

Tolerogenic T cells, Th1/Th17 cytokines and TLR2/TLR4 expressing dendritic cells predominate the microenvironment within distinct oral mucosal sites

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Abstract

Background: Most local oral vaccine strategies use the sublingual region for drug application. Only little is known about the cytokine microenvironment, the nature of T cell subtypes and expression of target structures for adjuvants at different oral mucosal regions (OMR). However, targeting the optimal OMR might ensure highest efficiency of drug uptake and lowest risk for adverse effects.

Methods: Expression of TGF- β 1, IL10 as well as Th1, Th2 and Th17 cytokines and transcription factors was investigated at different OMR and skin by quantitative real-time PCR, immunohistochemistry or flow cytometry.

Results: Highest number of T cells was located in vestibular/buccal region (VBR). In contrast to skin (SK), OMR T cells produced TGF- β 1, IL-10, IFN- γ and IL-17. Significantly higher TGF- β 1 mRNA expression in the VBR compared with the sublingual region (SLR) and skin could be detected, while equal transcripts of IL-10 and regulatory T cell-associated transcription factor FoxP3 could be demonstrated. Expression of Th17-associated IL-17A, IL-17F, IL-22 and IL-26 mRNA could be demonstrated in VBR and SLR but not in SK. Interestingly, compared to SK, significantly higher expression of TGF- β 1 and IFN- γ could be detected in OMR. Moreover, expression of toll-like receptor (TLR) 2 and TLR4 was highest in VBR with significant expression on dendritic cells in OMR.

Conclusion: From this data, we conclude that (i) VBR and SLR represent a protolerogenic microenvironment, (ii) both regions form a Th1 cytokine-predominated microenvironment, but also express mRNA for Th17 cytokines and (iii) TLRs detectable in VBR and SLR might serve as a target structures for adjuvants.

It is well known that antigen presenting cells (APCs) such as oral Langerhans cells (oLCs) are critically influenced by the composition of the microenvironment, which enforces either their inflammatory or their tolerogenic properties (1). The

oral mucosa is comprised of lining mucosa in the vestibular/buccal region (VBR) and masticatory mucosa in the gingival and palatal region. Moreover, the oral mucosa as a whole is considered as a protolerogenic site, because acute inflammation is rarely seen despite high frequency of contact to commensal bacteria or food (2). On the other hand and in contrast to acute inflammation, chronic inflammatory conditions such as chronic periodontitis (CP) involving the gingival epithelium are often observed (3). However, only little is known about the balance of proinflammatory and protolerogenic mechanisms within different oral mucosal regions (OMR) such as masticatory compared with lining mucosa

Abbreviations

FoxP3, Forkhead box P3; GT, gingival tissue; LC, Langerhans cells; RORC, Retinoic acid-related orphan receptor; SCIT, subcutaneous allergen-specific immunotherapy; SLIT, sublingual allergen-specific immunotherapy; SLR, sublingual region of oral mucosa; T-bet, T box expressed in T cells; T reg, T regulatory cells; VBR, vestibular/buccal region of oral mucosa.

and their impact on oLCs. Furthermore, it has been shown recently that distinct types of mucosal epithelia within the oral cavity harbour different numbers of oLCs and mast cells, which together with regional differences of the cytokine microenvironment, nature of T cell subtypes and expression of target structures for adjuvants, might profoundly impact on efficiency of local oral vaccines (4). This opens the question for new application sites for oral vaccines within the oral cavity, in particular for allergen-specific immunotherapy. Thus, various oral mucosal clinically noninflammatory epithelia were investigated for presence of T cells, protolerogenic and proinflammatory markers important for SLIT. In this context, human noninflammatory lining mucosa from the sublingual region (SLR) – the current allergen application site in SLIT – and VBR has been investigated. Moreover, the lining mucosal regions were compared with noninflammatory masticatory mucosa from gingival tissue (GT). Finally, the OMR were compared to normal skin to further gain insights into the characteristics of oral mucosal immunology in a steady-state situation.

Material and methods

Oral mucosa specimen

Punch-biopsies (Ø 8 mm) were taken simultaneously from different mucosal sites about 24 hours postmortem during routine autopsies. Lining mucosa was taken from the vestibular/buccal and sublingual region, while masticatory mucosa was taken from gingival region. Furthermore, epidermal skin was taken as control tissue. The tissue was obtained during human autopsies at the Department of Pathology, University of Bonn ($n = 20$). Individual's age ranged from 45 to 65 years (9 females; 11 males). Autopsies of individuals with malignancies of orofacial area, autoimmune diseases and undergoing chemotherapy or radiotherapy for disease were excluded. All specimens were obtained with the approval of the local ethics committee.

RNA isolation and quantitative real-time PCR analysis

For mRNA analysis, skin biopsies were homogenized using the Precellys 24 homogenizer (Peqlab, Erlangen, Germany) together with appropriate homogenization kit (Peqlab), and total RNA was extracted according to the trizol method. After phase separation through addition of chloroform and centrifugation, the aqueous phase containing RNA was selected and washed in 70% ethanol. RNA was cleaned up with RNeasy Mini Kit (Qiagen, Hilden, Germany) including digestion of genomic DNA and was subjected to cDNA synthesis with TaqMan reverse transcription reagents with random hexamers according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). The prepared cDNA was amplified using TaqMan assay Master Mix (Applied Biosystems, Foster City, CA, USA) according to the recommendations of the manufacturer in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Primers for IL10 (Hs 00174086_m1), FOXP3 (Hs 01085835_m1),

IFNG (Hs 00989291_m1), Tbet/TBX21 (Hs 00894392_m1), IL17A (Hs 00936345_m1), IL17F (Hs 00369400_m1), IL22 (Hs 00220924_m1), IL26 (Hs 00218189_m1), RORC (Hs 01076112_m1), IL6 (Hs 99999032_m1), TLR2 (Hs 00152932_m1), TLR4 (Hs 00152939_m1), CCL20 (Hs 00171125_m1) and TGFB (Hs 00998129_m1) as well as endogenous controls (18s rRNA) were from Applied Biosystems. All assays were performed according to the manufacturer's instructions. Relative quantification and calculation of the range of confidence was performed using the comparative $\Delta\Delta CT$ methods. All analyses were conducted in duplicates, and experimental samples were quantitated using sample dilutions ensuring that the Ct values were in the linear proportion of the standard curve.

Preparation of cell suspensions and immunolabelling

Oral mucosal tissue from different sites was collected in PBS buffer during autopsy and processed directly to obtain single cell suspension. Mucosal epithelial cell suspensions were prepared by trypsinization in a 0.5% trypsin buffer without Ca^{2+} for 2 hours at 37°C. Then, tissues were collected in PBS buffer and vortexed at high speed for 1 min. Afterwards, suspensions were passed through a 40 µm nylon mesh (BD Falcon, Heidelberg, Germany) to obtain single cells suspension. An indirect or direct triple-staining for a number of $0.5\text{--}2 \times 10^5$ unfixed, vital cells was performed as described in detail earlier (5). For intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm Solution Kit (BD Biosciences, Heidelberg, Germany). Prior to intracellular staining, T cells were activated by PMA and ionomycin for 12 hours. Monesin (GolgiStop, BD Biosciences) was added for the last 3 hours of culture following the instruction manual. Finally, the cells were washed twice and analysed by flow cytometry.

Flow cytometric analysis

Cells were analysed on a FACS Canto (BD Biosciences) as described in detail elsewhere. For quantitative evaluation, dead cells were excluded by 7-AAD (Sigma, Deisenhofen, Germany) staining, and dendritic cells were detected either by monoclonal antibody (mAb) phycoerythrin (PE)-labelled CD1a (T6RD1, IgG1, Beckman Coulter, Krefeld, Germany) or by Fluorescein isothiocyanate (FITC)-labelled CD1a (HI149, BD Biosciences). Expression of TLR2 was detected by mAb TL2.1 (eBiosciences, San Diego, CA, USA). TLR4 was either detected by mAb HTA125 (eBiosciences) or PE-labelled mAb 76B357.1 (Biomol, Hamburg, Germany). Allophycocyanin (APC)-conjugated goat anti-mouse (GaM/APC) or FITC-conjugated goat anti-mouse (GaM/FITC) from Jackson Laboratories (West Grove, PA, USA) were used as secondary antibodies in indirect staining protocols. Normal mouse serum for blocking purposes was obtained from Dianova (Hamburg, Germany), and 7-amino-actinomycin-D (7-AAD) to exclude dead cells was from Sigma. T cells were detected by mAb APC-Cy7-labelled CD3 (SK-7, IgG1, BD Biosciences). Cytokines were stained by PE- or FITC-labelled

mAb B27 (IFN- γ , IgG1, BD Biosciences), 8D4-8 (IL-4, IgG1, BD Biosciences), rat antibody (rAb) JES3-9D7 (IL-10, IgG1, BD Biosciences), mAb ebio64DEC17 (IL17A, IgG1, eBiosciences) and mAb 1D11 (TGF- β 1, IgG1, R&D Systems, Wiesbaden, Germany). MAb FITC- or PE-labelled MOPC-21 (IgG1, BD Biosciences) and rAb PE-labelled R35-95 were used as respective isotype controls.

Acquired data were analysed by FLOWJO software (Tree Star Inc., Ashland, OR, USA).

Immunohistochemistry

Serial paraffin wax sections (4 μ m) of oral mucosal tissue from VBR ($n = 5$), GT ($n = 5$), SLR ($n = 5$) and SK ($n = 5$) were stained by mouse monoclonal antibody specific for CD3 (Clone PS1, Novocastra, Newcastle upon Tyne, UK) to detect T cells for 1 hour at room temperature after heat-mediated antigen retrieval. LSAB2 kit (DAKO, Hamburg, Germany) was used for secondary labelling with fast red chromogen. Immunohistochemical image data were analysed and quantified by stereological image processing software IMAGE-PRO PLUS (Version 6.0; Media Cybernetics, Inc., Bethesda, MD, USA). Five random microscope fields were

selected from each slide ($\times 200$), and the number of immunopositive cell/field was counted by means of the five fields.

Statistical analysis

For statistical evaluation of statistical significances, the Wilcoxon (signed-rank) test was performed. These tests were realized using the SPSS 17.0 for Windows software (SPSS GmbH Software, an IBM Company, Munich, Germany). Results are shown as arithmetic mean $\pm$ standard error of the mean (SEM); * $P < 0.05$; no indication, not significant unless otherwise indicated.

Results

VBR harbours highest numbers of CD3⁺ cells

As T cells represent a significant source of proinflammatory and protolerogenic cytokines, various OMR were investigated for CD3⁺ T cells by immunohistochemistry. In this context, CD3⁺ T cells were localized within the mucosal dermal compartment and were significantly higher detected in OMR GT, VBR and SLR compared with skin. Within OMR, highest numbers of

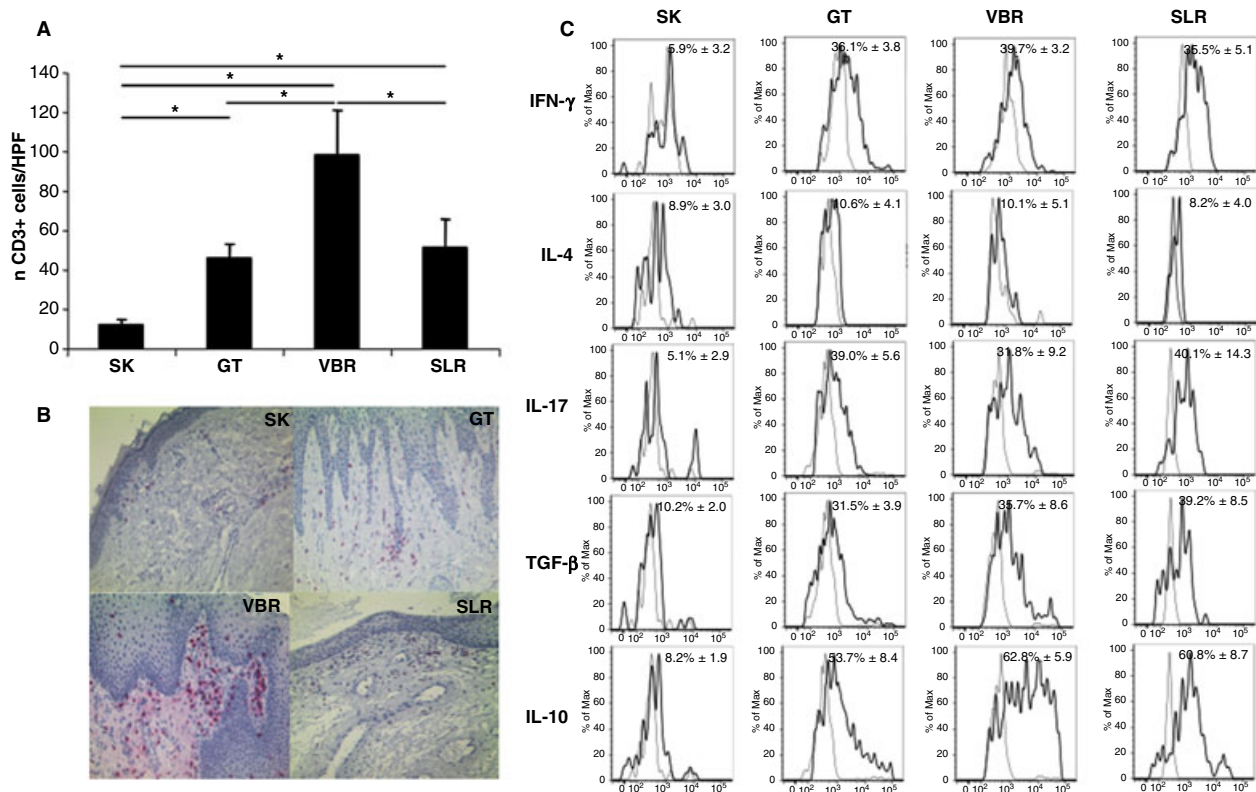


Figure 1 Highest numbers of T cells are present within VBR. (A) CD3⁺ T cells are located within papillar dermal compartment (SK, skin; GT, gingival tissue; VBR, vestibular/buccal region; and SLR, sublingual region) with significantly highest distribution within VBR. Mean value of $n = 5$ experiments of each region $\pm$ SEM as well as one representative immunohistochemical staining of each region is

depicted (B). (C) In contrast to SK, CD3⁺ T cells from GT, VBR and SLR produce IFN- γ , IL-17, TGF- β 1 and IL-10, while IL-4 is only expressed in little amounts (black line, respective cytokine; when compared with isotype control, grey line). Mean value in percentage of $n = 3$ experiments $\pm$ standard error of the mean (SEM) is given in the right corner.

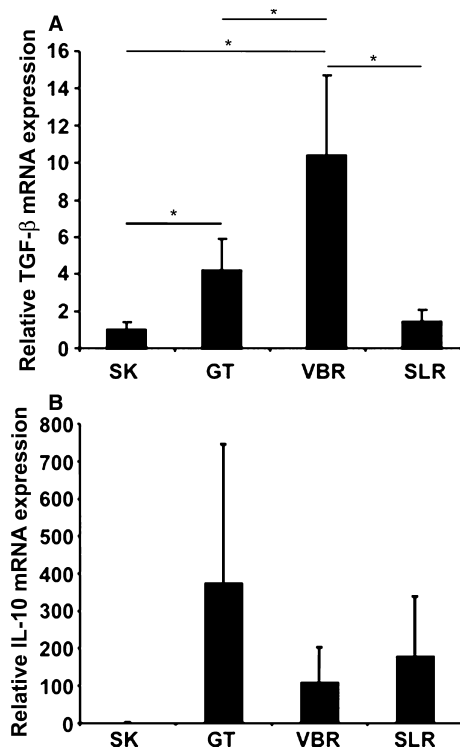


Figure 2 Oral mucosal microenvironment harbours protolerogenic cytokines. Transcripts for TGF-β1 (A) and IL-10 (B) were investigated by real-time PCR in skin (SK), gingival tissue (GT), vestibular/buccal region (VBR) and sublingual region (SLR). Mean value of $n = 7$ experiments $\pm$ SEM is shown. The highest TGF-β1 mRNA expression was detected in VBR, while IL-10 mRNA expression was comparable within all oral mucosal regions studied, but not detectable in the skin.

CD3⁺ T cells were present in VBR (Fig. 1A, B). Stimulation of single cells suspensions of SK, GT, VBR and SLR with PMA and ionomycin led to the induction TGF-β1, IL-10, IFN-γ and IL-17 producing T cells from OMR, while IL-4 could only be detected in little amounts. Moreover, T cells from SK displayed only low cytokine production after stimulation (Fig. 1C).

Higher TGF-β1 gene expression within VBR

Allergen-specific tolerance induction is thought to be a key mechanism of specific immunotherapy (6). As T cells from OMR produced comparable amounts of TGF-β1 and IL-10 upon stimulation, transcripts of the tolerogenic cytokines IL-10 and TGF-β1 were investigated in the different regions. Thereby, a significantly higher expression of TGF-β1 in VBR compared with SLR, GT and SK was observed (Fig. 2A). Transcripts of IL-10 could be detected in equal amounts in SLR and VBR (Fig. 2B). Further on, we could detect comparable mRNA expression of FoxP3 in SLR, VBR and GT, which was higher as in the skin (Fig. 3).

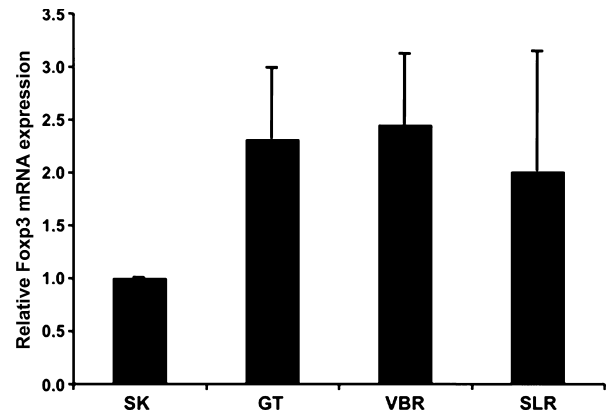


Figure 3 Higher expression of FoxP3⁺ T cells within oral mucosa compared to skin. (A) Transcripts for FoxP3 were investigated by real-time PCR in skin (SK), gingival tissue (GT), vestibular/buccal region (VBR) and sublingual region. Mean value of $n = 9$ experiments $\pm$ SEM is shown. FoxP3 expression was comparable within oral mucosal regions.

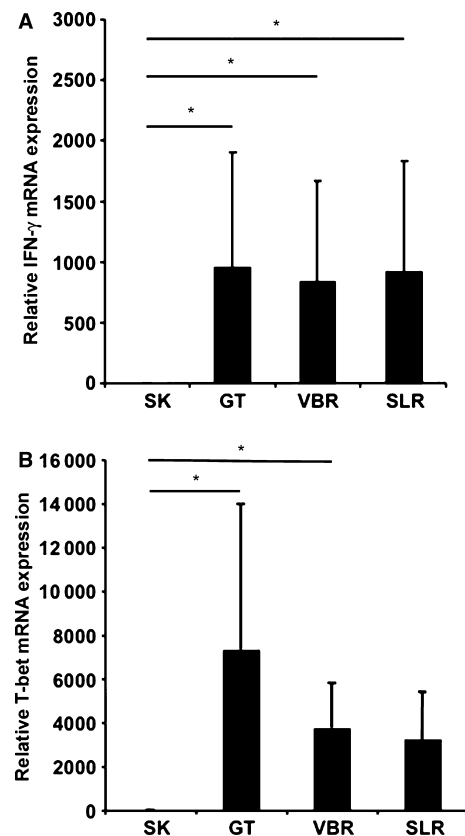


Figure 4 Preferential Th1 microenvironment within oral mucosa. Transcripts for IFN-γ ($n = 9$) and Th1 transcription factor T-bet ($n = 7$) were investigated by real-time PCR in the skin (SK), gingival tissue (GT), vestibular/buccal region (VBR) and sublingual region (SLR). IFN-γ as well as T-bet expression was comparable within oral mucosal regions, but not detectable in skin. Mean values $\pm$ SEM are shown.

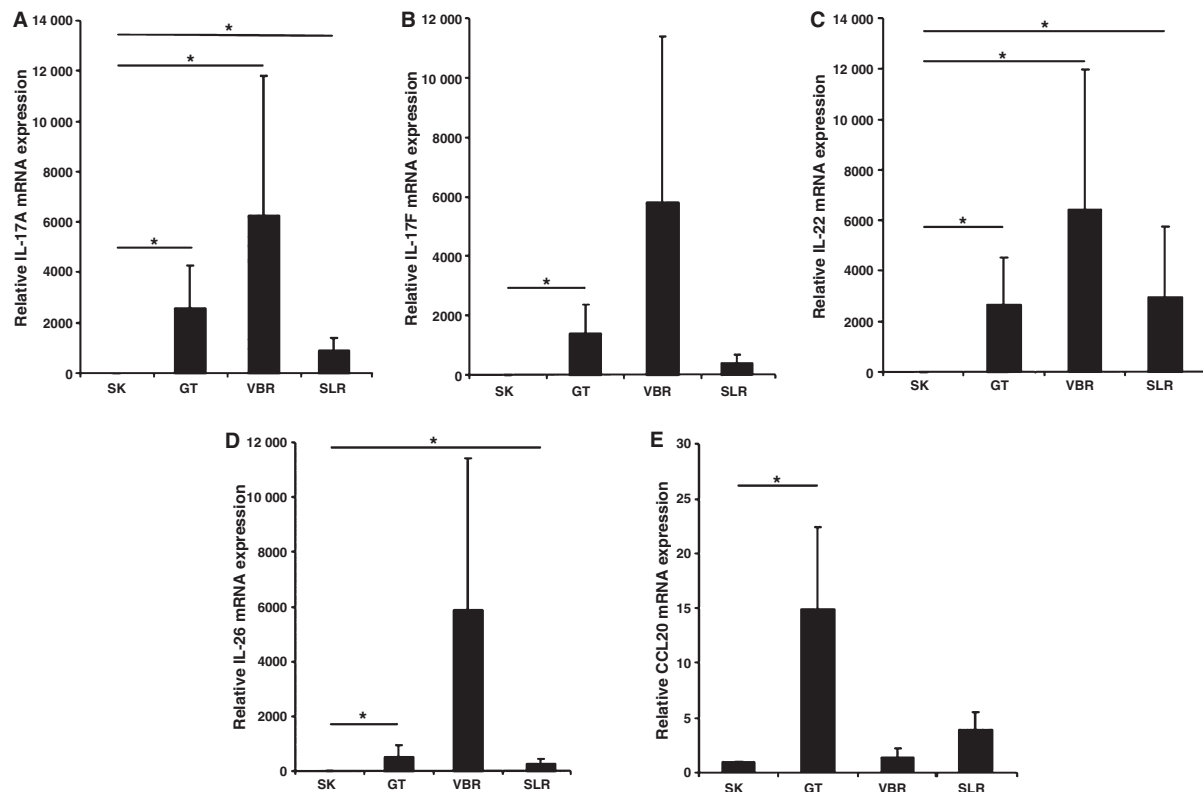


Figure 5 Th17-associated cytokines are detectable within oral mucosa. Transcripts for IL-17A (A), IL-17F (B), IL-22 (C), IL-26 (D) and CCL20 (E) were investigated by real-time PCR in skin (SK), gingival tissue (GT), vestibular/buccal region (VBR) and sublingual

region (SLR) ($n = 9$). Significant expression could be detected in VBR, GT and SLR, while IL-17A (A), IL-17F (B), IL-22 (C) and IL-26 were absent from the skin. Mean value $\pm$ SEM is shown.

High Th1 cytokine gene expression within vestibular/buccal and sublingual region

A shift from an allergy accelerating Th2 towards a Th1 immune response is thought to be another key mechanism in specific immunotherapy (7). As T cells from OMR produced comparable amounts of IFN- γ but no IL-4 upon stimulation, VBR, SLR and GT were analysed for Th2- and Th1-associated cytokine and transcription factor gene expression in comparison to SK. Significantly enhanced mRNA expression of Th1-associated IFN- γ and transcription factor T-bet could be observed in SLR, VBR and GT (Fig. 4A, B). In contrast, transcripts for Th2 related IL-4 and GATA-3 could not be detected in any of the investigated regions (data not shown).

Different distribution of Th17 cells within the oral mucosal tissue

Recently, another proinflammatory T cell subset apart from Th1 and Th2 has been described, expressing IL-17A and IL-17F and designated as Th17 cells (8). Th17 cells are also characterized by the release of IL-22, IL-26, CCL20 and expression of the transcription factor RORC. Th17 are closely related to Tregs as TGF- β 1 in combination with IL-6 propagates Th17 differentiation (9). In contrast to normal

skin, strong expression of IL-17A, IL-17F, IL-22, IL-26 and CCL20 genes as well as RORC could be detected within all investigated OMR (Figs 5A–E and 6A). Within OMR, VBR and GT displayed highest expression of Th17-related cytokines. Strongest gene expression of Th17 propagating IL-6 could be detected within GT (Fig. 6B).

Strongest gene expression of TLR4 within the VBR

Innate immune receptors such as TLRs might critically influence T cell immune responses (10). In this context, transcripts for TLR2 and TLR4 could be detected within oral mucosa at all sites studied. However, a significantly higher mRNA expression of TLR2 could be demonstrated within SLR, VBR and GT compared with the skin. Transcripts for TLR2 were equally expressed within investigated oral mucosal sites (Fig. 7A). In contrast, TLR4 mRNA expression differed profoundly within oral mucosal sites (Fig. 7B). The highest TLR4 gene expression could be detected within VBR, while expression in SLR, GT and SK was low.

Dendritic cells within oral regions express TLR2 and TLR4

It has been shown that various cell types express TLRs such as keratinocytes, fibroblasts and APCs (11). Especially, APCs

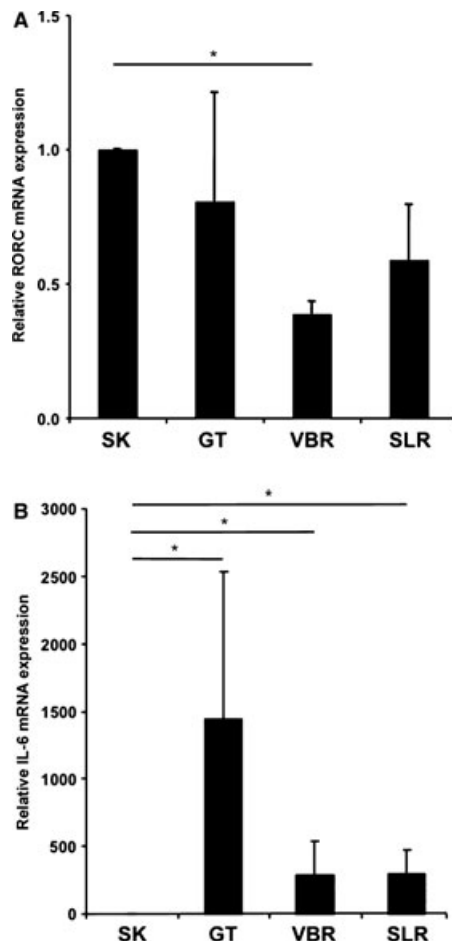


Figure 6 Th17-related transcription factor RORC and IL-6 are expressed in oral mucosa. Transcripts for Th17 transcription factor RORC (A) and IL-6 (B) were investigated by real-time PCR in skin (SK), gingival tissue (GT), vestibular/buccal region (VBR) and sublingual region ($n = 9$). Significant transcripts for IL-6 could be detected in mucosal regions in contrast to skin, while RORC was detectable in the skin as well. Mean value $\pm$ SEM is shown.

such as DCs are thought to play a central role in specific immunotherapy (12). Thus, we investigated the expression of TLR2 and TLR4 on CD1a⁺ DCs in different mucosal regions. In this context, TLR2 and TLR4 were significantly express on oral DCs but not skin DCs (Fig. 8A, B). In line with data from mRNA level, the highest expression of TLR4 could be detected within the VBR region.

Discussion

Recently, meta-analyses have provided strong support for the clinical efficiency of SLIT (13). Most scientific investigations in mucosal immunity are restricted to mucosal tissue of the gastrointestinal tract (GIT) where tolerance induction predominates through a sophisticated immunological network of inductive and effector sites (14). However, oral mucosal

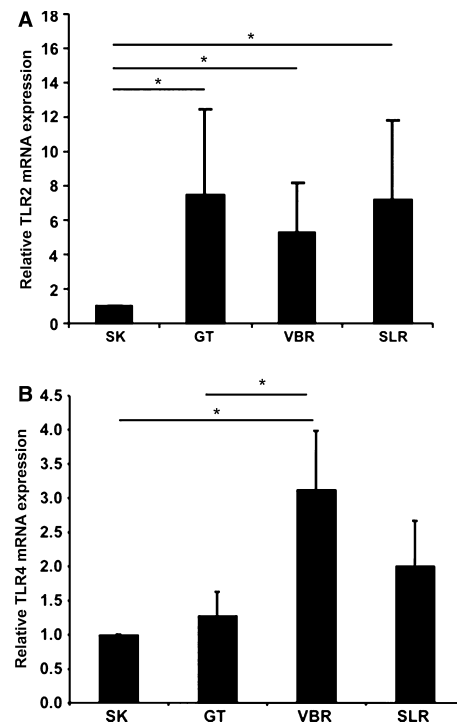


Figure 7 High expression of TLR2 and TLR4 within oral mucosa. Transcripts for TLR2 (A) and TLR4 (B) were investigated by real-time PCR in skin (SK), gingival tissue (GT), vestibular/buccal region (VBR) and sublingual region (SLR) ($n = 12$). Significant transcripts for TLR2 and TLR4 could be detected in mucosal regions in contrast to skin. The highest expression of TLR4 was detected within VBR. Mean value $\pm$ SEM is shown.

immunity still remains an area of uncertainty. Although the oral mucosa represents the entry port to the GIT, it is considered as part of a separate immunological network with many similarities to epidermal skin from the histological point of view despite of missing stratum granulosum (2). Moreover, oral mucosal tissue is subdivided into lining and masticatory mucosa with different histological and functional features, which could impact on the optimal allergen application region (2). This is further supported by a recent publication demonstrating diverse distribution of oLCs and mast cells within different OMR (4). Nevertheless, not much is known about normal oral mucosal immunity and differences within masticatory and lining mucosa.

In this report, we investigated and compared for the first time the distribution of proinflammatory and tolerogenic cytokine producing T cells as well as expression of proinflammatory and tolerogenic cytokines and transcription factors transcripts in noninflammatory lining and masticatory mucosa as well as skin. Furthermore, we performed a comparison of these markers in OMR most suitable for allergen application during SLIT – the VBR and the sublingual region. Thereby, we could demonstrate that compared to normal skin in lining and masticatory mucosa (i) CD3⁺ T cell preferentially produces TGF- β 1, L-10, IL-17 and

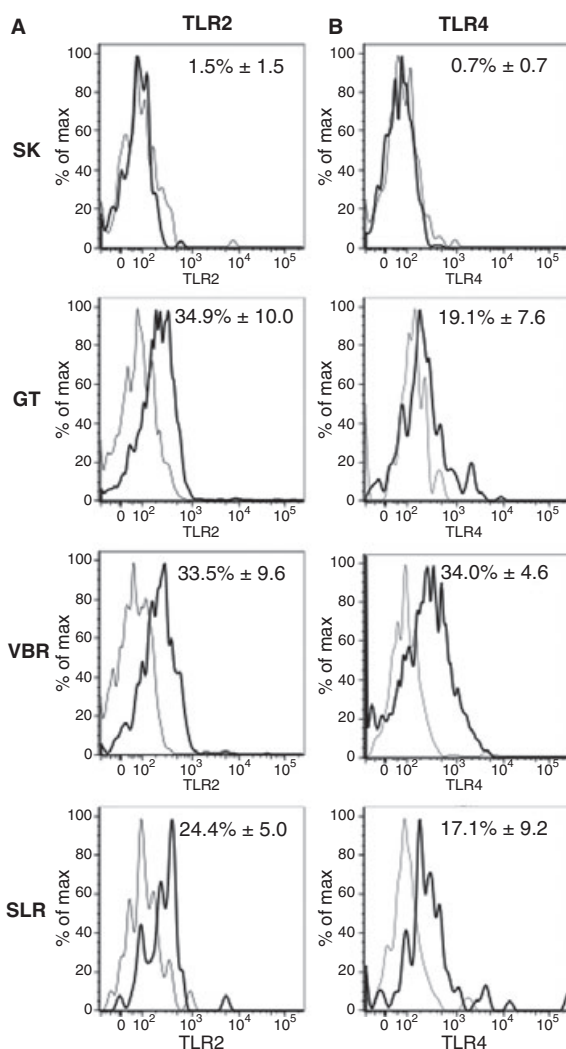


Figure 8 Expression of TLR2 and TLR4 on oral mucosal dendritic cells. Representative histograms with isotype control (grey graphs) of expression of TLR2 (A) (black graph; left column; $n = 3$) and TLR4 (B) (black graph; right column; $n = 3$) on CD1a⁺ DCs was investigated by flow cytometry in skin (SK; first row), gingival tissue (GT, second row), vestibular/buccal region (VBR, third row and sublingual region; SLR, fourth row). Percentage of positive CD1a⁺ DCs as mean $\pm$ standard error of mean (upper right corner in histograms) is shown. While TLR2 and TLR4 were strongly expressed on oral mucosal DCs in all investigated regions, TLR2 and TLR4 were virtually absent on skin epidermal DCs.

IFN- γ , (ii) TGF- β 1 and Foxp3 are significantly expressed, (iii) IFN- γ and T-bet expression are detectable, (iv) expression of Th17-related cytokines and transcription factor RORC are significant and (v) TLR4 transcripts were significantly detectable. Furthermore, although we could not demonstrate a difference in cytokine expression on protein level in CD3⁺ T cells after stimulation by comparing VBR and SLR, we observed highest detection of TGF- β 1 transcripts within VBR, reflecting a tolerogenic baseline microenviron-

ment of this oral mucosal tissue site. In turn, TGF- β 1 might influence DC phenotype and behaviour towards tolerance induction, as it has been reported that TGF- β 1 sustains tolerogenic DC potential (15, 16). Because it has been shown that the VBR region harbours the highest number of DCs within different OMR, the high TGF- β 1 expression within VBR further suggests that this region represents an attractive alternative allergen application site for SLIT. However, it is well accepted that TGF- β 1 is not only involved in tolerance induction but also critical in combination with IL-6 for Th17 cell development (9). It has been shown in various animal models that misleading Th17 immune responses play a crucial part in autoimmune diseases (8). Nevertheless, Th17 are also critically involved in protection towards microbial infection in mucosal immunity (17). As the oral mucosa is constantly exposed to microbial products, the herein reported detection of Th17 cytokines and RORC might express such protective immunity. In this context, it has been shown that especially gingival mucosa is highly exposed to various microbes such as *Porphyromonas gingivalis* causing chronic periodontitis (CP), and a recent publication indicates that Th17 responses lead to the protection of bone loss in CP (18, 19). Furthermore, the data presented here are in line with a recent published study investigating the distribution of Th17 cells and regulatory T cells within lingual region in BALB/c mice (20). Moreover, recent published data provide evidence that vaccination of mice with a single mycobacterial peptide induces IL-17 producing CD4⁺ T cells, which in turn recruit IFN- γ to restrict growth of *Mycobacterium tuberculosis* in the lung (21). In this model, IL-17 producing T cells appear to be critical not only for the recruitment but also for surveillance of Th1 cells – a mechanism that might have an impact on SLIT because an induction of Th1 cells producing IFN- γ is thought to be another key mechanism of specific immunotherapy (7). From the data obtained in this study, IFN- γ appears to play a role within normal oral mucosal immunity, as it could be detected within OMR but not the skin. However, not much is known about Th17 cells or its connection to Tregs and Th1 cells in allergen-specific immunotherapy and more studies are necessary to investigate the role of Th17 immune responses leading either to immune protection or autoimmunity.

Adjuvants have been used in vaccination strategies for long time. Recently, protocols were established using adjuvants such as the TLR4-ligand monophosphoryl lipid (MPL) in SCIT (22, 23). It has been shown that activation of TLR4 on oLCs leads to the production IL-10 and induction of FoxP3⁺ IL-10 and TGF- β 1 as well as IFN- γ producing T cells (24). Further on, a recent publication could demonstrate the presence of FoxP3⁺ Tregs in SLR upon sublingual immunotherapy (25). Thus, various TLR ligands are under investigation for potential use as adjuvants in SLIT to enhance efficacy. In this context, the data obtained from this study show an inhomogeneous TLR2 and TLR4 expression within the investigated OMR with the highest expression of TLR4 within VBR, suggesting that this region is highly susceptible for TLR4 ligands.

Nevertheless, as we were not able to distinguish between atopic and nonatopic individuals in this study, a final conclusion on differences in the microenvironment of different oral mucosal sites in such individuals can not be drawn. However, it has been published recently with the help of an *ex vivo* model that oral mucosal Langerhans cells (oLCs) of atopic and nonatopic individuals show comparable binding of grass pollen allergen *Phl p5* during resorption, production TGF- β 1 and IL-10 as well as induction of TGF- β 1 and IL-10 producing T cells. Nonetheless, further studies have to focus on this issue (26).

From this data, we conclude that (i) VBR and SLR display protolerogenic properties, (ii) both regions form a predomi-

nated Th1 microenvironment, but also express mRNA for Th17 cytokines and (iii) that TLR4 detectable on DCs in VBR and SLR might serve as a target structure for adjuvants. Thereby, VBR might represent an attractive alternative application site for vaccines, which use the oral mucosal route.

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