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ORIGINAL ARTICLE

Expression of Th1/Th2/Th3/Th17-Related Genes in Recurrent Aphthous Ulcers

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Abstract The pathogenesis of recurrent aphthous ulceration (RAU) is unknown, although an abnormal immune reaction appears to be involved. RAU may result from oral epithelium damage caused by T cell-mediated immune response. To improve understanding of the role of T cells in RAU, the present study analyzed. the expression of T cell-related genes in oral ulcers from patients with RAU, as well as in healthy non-keratinized oral mucosa from aphthae-free volunteers. Biopsies from RAU patients and healthy individuals were analyzed using Human Th1-Th2-Th3 RT^2 Profiler PCR Array and qRT-PCR that allowed to quantify the transcript levels of 86 genes related to T cell activation. We found that cells present in aphthous ulcers express a characteristic Th1-like gene profile. The majority of genes up-regulated in aphthous lesions such as IFN-γ, TNF, IL-15, IRF1, STAT-1 and STAT-4 were Th1-associated. Th2-realated genes were not overexpressed in RAU tissues, with the exception for CCR3. Th3- and Th17-related gene expression patterns were not demonstrated in RAU. These findings clearly reveal that aphthous ulcer formation is predominantly dependent on the activation of the Th1-type immune response.

Keywords Recurrent aphthous ulcers · Oral mucosa · Th-related gene expression

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Introduction

Recurrent aphthous ulcers (RAU) are the most common type of ulceration in oral cavity that affects about 20% of the world's population (Rivera-Hidalgo et al. 2004). The pathogenesis of RAU is unknown, there is, however evidence for the involvement of genetic, immunological and infectious factors (Jurge et al. 2006). Recently, it was proposed that imbalance of the $CD4^+$ Th1/Th2 immune response may contribute to loss of immune tolerance in oral mucosa causing inflammatory reaction and appearance of aphthae. Local and systemic predominance of Th1 cytokine production interferon (IFN)-γ, tumor necrosis factor (TNF), interleukin (IL)-2 and IL-12 in RAU have been reported in several studies (Albanidou-Farmaki et al. 2007; Buno et al. 1998; Dalghous et al. 2006; Lewkowicz et al. 2005; Natah et al. 2000). An increased Th1 activity in aphthous lesions has been confirmed by cDNA microarray study that revealed more intense expression of Th1-related genes (Borra et al. 2004). However, elevated levels of IL-4 and IL-5 were also demonstrated in RAU, while IL-10 production appeared to be unaffected or decreased (Buno et al. 1998; Dalghous et al. 2006; Lewkowicz et al. 2005). Recent report has also described the increased expression of chemokine RANTES and chemokine receptors CCR3 (linked with Th2 cells), CCR5 and CXCR3 (linked with Th1 cells) in aphthous ulcers (Dalghous et al. 2006). These findings together suggest a mixed Th1/Th2 response in RAU.

T helper cell subtypes, Th1 and Th2, originate from common naive precursor cells in response to antigen and cytokine stimulation. Th cells are functionally divided according to their cytokine profiles and type of mediated immune reaction. It is well recognized that disturbances in the balance between Th1 and Th2 responses can promote immune-mediated diseases. Enhanced Th2 response is involved in atopic diseases, such as asthma, whereas a dominating Th1 response is implicated in chronic autoimmune diseases, like type 1 diabetes or rheumatoid arthritis (Romagnani 1996). Recent studies have provided evidence for a third effector Th cell pathway, namely Th17, based on their production of IL-17 which is not produced by either Th1 or Th2 cells (Park et al. 2005). Th17 cells produce a range of other factors known to drive immune response, including TNF, IL-6, granulocyte–macrophage colony-stimulating factor, CXCL1 and CCL20 (Kikly et al. 2006). Proinflammatory Th17 cells appeared to be implicated in autoimmune tissue damage and had a dominant role in the induction of chronic autoimmune inflammation of the central nervous system and joints (Langrish et al. 2005).

Differentiation and expansion of $CD4^+$ effector cells is tightly controlled by T regulatory cells. Several types of T $CD4^+$ regulatory cells have been described including IL-10 producing T regulatory-1 (Tr1) cells, transforming growth factor (TGF)-β secreting Th3 cells and $CD4^+CD25^+$ $FOXP3^+$ regulatory cells. Tr1 cells exert their suppressive function mainly through IL-10 release that interferes with CD28 and inducible costimulator costimulatory signals (ICOS), and induces suppressor of cytokine signaling (SOCS) molecules (Taylor et al. 2007). Th3 regulatory cells are able to induce mucosal tolerance by migration to lymphoid tissues or target organs and suppression of immune responses through TGF-β release (Faria and Weiner 2006; Weiner 2001).

To better understand the mechanisms driving the pathogenesis of RAU, it is important to elucidate what is happening at the site of inflammation. Therefore, to address the relative contribution of different Th pathways in the pathogenesis of RAU, we evaluated the expression of genes that are associated with Th cell responses in oral ulcers form RAU patients, and for comparison, oral tissues from healthy individuals.

Materials and Methods

Patients and Samples

The study population consisted of 15 RAU patients (7 men, 8 women; mean age: 33.2 ± 5.24 years) who did not have any other systemic or inflammatory diseases and 12 healthy individuals, matched for age and sex (5 men, 7 women; mean age: 31.5 ± 6.35 years). A full blood cell count, red blood cell folate, serum levels of vitamin B12 and ferritin were performed to rule out a possible underlying haematological or gastrointestinal disorder. All RAU patients and controls, recruited from the outpatient clinic of the Department of Periodontology and Oral Medicine, Medical University (Lodz, Poland), were non-smokers and did not take any medication during the previous 2 months. The diagnosis of RAU was based on accepted clinical criteria (Porter and Scully 1999). All patients included in this study have been investigated in our clinic during previous episodes of ulceration that helped us to rule out a possible systemic cause. RAU patients had minor aphthous lesions that recurred at least one time per month during the six-month period prior to the study. Patients did not receive any treatment and their lesions healed spontaneously. Excisional mucosal biopsies were taken from healthy individuals and patients in the active stage of the disease (2–3 day-old ulcers). Normal non-keratinized lip or alveolar lining mucosa was taken during frenuloplasty or vestibuloplasty. Aphthous lesions were biopsied from the lip or buccal mucosa. The experimental protocol was approved by the Ethics Committee of Medical University of Lodz, and informed consent was obtained from all participants of the study.

RNA Isolation and cDNA Transcription

Total RNA was isolated from mucosal tissue with the use of Trisol (Invitrogen) and RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions, and treated with RNase-free DNase I (Qiagen, USA) to remove contaminating genomic DNA. One µg of the total RNA was reverse transcribed to cDNA with random hexamers and M-MMLV reverse transcriptase using ReactionReady™ First Strand cDNA Syntesis Kit (SuperArray Bioscience Corporation, Frederick, USA).

Multiple Gene Profiling Microarray

Gene expression was analyzed using Human Th1-Th2-Th3 RT^2 Profiler PCR Array (SuperArray, USA). cDNA was amplified in the presence of 84 specific primers (RefSeq accession numbers presented in Table 1), coated in 96-well microtiter plates on a Bio-Rad iCylcer® according to the following program: 95°C, 10 min (activation of HotStart DNA polymerase); 50 cycles of (95°C, 15 s; 60°C, 60 s). Separate samples from RAU individuals and controls were measured in a single run. We used RT^2 Real-Time™ SYBR Green/PCR Master Mix (SuperArray, USA) that contains all of the reagents and buffers required for qRT-PCR. The mean expression levels of the following housekeeping genes were used for the normalization of the cDNA

Table 1 Fold differences in relative gene expression in aphthous ulcers compared to normal oral mucosa

Gene symbol	Gene name	GeneBank	Fold change RAU/normal	p value
Chemokines and their receptors				
CCL11	Chemokine (C–C motif) ligand 11	NM_002986	–1.2	0.629
CCL5	Chemokine (C–C motif) ligand 5	NM_002985	–5.6	0.216
CCL7	Chemokine (C–C motif) ligand 7	NM_006273	37.8	**<0.001**
CCR2	Chemokine (C–C motif) receptor 2	NM_000648	18.9	**0.044**
CCR3	Chemokine (C–C motif) receptor 3	NM_001837	16.9	**0.009**
CCR4	Chemokine (C–C motif) receptor 4	NM_005508	8.5	0.459
CCR5	Chemokine (C–C motif) receptor 5	NM_000579	10.2	0.128
CXCR3	Chemokine (C-X-C motif) receptor 3	NM_001504	1.1	0.532
Cytokines, cytokine-related ligands, growth factors and their receptors				
TNF	Tumor necrosis factor	NM_000594	18.9	**0.034**
IFN-γ	Interferon, gamma	NM_000619	59.7	**0.004**
CSF-2	Colony stimulating factor 2	NM_000758	33.7	**<0.001**
IL-1R1	Interleukin 1 receptor, type I	NM_000877	2.2	0.541
IL-1R2	Interleukin 1 receptor, type II	NM_004633	1.9	0.673
IL-2	Interleukin 2	NM_000586	1.0	0.829
IL-2Rα	Interleukin 2 receptor, alpha	NM_000417	1.1	0.677
IL-4	Interleukin 4	NM_000589	–2.2	0.592
IL-4R	Interleukin 4 receptor	NM_000418	–2.3	0.220
IL-5	Interleukin 5	NM_000879	1.5	0.773
IL-6	Interleukin 6	NM_000600	18.4	**0.047**
IL-6R	Interleukin 6 receptor	NM_000565	1.2	0.339
IL-7	Interleukin 7	NM_000880	2.5	0.227
IL-9	Interleukin 9	NM_000590	–1.5	0.599
IL-10	Interleukin 10	NM_000572	–1.1	0.607
IL-12β	Interleukin 12 beta	NM_002187	2.2	0.543
IL-12Rβ2	Interleukin 12 receptor, beta 2	NM_001559	2.0	0.549
IL-13	Interleukin 13	NM_002188	1.4	0.867
IL-13Rα1	Interleukin 13 receptor, alpha 1	NM_001560	1.4	0.986
IL-15	Interleukin 15	NM_000585	21.1	**0.013**
IL-17α	Interleukin 17alpha	NM_002190	3.2	0.563
IL-18	Interleukin 18	NM_001562	6.4	0.254
IL-18R1	Interleukin 18 receptor 1	NM_003855	12.9	**0.045**
IL-23α	Interleukin 23, alpha subunit p19	NM_016584	–1.2	0.559
TGF-β3	Transforming growth factor, beta 3	NM_003239	1.5	0.544
INHα	Inhibin, alpha	NM_002191	0.7	0.943
INHβA	Inhibin, beta A	NM_002192	18.7	**0.027**
IRF1	Interferon regulatory factor 1	NM_002198	13.4	**0.032**
IRF4	Interferon regulatory factor 4	NM_002460	1.3	0.455
Cell receptors and costimulatory molecules				
CD4	CD4 molecule	NM_000616	27.3	**0.034**
CD27	CD27 molecule	NM_001242	–5.6	0.337
CD28	CD28 molecule	NM_006139	–45.6	**0.008**
CD40LG	CD40 ligand	NM_000074	12.3	**0.045**
CD80	CD80 molecule	NM_005191	21.1	**0.033**
CD86	CD86 molecule	NM_006889	27.4	**0.017**
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4	NM_005214	15.2	**0.042**

Table 1 continued

Gene symbol	Gene name	GeneBank	Fold change RAU/normal	*p* value
FASL	Fas ligand	NM_000639	0.5	0.772
HAVCR2	Hepatitis A virus cellular receptor 2, T cell Ig mucin-3	NM_032782	1.3	0.559
ICOS	Inducible T-cell co-stimulator	NM_012092	18.6	**0.040**
IGSF6	Immunoglobulin superfamily, member 6	NM_005849	18.0	**0.013**
SFTPD	Surfactant, pulmonary-associated protein D	NM_003019	–2.1	0.447
TLR4	Toll-like receptor 4	NM_138554	–1.2	0.884
TLR6	Toll-like receptor 6	NM_006068	12.4	**0.033**
TNFSF4 (OX40L)	Tumor necrosis factor (ligand) superfamily, member 4	NM_003326	–2.8	0.664
TNFRSF8	Tumor necrosis factor receptor superfamily, member 8 (CD30)	NM_001243	1.9	0.455
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	NM_001561	0.5	0.932
Transcription/signaling molecules				
CEBPB	CCAAT/enhancer binding protein, beta	NM_005194	1.1	0.848
CREBBP	CREB binding protein	NM_004380	0.8	0.975
GATA3	GATA binding protein 3	NM_002051	0.6	0.933
GFI1	Growth factor independence 1	NM_005263	1.1	0.866
GLMN	Glomulin, FKBP associated protein	NM_053274	2.8	0.730
GPR44	G protein-coupled receptor 44	NM_004778	–1.1	0.556
JAK1	Janus kinase 1	NM_002227	0.9	0.655
JAK2	Janus kinase 2	NM_004972	19.4	**0.028**
LAG3	Lymphocyte-activation gene 3	NM_002286	–0.7	0.883
LAT	Linker for activation of T cells	NM_014387	21.9	**0.033**
MAF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog	NM_005360	0.5	0.895
MAP2K7	Mitogen-activated protein kinase kinase 7	NM_145185	1.2	0.833
MAPK8	Mitogen-activated protein kinase 8	NM_002750	0.9	0.912
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	NM_172390	23.8	**0.017**
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	NM_012340	1.7	0.872
NFATC2IP	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein	NM_032815	27.1	**0.007**
PCGF2	Polycomb group ring finger 2	NM_007144	–1.3	0.651
PTPRC	Protein tyrosine phosphatase, receptor type, C (CD45)	NM_002838	16.3	**0.012**
SOCS1	Suppressor of cytokine signaling 1	NM_003745	21.2	**0.021**
SOCS2	Suppressor of cytokine signaling 2	NM_003877	0.3	0.779
SOCS3	Suppressor of cytokine signaling 3	NM_003955.3	12.4	**0.012**
SOCS5	Suppressor of cytokine signaling 5	NM_144949	–0.3	0.779
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	NM_000582	0.2	0.882
STAT1	Signal transducer and activator of transcription 1	NM_007315	21.9	**0.016**
STAT4	Signal transducer and activator of transcription 4	NM_003151	25.3	**0.009**
STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	NM_003153	2.1	0.442
T-bet	T-box 21 (TBX21)	NM_013351	3.1	0.339
TFCP2	Transcription factor CP2	NM_005653	0.4	0.809
TMED1	Transmembrane emp24 protein transport domain containing 1	NM_006858	1.2	0.445
TYK2	Tyrosine kinase 2	NM_003331	–2.9	0.592
YY1	YY1 transcription factor	NM_003403	–1.1	0.329

Total RNA from aphthous ulcers and normal mucosa was subjected to reverse transcription and real-time PCR for the reference genes (hypoxanthine phosphoribosyltransferase 1, β-actin and glyceraldehyde-3-phosphate dehydrogenase) and the relevant Th1/Th2/Th3/Th17-related genes. The fold changes in gene expression were calculated using the ΔΔCt method. The statistically significant *p* values are marked in bold type

samples: hypoxanthine phosphoribosyltransferase 1, β-actin and glyceraldehyde-3-phosphate dehydrogenase.

Analysis of SOCS-3 mRNA Using Quantitative Real-Time RT-PCR

All the reagents were provided by SuperArray, USA. cDNA was amplified in the presence of specific primers for SOCS-3 (NM_003955.3) in 96-well microtiter plates on a Bio-Rad iCylcer® according the following program: 95°C, 15 min (activation of HotStart DNA polymerase); 50 cycles of (95°C, 30 s; 55°C, 30 s; and 72°C, 30 s). Separate samples from RAU individuals and controls were measured in triplicate. As a positive control cDNA synthesized from Human XpressRef™ Universal Reference Total RNA was used. Specificity of the reaction was checked by melting curve analysis, and relative expression of the genes was determined by the means of software program supplied by BioRad, USA.

Statistics

Data from real-time PCR were calculated using the $\Delta\Delta C_t$ method and the PCR Array Data Analysis Template v3.0 (SuperArray, USA) as previously described (Hansel et al. 2008).

The fold differences in relative gene expression between normal oral mucosa and aphthous ulcers were calculated according to the formula:

$$\frac{\frac{2^{-C_t(\mathrm{GOI})}\mathrm{RAU}}{2^{-C_t(\mathrm{HKG})}\mathrm{RAU}}}{\frac{2^{-C_t(\mathrm{GOI})}\mathrm{Normal}}{2^{-C_t(\mathrm{HKG})}\mathrm{Normal}}} = \frac{2^{-[C_t(\mathrm{GOI})-C_t(\mathrm{HKG})]}\mathrm{RAU}}{2^{-[C_t(\mathrm{GOI})-C_t(\mathrm{HKG})]}\mathrm{Normal}} = \frac{2^{-\Delta C_t}\mathrm{RAU}}{2^{-\Delta C_t}\mathrm{Normal}} = 2^{\Delta\Delta C_t}$$

where GOI is the gene of interest, HKG the housekeeping gene, C_t the threshold cycle; RAU–aphthous ulcers; Normal–mucosa of healthy individuals. $\Delta\Delta C_t$ is equal to ΔC_t (RAU) $-$ ΔC_t (normal).

Statistical significance of differences among the groups was determined by the-Student's t test (for normal distribution and equal variances), Cochran Q test (for normal distribution and unequal variances) or Wald-Wolfowitz runs test (for non parametric distribution), following calculation of the mean $\pm$ SD of each gene for both groups. The verification of normal distribution and analysis of variances were made using the Kolmogorov–Smirnov test and the Fisher's test. $p \leq 0.05$ was considered as the significant difference.

Results

The gene expression results identify one down-regulated and 28 up-regulated genes ($p \leq 0.05$) in aphthous ulcers compared to healthy mucosa (Table 1). Further seven genes demonstrated at least a 3-fold difference in expression between aphthous tissue and normal tissue, but p values did not reach statistical significance. In this case, up-regulation was observed in five genes, while two genes appeared to be down-regulated in the aphthous ulcer samples.

A subset of eight genes represented chemokines and their receptors (Table 1). CCL7, CCR2 and CCR3 were significantly up-regulated ($p < 0.001$, $p = 0.044$ and $p = 0.009$ respectively). Further three genes demonstrated a non-significant difference of at least 3-fold: the genes for CCR4 and CCR5 were up-regulated, while CCL5 was down-regulated.

Another predominant functional group consisted of genes coding for cytokines, cytokine-related ligands, growth factors and their receptors. Significant up-regulation was observed in eight genes: TNF ($p = 0.034$), IFN-γ ($p = 0.004$), IL-6 ($p = 0.047$), IL-15 ($p = 0.013$), IL-18R ($p = 0.045$), INHBA ($p = 0.027$), CSF-2 ($p < 0.001$) and IRF1 ($p = 0.032$). Further, two genes demonstrated a non-significant up-regulation of at least 3-fold: IL-17α and IL-18.

A total of nine genes related to cell receptors and costimulatory molecules had significantly different level of expression in the aphthous ulcers than in the normal mucosa tissues, with eight genes showing up-regulation: CD4 ($p = 0.034$), CD40L ($p = 0.045$), CD80 ($p = 0.033$), CD86 ($p = 0.017$), ICOS ($p = 0.040$), CTLA-4 ($p = 0.042$), IGSF6 ($p = 0.013$) and Toll-like receptor (TLR)6 ($p = 0.033$) and one gene showing down-regulation: CD28 ($p = 0.008$). Additionally, the expression of CD27 mRNA was 5.6 times lower in ulcerative tissues, but the difference was not statistically significant.

A subset of 31 genes represented transcription factors and signaling molecules. Up-regulation was shown in nine genes: JAK2 ($p = 0.028$), LAT ($p = 0.033$), NFATC1 ($p = 0.017$), NFATC2IP ($p = 0.007$), PTPRC ($p = 0.012$), STAT1 ($p = 0.016$), STAT4 ($p = 0.009$), SOCS-1 ($p = 0.021$) and SOCS-3 ($p = 0.012$). We also demonstrated 3.1-fold up-regulation of T-bet, but the difference was not statistically significant.

Discussion

We have conducted a gene expression profiling using Th1-Th2-Th3 RT^2 Profiler PCR Array to compare the relative expression of genes involved in Th cell responses in aphthous ulcers from RAU patients and non-keratinized mucosa from healthy individuals. Aside from analysis of Th1, Th2, Th3 and Th17-related genes, the PCR array allowed us to have an insight in wider aspects of immune

regulation in RAU. Additionally, we performed a separate analysis of SOCS-3 mRNA expression to better determine immunoregulatory mechanisms that help to maintain a local and self-limited character of aphthous ulcers.

In this study we demonstrated that genes up-regulated in aphthous ulcers predominantly encoded factors associated with Th1 polarization such as IFN-γ and TNF. Elevated amounts of IFN-γ and TNF in RAU both at mRNA and protein were consequently demonstrated in previous studies (Albanidou-Farmaki et al. 2007; Buno et al. 1998; Dalghous et al. 2006; Lewkowicz et al. 2005; Natah et al. 2000). IFN-γ, primarily produced by Th1 lymphocytes and NK cells, not only stimulate cytotoxic immune responses, but also promotes Th1 cells differentiation and inhibit Th2 polarizations. The predominance of Th1 immune response is further confirmed by elevated levels of mRNA for transcriptional factors IRF1, STAT-1 and STAT-4. However, other Th1-related genes such as IL-2 or IL-12 were not up-regulated in our study, which is in dissonance with the previous investigation (Buno et al. 1998; Dalghous et al. 2006). These discrepancies may be explained by application of different techniques for cytokine measurement in our and other studies. Cytokines were detected at protein level using immunohistochemistry that refers to a percent of cytokine-positive cells (Dalghous et al. 2006), or at mRNA level using semi-quantitative southern blot analysis (Buno et al. 1998), while we determined mRNA levels using quantitative real-time PCR assay.

Activated Th1 lymphocytes promote cytotoxic immune response by stimulating CD8^{+} Tc cells and monocytes. Cytotoxity may be further supported by IL-6, IL-15 and CSF-2 that appeared to be up-regulated in aphthous ulcers. IL-15, which is mainly produced by dendritic cells (DCs) and monocytes/macrophages, is a strong inducer of NK cell differentiation. Thus, ulcer formation in RAU may result not only from Tc cell but also from NK cell cytotoxity. The importance of IL-15 in RAU pathogenesis is additionally supported by the recent findings showing an increased IL-15 serum level in RAU patients (Curnow et al. 2008).

In our study Th2-related genes were not up-regulated. No differences in IL-4, IL-5, IL-9, IL-10, IL-13, STAT-6, GATA-3, IRF4 or TNFSF4 mRNA expression were seen between RAU and healthy tissues. These findings are partially in agreement with the previous investigation. It was demonstrated that IL-4 and expression in RAU is comparable to healthy mucosa (Dalghous et al. 2006), or increased in aphthous ulcers (Buno et al. 1998). IL-10 expression was reported to be similar in RAU and healthy mucosa (Buno et al. 1998; Dalghous et al. 2006) or decreased in RAU (Miyamoto et al. 2008).

As Th3 cells are known for the production of large amounts of TGF-β, and their development is mainly dependent on TGF-β, we concluded based on the low expression of TGF-β mRNA that Th3 regulatory cells are not induced during inflammatory response in RAU. In spite of low TGF-β mRNA expression in RAU, we demonstrated other features of negative immune regulation such as elevated levels of CTLA-4, SOCS-1 and SOCS-3, and decreased levels of CD28 mRNA. Considering that aphthous biopsies were taken on the second or third day after formation of ulcers, activation of genes related to immune suppression was a surprising finding. The induction of suppressive mechanisms during an early immune response in aphthae may explain a self-limited character of aphthous ulcers that usually appear as small $<$1 cm lesions that heal within 7–10 days. However, it is not clear how the suppression is triggered. It is unlikely dependent on the action of CD4^{+}CD25^{+} T regulatory cells, as our previous study showed that only one ulcer biopsy of eight was positive for FOXP3 mRNA (Lewkowicz et al. 2008).

Analysis of cytokine genes such as IL-17 and IL-23 that are associated with Th17 cell development and function showed their unchanged expression in RAU compared to normal tissue. Thus, Th17 cell population seems not to be involved in RAU-associated inflammatory response.

The majority of lymphocytes present in the aphthous ulcers are T CD4^{+} and CD8^{+} cells that are encountered in similar proportions (Dalghous et al. 2006). We have analyzed CD4 expression only in our study, and demonstrated its 27-fold up-regulation in aphthous ulcers. Analysis of gene expression for costimulatory molecules showed up-regulation of CD40L and ICOS that are present on activated lymphocytes. Additionally, increased expression of CD80, CD86 and IGSF6 in aphthous ulcers, costimulatory molecules that are related to DCs, may point out to the antigen uptake by DC and their maturation.

Lymphocyte migration from peripheral lymphoid tissue to the site of inflammation is possible due to expression of chemokine receptors and their ligands. CXCR3 (chemokine receptor for IP-10 and MIG) and CCR5 (chemokine receptor for MIP-1 and CCL5) are predominantly expressed on the surface of Th1 polarized T cells (Sallusto et al. 1998). Despite the evidence that the expression of CXCR3 and CCR5 is up-regulated in RAU on protein level (Dalghous et al. 2006), we failed to detect the considerable increase in mRNA expression of both CXCR3 and CCR5. Further, we demonstrated the increased expression of CCR2 and CCR3 (linked with Th2 cells) that was in agreement with the previous immunohistochemical study (Dalghous et al. 2006). We also found a 38-fold up-regulation of CCL7 that is among the most pleiotropic chemokines since it activates all major leukocyte classes by binding to CCR1, CCR2 and CCR3, and can be produced by different cell types, including fibroblasts, epithelial cells

and monocytes (Menten et al. 1999, 2001). Our findings indicate the engagement of CCL7 and its receptors CCR2 and CCR3 in immune cell recruitment in RAU that unexpectedly highlights the role of Th2 cells. These findings might be explained by the stage at which the biopsy was taken. During the pre-ulcerative stage, the mRNA levels of CXCR3 and CCR5 might have been higher, as during this stage inflammatory cells start to migrate to the site of inflammation, while 2–3 days later the inflammatory response would subside. Therefore, transcription of CXCR3 and CCR5 will drop, while at protein level these receptors would still be detected. On the contrary, at the 2–3 day of ulcer Th2 cells may appear to attenuate the inflammatory reaction.

Another major observation made in our study is the significant up-regulation of TLR6 mRNA in RAU. TLRs are expressed by the variety of cells such as keratinocytes, fibroblasts, endothelial cells, DCs, macrophages, neutrophils (Miyake 2007). TLR6 is responsible for the recognition of different structures of Gram-positive bacteria that are abundant in the oral cavity and activation of Th1-like immune response (Nakao et al. 2005). Additionally, it seems that TLR6 overexpression did not only result from the bystander activation, as TLR4, that is responsible for the recognition of Gram-negative bacteria, was not up-regulated in RAU. Thus, we may speculate that overexpression of TLR6 in the oral mucosa may lead to excessive immune response towards commensals. Indeed, some previous studies suggested the role of Gram-positive bacteria in RAU pathogenesis (Hasan et al. 2002; Sun et al. 2002). More recently, an impaired signaling through TLR2 has been demonstrated in RAU patients which may inhibit Th2-type immune response and, in consequence, promote an abnormal Th1-type immune response (Borra et al. 2009).

In conclusion, we showed that Th1-associated genes are overexpressed in RAU in the absence of Th3- or Th17-type gene up-regulation. The role of Th2 cells in RAU pathogenesis remains unclear as the only increased marker of their presence demonstrated in our study was CCR3. The data also suggest that the acute inflammation in RAU is efficiently neutralized by simultaneous induction of anti-inflammatory response that promote resolution of inflammation and tissue healing. Further comparisons of the expression of most crucial T cell-related factors in aphthous ulcers using immunohistochemistry may reveal the specific pathways of the immune response that are responsible for ulcer formation.

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