enriched CD1a⁺ oLCs. **C**, oLCs were analyzed for their CD14 (plot, y-axis) and Langerin/CD207 (plot, x-axis) expression. All CD1a⁺ cells expressed LC-specific marker Langerin/CD207 (representative plot of $n = 3$). Next to a CD14^{neg}/CD207^{high} (blue oval) population, a CD14^{low}/CD207^{high} (green oval) and a CD14^{high}/CD207^{high} (red oval) population was detected with highest TLR4 expression on CD14^{high}/CD207^{high} (red line, upper histogram; $n = 3$). Black lines represent respective isotype controls. Values represent percentage of gated cells positive for investigated marker.

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 reported TLR4 involvement in induction of apoptosis,²⁰ we examined oLC apoptosis by Annexin-V staining in a dose-response study after stimulation with increasing MPL concentrations (from 5 to 50 μ g/mL). As a result, we could not detect a significant change of oLC apoptosis with the MPL concentration used herein compared with the control condition (data not shown).

Activation of TLR4 alters the expression of costimulatory and coinhibitory molecules of oLC

Costimulatory molecules on antigen-presenting cells (APCs) are important for the modulation of T-cell immune responses via direct APC–T-cell contact to transmit either costimulatory or coinhibitory signals to T cells. Thereby it has been shown that in particular the coinhibitory molecules B7-H1, B7-H2, and B7-H3 are involved in a tolerogenic mechanism silencing T-cell responses mediated via the immunologic synapse between APC and T cells.^{21,22} We were able to demonstrate that oLCs significantly express B7-H2 and B7-H3, whereas B7-H1 was expressed only in low amounts (Fig 2; upper row). After TLR4 activation, B7-H1 and B7-H3 were upregulated on oLCs in contrast with the control (CTR) condition, whereas B7-H2 was downregulated in both conditions (Fig 2). Furthermore, we were able to demonstrate that B7.1/CD80 was significantly upregulated whereas B7.2/CD86 expression decreased on oLCs on TLR4 activation compared with CTR (Fig 3). To investigate whether unspecific inflammatory signals lead to the same

regulation of coinhibitory molecules, oLCs were stimulated with 50 U/mL recombinant TNF- α . However, we could not detect a significant upregulation of B7-H1 and B7-H3 (data not shown). Because it has been shown previously that TLR4 activation may induce splenic dendritic cell (DC) maturation,²³ we investigated whether TLR4 activation on oLCs alters their maturation level and maturation speed, but we could not detect a significant difference in upregulation of major histocompatibility class II molecule HLA-DR of CD83 or oLCs (data not shown).

These data suggest that TLR4 activation via MPL confers oLCs into DCs with phenotypic features of tolerogenic DCs.

TLR4 activation upregulates IL-10 production by oLCs

It has been reported previously that tolerogenic DCs are preferentially induced *in vitro* under the influence of suppressive cytokines such as IL-10.^{24,25} Furthermore, it has been shown that tolerogenic DCs from the gut produce TGF- β 1. Thus we investigated whether TLR4 activation by MPL may alter IL-10 as well as TGF- β 1 production of oLCs. We were able to show that TLR4 stimulation led to a significant upregulation of the IL-10 production by oLCs (Fig 4, A). However, we could not demonstrate significant amounts of TGF- β 1 in supernatant of inactivated or in TLR4 activated oLCs (data not shown). In line with these findings, the stimulatory capacity of TLR4 activated oLCs toward allogeneic T cells was significantly reduced in comparison with unstimulated oLCs (Fig 4, B).

From this set of experiments, we conclude that TLR4 activated oLCs might have suppressive properties important for the maintenance of the characteristic immunostasis within the oral mucosa.

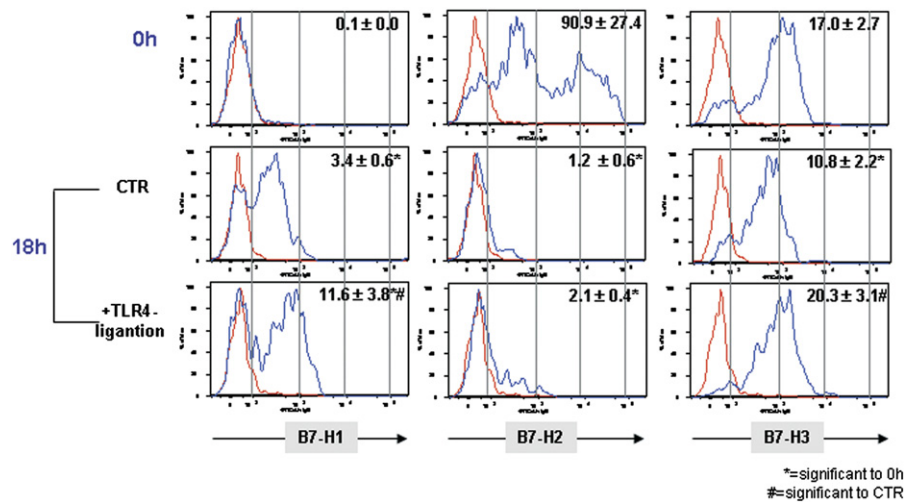


FIG 2. TLR4 ligation on oLCs increases expression of tolerogenic surface molecules. Freshly isolated oLCs (upper panel) express high amounts of B7-H2 (blue lines; second column) and B7-H3 (blue lines; third column), whereas B7-H1 was virtually absent (blue lines; first column). TLR4 ligation (lower panel) led to upregulation of B7-H1 (blue lines; first column) and B7-H3 (blue lines; third column) on oLCs (red lines, respective isotype control) compared with unstimulated oLCs (middle panel). Representative histograms of $n = 5$. Values represent rFI. *Significant to 0h; #significant to control/CTR.

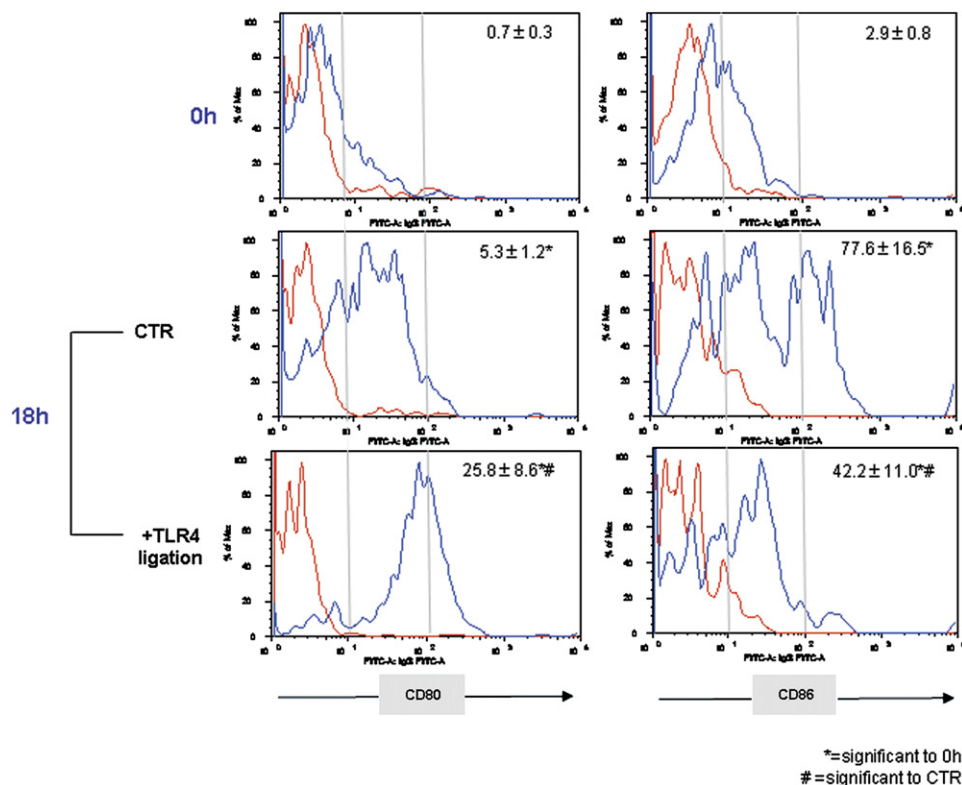


FIG 3. Differential costimulatory molecule expression on TLR4 ligation on oLCs. Representative histograms of CD80 (first column, blue line) and CD86 (second column, blue line) are shown. Red lines represent respective isotype controls. Compared with freshly isolated oLCs (upper panel), unstimulated oLCs (control, middle panel) and TLR4 ligation on oLCs led to upregulation of CD80 (first column, blue line) and CD86 (blue lines; second column; $n = 5$). Values represent rFI. *Significant to 0h; #significant to control/CTR.

TLR4 preactivated oLCs induce T cells producing IL-10, TGF- β 1, and T_H 1 cytokines

We investigated whether TLR4 ligation might induce regulatory T cells and analyzed whether TLR4 activation alters the

T-cell immune response in terms of their T_H 1, T_H 2, and T_H 3 cytokine production. We were able to demonstrate that coculture of allogeneic T cells with TLR4 preactivated oLCs led to an increased production of IL-10 as well as TGF- β 1 of the cocultured

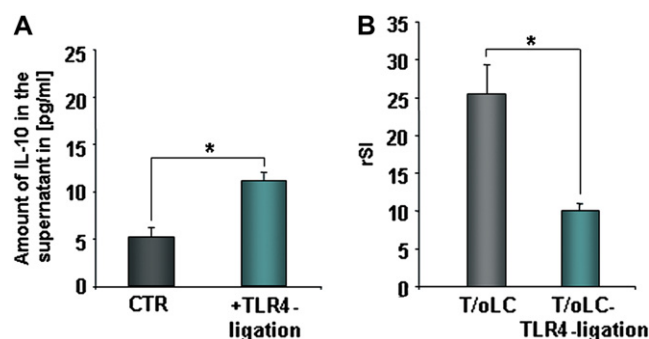


FIG 4. TLR4 ligation on oLCs increases their anti-inflammatory properties. **A**, Ligation of TLR4 led to an increase of IL-10 production by oLCs (blue bars) in comparison with oLCs that were left unstimulated (gray bars; $n = 10$). **B**, TLR4 ligation of oLCs (+oLC/+TLR4 ligation) reduced their stimulatory capacity toward T cells compared with T cells cocultured with unstimulated oLCs (T/oLC; $n = 5$).

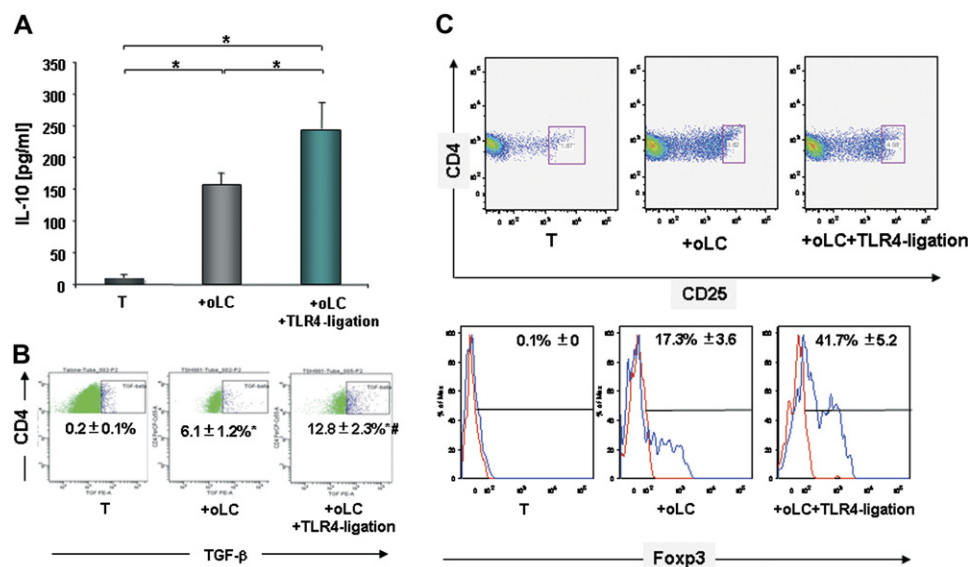


FIG 5. oLCs preactivated by TLR4 induce T cells with tolerogenic properties. Compared with T cells alone (T) and T cells cocultured with unstimulated oLC (+oLC), T cells cocultured with TLR4-activated oLCs (+oLC+TLR4-ligation) showed increased production of IL-10 (**A**) and TGF-β1 (**B**), $n = 5$. **C**, T cells were gated by CD4⁺ and CD25^{high} and further analyzed by Foxp3 expression ($n = 6$; representative dot plots and histograms).

T cells (Fig 5, A and B). Moreover, TLR4 preactivated oLCs induced significantly higher amounts of Foxp3⁺CD4⁺/CD25^{high} regulatory T cells (Fig 5, C). Furthermore, we detected a significant upregulation of the T_H1 cytokines IL-2 and IFN-γ compared with the control condition (Fig 6). Nevertheless, we could also demonstrate a low but significant rise in T_H2 cytokine IL-4 production, whereas we could not detect a significant increase of IL-5 production (Fig 6). However, because upregulation of T_H1 cytokines was relatively stronger compared with the increase of IL-4 (IL-2, 2.6-fold and IFN-γ, 2.1-fold vs IL-4, 1.5-fold), this finding suggests that TLR4 activation preferentially generates T_H1 T cells in addition to IL-10 and TGF-β1 producing T_H3 and regulatory T cells.

DISCUSSION

Several studies investigated the effect of TLR activation on the innate as well as adaptive immune response.³ It is obvious that TLR bearing LCs at immunologic sites exposed to a great number

of bacterial antigens such as the oral mucosa play a critical role in this context. Nevertheless, the immunologic mechanisms involved in TLR activation on cells of the adaptive immune response are not completely understood. Here we provide for the first time data showing that innate immunoreceptors such as TLR4 on oLCs not only transmit danger signals leading to an immunogenic reaction but also (1) induce coinhibitory molecules on oLCs involved in the alteration of immune responses toward tolerance, (2) lead to the production of the suppressive cytokine IL-10 by oLCs themselves, and (3) induce preferentially IL-10, TGF-β, and T_H1 cytokine-producing T cells in coculture experiments.

It is well known that costimulatory molecules on APCs such as LCs are critically involved in directing immune responses toward immunity or tolerance.²¹ It has been shown that plasmacytoid DCs are also critically involved in tolerance induction.⁷ However, we could previously show that in noninflammatory oral mucosa as used in this study, plasmacytoid DCs are virtually absent.¹² The newly described members of the B7 family, B7-H1, B7-H2, and

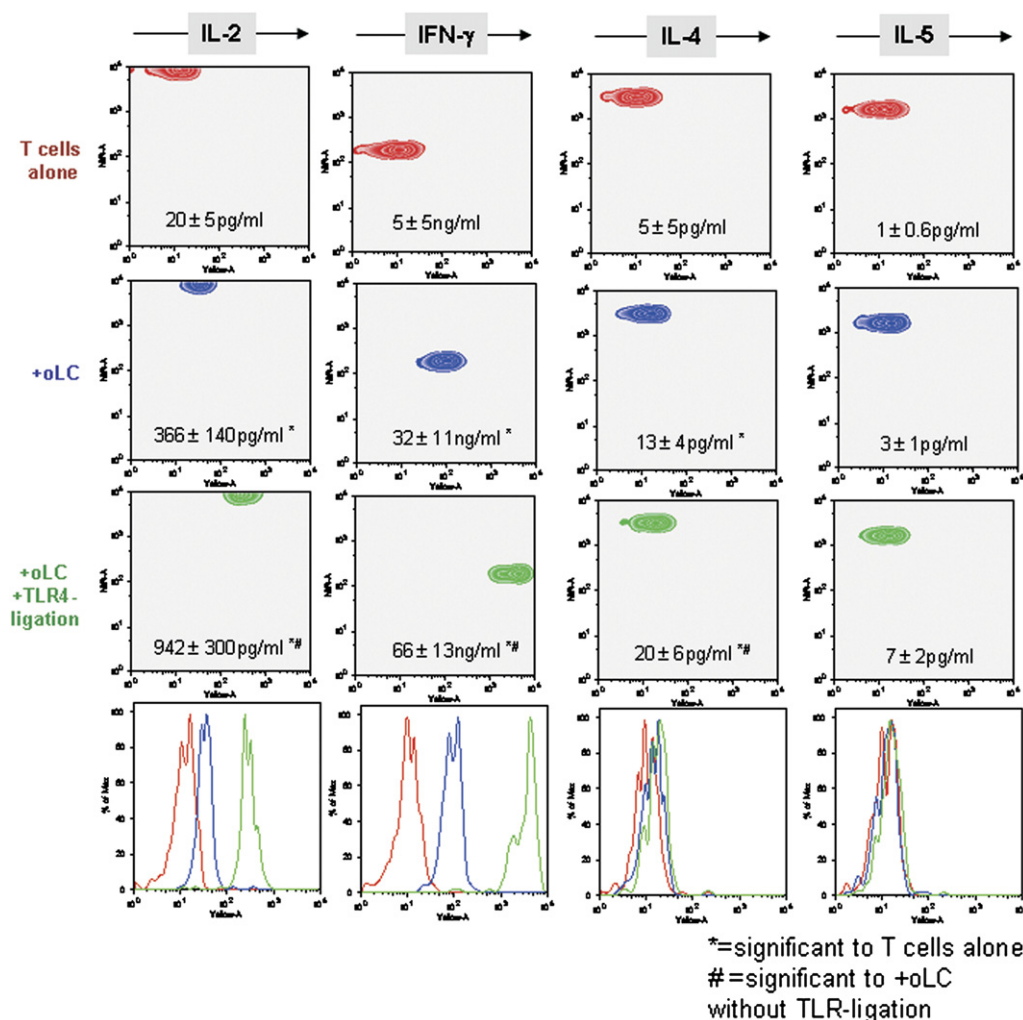


FIG 6. TLR4 ligation on oLCs induces preferentially T_H1 T cells. Cytokine production in supernatants of T cells cultured alone (upper panel, red plots), with unstimulated oLCs (second upper panel, blue plots), or TLR4 preactivated oLCs (third upper panel, green plots) was detected by beads in different near infrared channels (y-axis). Detected fluorescence (x-axis) correlated with cytokine concentrations. Histograms, lower panel, red graph = T cells alone; blue graph = T cells cultured with unstimulated oLCs; green graph = T cells cultured with TLR4 activated oLCs; n = 5, values in plots represent cytokine concentrations $\pm$ SEMs. *Significant to T cells alone; #significant to oLCs without TLR ligation.

B7-H3, have been linked to modulation of T-cell responses toward tolerance²¹ via direct cell-cell contact. The herein described upregulation of B7-H1 and B7-H3 after TLR4 stimulation might enable oLCs to induce tolerance because it has been shown that B7-H1 activates apoptosis of T cells, whereas B7-H3 induces T-cell suppression.^{21,26} In fact, we could demonstrate that blocking B7-H1 and B7-H1/B7-H3 on oLCs led to an upregulation of the T-cell response, whereas blockage of B7-H3 did not alter the T-cell response compared with the control condition, suggesting that B7-H1 contributes to the herein observed downregulation of T-cell response (data not shown). In view of the high expression of B7-H2 on oLCs in contrast with epidermal LCs (data not shown), the rapid downregulation of B7-H2 on untreated as well as TLR4 stimulated oLCs implies that it might be critical for peripheral tolerance induction of oLCs resting in an immature state, whereas B7-H1 might play a central role in maturing oLCs. In regard to the costimulatory molecules, a recent study reported that blocking B7.2/CD86 on DCs leads to tolerance induction via

B7.1/CD80/CD28 interaction.²⁷ It is therefore tempting to speculate that the observed reduced expression of B7.2/CD86 and increased expression of B7.1/CD80 on oLCs after TLR4 ligation might allow B7.1/CD80/CD28 interaction to silence the ongoing T-cell immune response. The detected decrease in T-cell proliferation and the increase of IL-10 production after coculture of oLCs prestimulated with MPL support this view. Further, MPL induced IL-10 production of oLCs might act in an autocrine way, as has been shown for IL-10 pretreated DCs *in vitro*, and transfer oLCs in an endogenous way to tolerogenic DC subtypes.²⁵ Beside that, there is some evidence that tolerogenic DCs arrest in a semi-mature state. Nevertheless, we could not detect a consistent decelerated expression of maturation markers such as CD83 on oLCs (data not shown). This observation goes along with previously published data in which MPL stimulation of *in vitro* generated DCs also showed inconsistent regulation of maturation markers.²⁸ However, compared with LPS stimulation, MPL stimulated DCs showed decelerated maturation in these studies.²⁸ Because oLCs

are constantly exposed to microbial agents such as TLR4 agonists, it seems to be reasonable that oLCs do not fully mature in response to these stimuli to prevent constant induction of inflammation and strong T-cell activation. We were able to detect an increase of IL-2 and IFN- γ production on TLR4 activation assuming an attenuated T_H1 immune response induced by MPL-triggered oLCs, which is in line with previously published data on the effect of MPL on APCs.^{29,30} It has been published that MPL is capable of downregulating inflammatory cytokine response such as IL-1 β production.^{31,32} We could demonstrate a higher production of the anti-inflammatory cytokines IL-10 and TGF- β 1 of T cells associated with an ameliorated T-cell proliferation, suggesting that the concomitant induction of regulatory T cells might lead to inhibition of the T-cell proliferation. This observation goes along with previous data showing upregulation of IL-10 production in response to LPS and the presence of regulatory T cells in chronic gingival diseases such as periodontitis.³² Taken together, the properties of MPL in the context of oLC stimulation shown here might form the basis for its successful use as adjuvant in allergen-specific immunotherapies that use the sublingual route. In view of the recently demonstrated efficacy of SLIT in treating allergic diseases,³³ it is tempting to speculate that constant exposure to LPS within the oral cavity might serve as an additional naturally tolerance-inducing adjuvant. Moreover, the data demonstrated here hold promise that TLR ligands such as MPL added to the allergen extracts could be helpful to target and enforce the immunoregulatory properties of oLCs while optimizing the efficacy of SLIT and minimizing the risk for side effects of novel immunotherapeutic approaches.

Clinical implications: The data indicate that prodefensive and protolerogenic capacities of oLCs mediated via TLR4 might be used for adjuvants in SLIT.

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METHODS

CD1a was detected by monoclonal antibody (Mab) Phycoerythrin (PE)-labeled T6RD1 (IgG1, Beckman-Coulter, Krefeld, Germany) and PE-Carbocyanin (Cy) 5-labeled HI149 (IgG1, BD Biosciences, Heidelberg, Germany). Unconjugated mAb BB-1/B7 (IgG1) reacting with B7.1/CD80, mAb B70/B7-2 (IgG1) directed against B7.2/CD86, mAb M5E2 (IgG2a) and allophycocyanin (APC)-Cy7-labeled mAb (IgG2b) MfP9 detecting CD14 and Annexin-I staining kit were purchased from BD Biosciences. Mab DCGM4 detected Langerin/CD207 (IgG1, Beckman-Coulter). Unconjugated mAb 130002 (IgG2a) detecting B7-H1, mAb 136716 (IgG2b) reacting with B7-H2, mAb 185504 (IgG1) detecting B7-H3 and PE-labeled mAb 9016 (IgG1) directed against TGF- β 1 were from R&D Systems, Wiesbaden, Germany. Unconjugated mAb HTA125 (NatuTec, GmbH, Kranenburg, Germany) and mAb PE-labeled 76B357.1 (Biomol, Hamburg, Germany) detected TLR4. MOPC-141 (IgG2b), MOPC-21 (IgG1), UPC10 (IgG2a, Sigma, Deisenhofen, Germany) and IgG1RD1 (Beckman-Coulter, Krefeld, Germany) were used as appropriate isotype controls. Fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse (GaM/FITC) antibody was from Jackson Laboratories (West Grove, PA, USA). Recombinant tumor necrosis factor- α was from R&D Systems. Normal mouse serum for blocking purposes was obtained from Dianova (Hamburg, Germany) and 7-Amino-Actinomycin-D (7-AAD) was

from Sigma. Foxp3 staining was performed by using FoxP3 staining buffer set and FITC labeled rat antibody to Foxp3 (IgG2a, clone PCH101) with respective isotype control from ebioscience (San Diego, CA., USA) following user manual. PE-Cy5 labeled mAb RDA4 (IgG1) detected CD4 and PE-labeled M-A251 (IgG1) identified CD25 (BD Biosciences).

Total RNA was extracted from oral mucosa specimen as described in detail elsewhere.¹⁷ Denaturation at 94°C for 40 s was followed by annealing of the primers at 55°C for 30s and extension at 72°C for 30s. A final extension phase of 5 min was added. Specific primer sequences for each gene were as follows: human β -actin: sense, 5'-GAG CGG GAA ATC GTG CGT GAC ATT-3'; antisense, 5'-GAT GGA GTT GAA GGT AGT TTC GTG-3', yielding a fragment of 240 bp; human TLR4 sense, 5'- CTG CAA TGG ATC AAG GAC CA -3'; antisense, 5'- TCC CAC TCC AGG TAA GTGT T-3' (fragment of 623 bp). Amplification was performed on Master Cycler Gradient (Eppendorf GmbH, Wesseling-Berzdorf, Germany). The PCR cycle numbers for the amplification of the respective cDNAs were 27 for β -actin and 32 for TLR4. Specific PCR fragments were separated on a 1% agarose gel and visualized using ethidium bromide staining. The PCR products were evaluated semiquantitatively by comparing the ratio of the specific products versus the β -actin band by digital image analysis using the WinCam system (Cybertech, Berlin, Germany).