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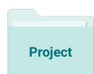

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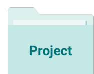

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METHYL MERCAPTAN AND HYDROGEN SULFIDE PRODUCTS STIMULATE PROINFLAMMATORY CYTOKINES IN PATIENTS WITH NECROTIC PULP TISSUE AND ENDODONTICALLY TREATED TEETH

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Bacterial infections of the residual dentin or infected pulp tissue are responsible for most cases of endodontic treatment failures. Persisting microorganisms in necrotic pulp tissue produce sulphur components such as methyl mercaptan and hydrogen sulfide as well as thioether derivatives. Although there is emerging evidence that these sulphur compounds stimulate immune cells and induce the inflammatory cascade, the immunological mechanisms of local and systemic inflammation have not been described. In this retrospective study we evaluated the *ex-vivo* immune response of peripheral blood mononuclear cells to sulphur compounds in 53 patients with clinical or radiologic endodontic treatment failure, 20 patients with clinical discomfort or radiological findings without previous endodontic treatment and a control group of 31 patients who had received successful endodontic treatment at least five years previously. Patients with endodontic abnormalities showed significantly higher *ex-vivo* sulphur compound-stimulated interferon-gamma (IFN-γ) and interleukin-10 (IL-10) levels as compared to the control group. The association between *ex-vivo*-stimulated cytokines and endodontically derived sulphur compounds was further substantiated by the fact that the number of IFN-γ and/or IL-10-positive patients decreased significantly 3-8 months after re-treatment of the root canal or tooth extraction. Furthermore, serum tumor necrosis factor-alpha (TNF-α) levels were higher in patients than in controls, and at the same time, the TNFA -308 G/A polymorphism was associated with endodontic treatment failure in our study population. We conclude that a cellular immune response to sulphur compounds contributes to the inflammatory process observed in relation to endodontic treatment failures.

PROOF

According to recent studies, state-of-the-art endodontic treatments are successful in up to 90% of cases. In cases of recurrent inflammation revisions by endodontic specialists can achieve success rates of about 80%, which is significantly better than the success rates of dentists without respective specialization (1). Despite these achievements, even today 12% of patients suffer from persisting low-to-moderate grade adverse effects post-endodontic surgery, despite adherence to the approved protocol (2). The re-treatment success rate for primarily unfound and untreated canals is approximately 80% (3, 4). A recently published meta-analysis comparing the literature published between 1922 and 2002

Key words: pulp inflammation, methyl mercaptan, hydrogen sulfide, thioether, interferon-γ, interleukin-10, tumor-necrosis-factor-α

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yielded an average success rate of 75% for re-treated root canals with apical periodontitis (5). According to the statements of the German Society of Dental, Oral and Craniomandibular Sciences (DGZMK) and the German Society for Conservative Medicine (DGZ), the available data, however sparse, support a full recovery rate of 90% within 3 to 4 years. At the same time, the frequency of post-endodontic apical periodontitis may have been underestimated due to inadequate radiological imaging (6).

It is generally accepted that persisting bacterial infections in dentin or pulp tissue account for most cases of treatment failure (7). Complete elimination of bacterial infections is limited by currently available techniques, and persisting microorganisms located in necrotic pulp tissue produce a wide range of metabolites. Sulphur compounds, including methyl mercaptan and hydrogen sulfide as well as thioether derivatives, such as dimethyl sulfide and diethyl sulfide, are metabolic products mainly generated by anaerobic bacteria through desulfuration of cysteine-rich glutathione, L-methionine and L-methionyl-containing peptides. The main producers of hydrogen sulfide products are *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Treponema denticola* and *Veillonella alcalescens*. Methyl mercaptan is predominantly produced by *Porphyromonas gingivalis*, *Porphyromonas intermedia* and *Fusobacterium nucleatum* (8).

Sulfate-reducing bacteria are etiologically involved in destructive periodontal diseases and promote disease progression (9). Sulfide compounds are detectable in gingival fluid of periodontal pockets (8). It has been described that these sulphur compounds can exert toxic effects with the central nervous system as primary target. Hydrogen sulfide can severely alter the neural architecture and growth characteristics, as shown for Purkinje cells (10). In the oral cavity, hydrogen sulfide damages epithelial cells and increases mucosal permeability (11). Methyl mercaptan decreases collagen synthesis by human fibroblasts inhibiting cell migration to periodontal ligament cells, potentially contributing to the pathogenesis of periodontal disease (12).

Sulphur compounds can also exert activating or modulating effects on immune cells, contributing to inflammatory tissue degradation. Methyl mercaptan has been shown to promote the production of interleukin 1 (IL-1) by human mononuclear cells. Similarly, the production of interleukin 6 (IL-6) stimulated by lipopolysaccharides (LPS) is increased in the presence of methyl mercaptan (13). Furthermore, methyl mercaptan alone or in combination with IL-1 or LPS, can significantly enhance the secretion of PGE2 and cAMP, which are required for the synthesis of matrix metalloproteinases and other procollagenases in human gingival fibroblasts and polymorphonuclear leucocytes (14). These effects may promote production of collagenase and subsequent tissue destruction in human periodontal disease. Methyl mercaptan also enhances the activity of aspartate protease cathepsin B in gingival infiltrated leucocytes, increasing the rate of collagen degradation (14).

The majority of data on sulphur compounds in tissue degradation have been derived from studies on periodontal diseases. Conversely, a putative role of methyl mercaptan and thioethers in necrotic pulp tissue and chronic pulpitis has not been substantiated, even if several studies have shown that anaerobic bacteria in the root canal are the predominant cause for endodontic treatment failures. Lin and coll. demonstrated the presence of stainable bacteria in root canals in 69% of patients suffering from endodontic treatment failures. Furthermore, the severity of periradicular inflammation correlates to presence of bacteria in the root canal (15). Non-vital teeth are less resistant to bacterial invasion into dentinal tubules than vital teeth (16). Anaerobes most frequently isolated from primarily and secondarily infected root canals are sulfate-reducing bacteria similar to those found in periodontal disease, including *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, the main producers of methyl mercaptan, dimethylsulfide und diethylsulfide (17). Notwithstanding this evidence, apical periodontitis can reportedly affect even radiologically impeccable endodontic treatments without correlation to any residual microbiological load. This observation suggests a contribution of host factors, such as a generally increased inflammatory predisposition or individual differences in the immune response to vital bacteria, persisting bacterial antigens or sulphur compounds.

The aims of this retrospective study were firstly to investigate the relation of the *ex-vivo*

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immune response to sulphur compounds to clinical and radiologic findings, and secondly to identify predisposing factors for endodontic treatment failure. Data were obtained from a group of patients with clinical or radiologic endodontic treatment failure and from a control group of individuals who had received successful endodontic treatment more than five years previously, as defined by sufficiently filled canals and the absence of radiologic abnormalities or clinical symptoms.

MATERIALS AND METHODS

Study population

One hundred and four patients presenting only one single- or multi-rooted avital tooth in the upper or lower jaw were examined in a dental practice with endodontic specialization between March 2010 and October 2012. Patients with periodontitis or several affected teeth were excluded. All patients received dental treatments and laboratory diagnostics in the course of routine medical care. For retrospective evaluation of the data, patients were grouped into a patient group (n=73) and a control group (n=31) based on presence or absence of clinical symptoms and radiological findings, respectively. 20 out of 73 patients showed clinical discomfort or radiological findings without previous endodontic treatment measures. Fifty-three out of 73 patients had undergone previous endodontic treatment that proved to be insufficient by radiological imaging (inhomogeneous filling, fully or partially unfilled root canals) or had developed apical periodontitis. Scoring of clinical symptoms was based on the sensitivity to percussion or occlusal load, local pain or signs of periodontal inflammation such as mucosal redness and swelling. Anamnestic data were collected at the first consultation for pain sensation or at the time of endodontic treatment. Available imaging data (dental x-ray, panoramic x-ray, 3D –cone beam) were taken into consideration.

The control group of 31 symptom-free patients had undergone successful endodontic treatment of one tooth at least 5 years previously (>5 - 24 years). By means of radiological imaging, the treated teeth showed sufficiently filled root canals and an intact periodontal space.

According to the Declaration of Helsinki, all patients gave written informed consent for the scientific evaluation of their data. The local IRB waived the demand of an approval as all diagnostic tests and treatment protocols were part of routine care.

Dental treatment and laboratory diagnostics

Out of 73 patients, 45 and 28 patients underwent re-treatment of the existing root canal filling and tooth extraction, respectively. Tooth extraction was performed in the presence of severe endodontic and/or periodontal lesions, inadequate removal of filling material and unfeasible tooth restoration.

Routine laboratory diagnostics were conducted for the entire study population and comprised genotyping of IL1A, IL1B, IL1RN and TNFA genes, lymphocyte transformation tests in the presence of sulphur compounds and the determination of TNF-α, IP-10 and MBL levels in serum. Except for genotyping, all diagnostic tests were performed when the patient presented to the dentist for the first time and 3-8 months after initial treatment when follow-up samples were taken.

Revision

Upon generation of an access cavity using rubber dam and microscopic imaging (Kaps Typ SOM 32, Asslar), probing and preparation of the coronal third of the canal was performed, as well as probing of the glide path and preparation of canals up to the apical constriction to minimum ISO 30. Endometrical length measurement was controlled using an electronical apexfinder (ROOT-ZX, J. Morita Corporation, Tokyo, Japan The rinse protocol comprised of 5% sodium hypochlorite, EDTA-solution and Chlorhexidin. Irrigation was performed using ultrasonic procedure (30 THz). Shaping and cleaning of the root canal were finalized by applying Ca(OH)2 and a bacteria tight seal using cement or composite. After 1-2 weeks, obturation was carried out by vertical condensation of gutta-percha cones (Roeko-Coltene/ Whaledent, Switzerland) sealed with AH plus (Dentsply DeTrey, Konstanz, Germany). In case of immunological sensitizations, standard materials were replaced by individually tolerated alternative materials such as Epiphany (Pentron Clinical Technologies, Wallingford USA) and MTA (Dentsply DeTrey, Konstanz, Germany). A bacteria tight composite filling was applied to prevent microbial recontamination. Treatment outcome was controlled by dental X-ray or 3D-cone beam.

Extraction

All tooth extractions were performed under local anesthesia. To minimize the risk of contamination, alveoles were gently reamed out by a surgical ball cutter and irrigation was performed by sterile sodium chloride-solution to facilitate uncontaminated bone regeneration. Extraction sites were never stabilized by augmentation.

Genotyping

For genetic analyses, genomic DNA was extracted from 2 ml EDTA-whole blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Genotyping of

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polymorphisms rs1800587 (IL1A), rs1143634 (IL1B) and rs419598 (IL1RN) was performed by multiplex PCR and subsequent reverse hybridization, according to the manufacturer's protocol (GenoType IL-1; VER 1.0, HAIN Lifescience, Nehren, Germany). Genotyping of rs1800629 (TNFA) was carried out by real-time PCR and melting curve analysis using a Light Cycler 1.5 (Roche Diagnostics, Mannheim, Germany) as published elsewhere (18).

Cytokine analyses

Serum samples were obtained by centrifugation of coagulated whole blood and stored at -80°C for further analysis. Cytokine concentrations were determined by commercially available enzyme-linked immunosorbent assays (ELISA), including TNF-α Immulite (DPC Biermann, Bad Nauheim, Germany), IP-10 (MERCK/Millipore, Darmstadt, Germany) and MBL (AntibodyShop, Gentofte, Denmark). Analyses were performed according to the manufacturer's instructions. The MBL assay detects functional MBL oligomers, excluding the measurement of inactive MBL monomers.

Sulphur compound ex- vivo stimulation assay

Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml venous heparinized whole blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. PBMC were resuspended in RPMI 1640 (Biochrom KH, Berlin, Germany), supplemented with 2 mM L-glutamine (Sigma) and 10% heat-inactivated autologous serum. PBMC at the concentration of 1×10^6 / ml were seeded in flat-bottom 24-well microtiter plates (Nunclon, Wiesbaden, Germany) and stimulated with 500 ng/ml methanethiol (CH3SH, methyl mercaptan, Sigma, Germany) and dimethyl sulphide each (CH3SCH3, Dimethylthioether, Sigma, Germany). For comparison to baseline cytokine levels, PBMCs cultured in the absence of sulphur compounds served as control. Cells were cultured in a humidified incubator with 5% CO2 at 37°C for 24 hours. After collection of cell supernatants by centrifugation, secreted IFN-γ and interleukin 10 were determined using Human Cytokine Panel I (MPXHCYTO-60K; Merck, Darmstadt, Germany), according to the manufacturer's instructions. The Luminex® 200™ with xPonent® Software (Luminex, Austin, USA) was used for detection. Final IFN-γ and IL-10 values were obtained by subtracting unstimulated control values from sulphur compound stimulated values. In addition, Trypan blue staining was performed to confirm that applied doses of sulphur compounds were not cytotoxic after 24-h stimulation.

Statistics

The *Chi*-squared test was used to compare anamnestic and clinical parameters, as well as genotype frequencies between patient and control groups. Patients' ages, *ex-vivo* stimulation test results and cytokine levels were analyzed by Mann Whitney U-Test. Comparison of positive or negative scores of the *ex vivo* stimulation test was done by *Chi-* squared test. A positive score was defined as IFN-γ > 0.3 IU/ml or IL-10 >10 pg/ml (upon subtraction of basal values). p values <0.05 were considered as statistically significant. Outcomes of primary and follow-up testings were compared by Mc-Nemar test. Statistical analyses were performed using IBM SSP Statistics, version 19.

RESULTS

Endodontic abnormalities were associated with IFN-γ and IL-10 stimulation by sulphur compounds

In order to assess potential differences in the immune responses to sulphur compounds in patient and control groups, the production of cytokines in isolated PBMC was analyzed after 24 h stimulation with and without sulphur compounds. Whereas IFN-γ served as marker for TH1 based immune responses, interleukin 10 (IL-10) was used to detect TH2 responses. PBMCs in the patient group produced significantly higher levels of IFN-γ and IL-10 after stimulation with sulphur compounds as compared to control group (IFN-γ 1.53 IU/ml *vs* 0.1 IU/ml, p<0.0001; IL-10: 46.09 pg/ml *vs* 27.41 pg/ml; p<0.0001; Fig. 1). Detailed evaluation of the data revealed that out of 73 patients, 9 showed increased IFN-γ levels, 19 increased IL-10, and 11 both increased IFN-γ and IL-10 concentrations. In 34 patients neither increased IFN-γ nor IL-10 levels could be detected. In contrast to the patient group, increased IL-10 levels were only observed in 1 patient of the control group. In addition, IFN-γ levels were not altered in PBMCs isolated from the control group upon stimulation with sulphur compounds. Defining a positive result of the *ex-vivo* stimulation assay as IFN-γ > 0.3 IU/ml and or IL-10 < 10 pg/ml, 39/73 patients (53.4%) scored positive, as compared to 1/31 controls (3.2%). The difference proved to be statistically significant by Mann-Whitney U-test (p<0.001).

The outcome of ex-vivo sulphur compound stimulation test was independent on age and gender as well as smoking and medical preconditions of patients

We tested potential confounders of the correlation

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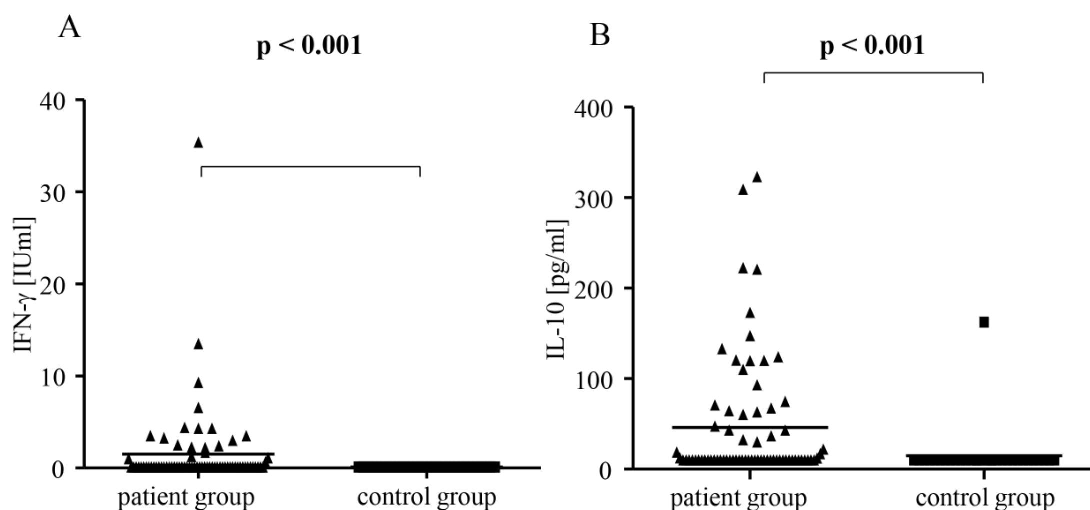

Fig. 1. *Levels of ex-vivo-stimulated IFN-γ (**A**) and IL-10 (**B**) in patients (n=73) and controls (n=31). Values were obtained by subtraction of baseline cytokine levels from stimulated cytokine levels. Patients display significantly higher values for both cytokines (Mann-Whitney U-Test).*

Table I. *Evaluation of potential confounders.*

	patient group (n = 73)		**control group (n = 31)**		**p-value**
Average age (years)	50.6		53.0		0.22
Range (years)	22-76		33-73		
Gender (F/M)	57/16		25/6		1.000
	n	**%**	**n**	**%**	
Smokers	13	17.8	3	9.7	0.382
General medical conditions					
hypertension	16	21.9	7	22.6	1.000
Sensitizations	38	52.8	12	38.7	0.205
Allergic rhinitis	16	21.9	9	29.0	0.459
autoimmune diseases	14	19.7	4	12.9	0.574
cancer history	8	11.3	2	6.5	0.719
Daily oral hygiene					
good	72	98.6	31	100	1.000
poor	1	1.4	0	0	
Bruxism					
yes	25	34.2	9	29.0	0.654
no	48	65.8	22	71.0	
Alcohol					
yes	5	6.8	2	6.5	1.000
no	68	93.2	29	93.5	

Age, gender, smoking habits and medical preconditions of patients (n=73) and controls (n=31). Statistics were evaluated by Mann Whitney U-Test with exception of patients' ages (Chi-squared test).

Table II. *TNFA -308 G/A is associated with endodontic treatment failure.*

Genotype	patient group n=73 n (%)	control group n=31 n (%)	p-value
IL1A -889 C/T			
CC	42 (57.5)	19 (61.3)	
CT or TT	31 (42.5)	12 (38.7)	0.829
IL1B +3953 CT			
CC	45 (61.6)	20 (64.5)	
CT or TT	28 (38.4)	11 (35.5)	0.828
IL1RA +2018 T/C			
TT	37 (50.7)	19 (61.3)	
CT or CC	36 (49.3)	12 (38.7)	0.392
TNFA -308 G/A			
GG	41 (56.2)	26 (83.9)	
GA or AA	32 (43.8)	5 (16.1)	**0.007**

Allele frequencies of IL1A rs1800587 (-889 C/T), IL1B rs1143634 (+3954 C/T), IL1RN rs419598 (+2018 T/C) and TNFA rs1800629 (-308 G/A). Only TNFA rs1800629 (-308 G/A) shows a significant association (Chi-squared test, p=0.007).

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between endodontic treatment failure and positive sulphur compound stimulation test outcome. Logistic regression analysis showed however, that the association was independent on age, gender and smoking habits (Table I). Similarly, there was no significant correlation with medical conditions such as hypertension, pollen and other allergies, autoimmune disease and cancer, as well as the status of personal oral care, bruxism and alcohol consumption (Table I).

TNFA -308 G/A polymorphism predisposes for endodontic treatment failure

IL-1, interleukin 1 receptor antagonist (IL-1RN) and tumour necrosis factor alpha (TNF-α) represent key cytokines of the innate immune responses. In order to analyze the potential contribution of functional polymorphisms in these cytokine genes to the outcome of endodontic treatment, the polymorphisms IL1A rs1800587 (-889 C/T), IL1B rs1143634 (+3954 C/T), IL1RN rs419598 (+2018 T/C) and TNFA rs1800629 (-308 G/A) were genotyped in patients and controls (Table II).

For interpretation of data, the presence of at least one minor allele was defined as the "risk genotype" for the respective polymorphism. The comparison of TNFA genotypes in the different groups revealed a significant increase in the prevalence of the TNFA risk genotype among patients as compared to controls (43.8% *vs* 16.1%, p= 0.007). Conversely, IL1A -889 C/T- , IL1B +2953 C/T and IL1RA +2018 T/C risk genotypes showed no significant differences between either group (Table II).

Endodontic treatment failure was associated with increased TNF-α levels

In order to examine levels of systemic inflammation, we measured TNF-α concentrations in serum samples obtained from the patient and control groups. The patient group displayed increased TNF-α concentrations in serum as compared to the control group (14.51pg/ml *vs* 11.90pg/ml, $p < 0.05$, Fig. 2). Mannose binding lectin (MBL) involved in mucosal immune defence and Interferon-inducible protein-10 (IP-10), a chemokine promoting the

Table III. *Stimulation test outcome before and after treatment, extraction group.*

	time point 1			time point 2		
patient ID	IFN-γ	IL-10	result	IFN-γ	IL-10	result
61	35.4	74.7	**positive**	<0.1	<10	negative
45	9.3	<10	**positive**	<0.1	54	**positive**
53	4.4	<10	**positive**	<0.1	<10	negative
69	4.3	124.2	**positive**	<0.1	<10	negative
46	3.5	22.3	**positive**	<0.1	<10	negative
52	3	<10	**positive**	<0.1	<10	negative
65	2.2	12.4	**positive**	<0.1	<10	negative
48	1	<10	**positive**	<0.1	<10	negative
43	<0.1	<10	negative	<0.1	<10	negative
44	<0.1	<10	negative	<0.1	<10	negative
47	<0.1	173.3	**positive**	<0.1	<10	negative
49	<0.1	<10	negative	<0.1	<10	negative
50	<0.1	309	**positive**	<0.1	<10	negative
51	<0.1	18.8	**positive**	<0.1	<10	negative
54	<0.1	120.5	**positive**	<0.1	<10	negative
55	<0.1	<10	negative	<0.1	<10	negative
56	<0.1	43	**positive**	<0.1	<10	negative
57	<0.1	<10	negative	<0.1	99	**positive**
58	<0.1	32.6	**positive**	<0.1	<10	negative
59	<0.1	<10	negative	<0.1	<10	negative
60	<0.1	<10	negative	<0.1	<10	negative
62	<0.1	120.3	**positive**	<0.1	<10	negative
63	<0.1	<10	negative	<0.1	<10	negative
64	<0.1	<10	negative	<0.1	<10	negative
66	<0.1	<10	negative	<0.1	<10	negative
67	<0.1	60.6	**positive**	<0.1	240	**positive**
68	<0.1	<10	negative	<0.1	26.6	**positive**

migration of activated T cells, did not show any statistically significant difference between patients and controls, albeit a tendency to higher IP-10 values in patients (MBL: 1438pg/ml *vs* 1541pg/ml; IP-10: 733.3pg/ml *vs* 661.7pg/ml; Mann-Whitney U-Test).

Stimulation test results normalize upon revision or extraction

We hypothesized that removal of the source of sulphur compounds may reduce cytokine production in the sulphur compound *ex-vivo* stimulation test. Therefore, we repeated the test 3-8 months after tooth extraction or re-treatment of the root canal at two time points. Sixty-nine of the initial 73 patients were available for follow-up testing (extraction group: 27/69 patients; re-treatment group: 42/69 patients). Patients who showed a negative outcome (IFN-γ < 0.3 IU/ml and IL-10 < 10 pg/ml) at either time point were scored as negative. As shown in Tables III and IV, both re-treatment and extraction are followed by a significantly reduced frequency of positive stimulation test outcomes.

In the extraction group, 16/27 patients (59.3%) scored positive before treatment, while only 4/27 (14.8%) scored positive after extraction (p= 0.004; Table III). Detailed evaluation showed that out of 16 patients who had been positive before treatment, 14 turned negative upon extraction. Two patients remained positive, one of which (ID 45) normalized IFN-γ secretion from 9.3 to 0.1 pg/ml but remained IL-10 positive. The other persistently positive patient (ID 67) remained IFN-γ negative but increased IL-10

Table IV. *Stimulation test outcome before and after treatmen*

	time point 1			time point 2		
patient ID	IFN-γ	IL-10	result	IFN-γ	IL-10	result
1	13.5	<10	**positive**	<0.1	<10	negative
15	6.6	43	**positive**	3.4	<10	**positive**
16	4.3	323	**positive**	<0.1	<10	negative
22	3.5	222.4	**positive**	<0.1	<10	negative
12	3.3	110	**positive**	<0.1	<10	negative
8	2.5	<10	**positive**	<0.1	<10	negative
5	2.4	<10	**positive**	<0.1	<10	negative
21	2.3	<10	**positive**	<0.1	<10	negative
7	1.7	36.7	**positive**	<0.1	<10	negative
3	1.1	<10	**positive**	<0.1	10.1	**positive**
9	0.7	147.4	**positive**	<0.1	87.3	**positive**
2	<0.1	17.2	**positive**	<0.1	<10	negative
4	<0.1	93.2	**positive**	<0.1	<10	negative
6	<0.1	<10	negative	<0.1	<10	negative
10	<0.1	63.3	**positive**	<0.1	<10	negative
11	<0.1	<10	negative	<0.1	<10	negative
13	<0.1	<10	negative	<0.1	14.4	**positive**
14	<0.1	<10	negative	<0.1	<10	negative
17	<0.1	<10	negative	<0.1	<10	negative
18	<0.1	47.4	**positive**	<0.1	<10	negative
19	<0.1	64.6	**positive**	<0.1	<10	negative
20	<0.1	<10	negative	<0.1	<10	negative
23	<0.1	<10	negative	<0.1	<10	negative
24	<0.1	<10	negative	<0.1	14.1	**positive**
25	<0.1	220.8	**positive**	<0.1	<10	negative
26	<0.1	<10	negative	12.3	<10	**positive**
27	<0.1	<10	negative	<0.1	<10	negative
28	<0.1	67.5	**positive**	<0.1	<10	negative
29	<0.1	30.1	**positive**	1	<10	**positive**
30	<0.1	<10	negative	<0.1	<10	negative
31	<0.1	<10	negative	<0.1	<10	negative
32	<0.1	<10	negative	<0.1	<10	negative
33	<0.1	<10	negative	<0.1	<10	negative
34	<0.1	<10	negative	<0.1	14.4	**positive**
35	<0.1	<10	negative	<0.1	<10	negative
36	<0.1	<10	negative	<0.1	11.5	**positive**
37	<0.1	12.3	**positive**	<0.1	<10	negative
38	<0.1	<10	negative	<0.1	<10	negative
39	<0.1	<10	negative	<0.1	<10	negative
40	<0.1	<10	negative	<0.1	<10	negative
41	<0.1	119.9	**positive**	<0.1	<10	negative
42	<0.1	70.9	**positive**	3.7	36.5	**positive**

secretion. Two patients scored negative in the initial testing but turned IL-10 positive during follow-up (ID 57 and ID 68). No IFN-γ negative patient turned IFN-γ positive.

In the revision group, 22/42 patients (52.4%) were positive for at least one cytokine in the sulphur compound stimulation test before treatment, while 10/42 scored positive during follow-up after re-

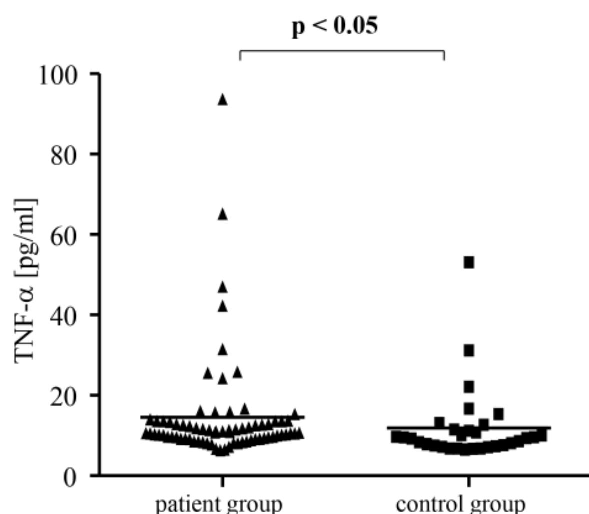

Fig. 2. *Distribution of TNF-α serum levels in patients and controls. Each symbol represents an individual. The horizontal lines represent the mean TNF-α serum value of the group. Mann-Whitney U-Test shows a significant group difference ($p < 0.05$).*

treatment (23.8%, p=0.023; Table IV). Detailed evaluation shows that 17 of the initially 22 positive patients had turned negative. Five patients maintained positive test results (IDs 3, 9, 15, 29, 42) and five initially negative patients turned positive during follow-up (IDs 13, 24, 26, 34, 36). Of these five however, four showed only marginal elevation of IL-10 (up to 14.4 pg/ml). Only one initially negative case showed strong cytokine induction (ID 26: IFN-γ 12.3 IU/ml, IL-10 negative).

Stimulation test results remain unchanged in controls

To test both the biological variability and the inter-assay reproducibility we repeated the sulphur compound stimulation test also for 28 of the initial 31 controls, who had not received any endodontic treatment in the meantime. Testing was performed in the same time window as for the patient group. Twenty-five out of 28 patients did not score positive at any time point, indicating high inter assay stability. The only initially positive patient scored negative six months later. However, two initially negative patients showed marginally increased IL-10 secretion at the follow-up time point (13.1 pg/ml and 27.7 pg/ml).

DISCUSSION

The present study demonstrates that immunological sensitization to methyl mercaptan and thioethers is associated with endodontic treatment failure. *Ex-vivo* stimulation of patient lymphocytes with sulphur compounds showed significantly increased cytokine release for patients with clinical complaints or radiological abnormalities. The fact that analyses were performed with systemically circulating mononuclear blood cells argues for a type IV immune sensitization to sulphur compounds. As T-lymphocytes produce both IFN-γ and IL-10, they likely account for the observed increase in cytokine levels. Still, other cellular components of PBMCs represent potential sources, such as natural killer cells, which have been shown to secrete IFN-γ (19) as well as blood monocytes and B-lymphocytes, which reportedly both release IL-10 (20). However, these cells lack antigen specific recognition sites and are therefore activated only by unspecific activation signals. Unspecific activation by methyl mercaptan and/or dimethyl sulfide is in fact highly unlikely due to the consistently negative cytokine scores in the control group.

The observation that sulphur compounds can stimulate immune cells to produce proinflammatory cytokines is well documented in the literature. Methyl mercaptan has been shown to induce the production of IL-1 by human mononuclear cells (14) and LPS-stimulated IL-6 production is augmented when methyl mercaptan is present (13). Furthermore, methyl mercaptan alone or in combination with IL-1 or lipopolysaccharide can significantly enhance the secretion of PGE2 and cAMP, which are required for induction of local inflammatory processes (14). Proinflammatory cytokines like IL-1, interleukin-8 (21), TNF-α (22) or even the T-cell mediator interleukin-2 (23) are increased in inflamed pulp tissue specimens of patients with irreversible pulpitis. Even if none of our patients suffered from irreversible pulpitis but had clinical signs of an avital tooth, it is tempting to speculate that the proinflammatory pathomechanism may be comparable.

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A putative causal link between immunological sensitization to methyl mercaptan and apical inflammation is supported by the observation that elimination of methyl mercaptan/thioether exposition by re-treatment of the root canal or tooth extraction leads to a significant reduction of sulphur compound-induced cytokine production. It is well known that removal of a stimulating antigen reduces the number of the respective antigen specific precursor T cells in the blood. The fact that both in the re-treated and in the extraction group a minority of primarily negative patients turned IL-10 positive during follow-up may be explained by regulatory mechanisms. These patients are exposed to sulphur compounds and may develop immune tolerance based on the action of regulatory T-lymphocytes. In *ex-vivo* stimulation set-ups IL-10 is considered a marker for regulatory T-cells and IFN-γ indicates the TH1 mediated effector cell response that is associated with local and/or systemic inflammation. As we focused the study on sensitization to sulphur compounds, we limited our analysis to these cytokines. Subsequent endodontic surgery may reactivate a latent tolerance reaction against sulphur compounds. It is important to note that this study was not designed to examine the influence of re-treatment *vs* extraction on the long-term success rate based on sulphur compounds associated immune phenomena.

While IL-10 upregulation typically occurs along with INF-γ production in T-cell mediated immune activation, the fact that three control individuals without signs of clinical findings scored IL-10 positive is in line with published data showing that isolated IL-10 does occur in absence of manifest inflammation or allergic response. This has been reported for allergens, especially for drug sensitized patients (24). Whether positive IL-10 scores designate the beginning of immune processes or, alternatively, demarcate stable immune tolerance cannot be determined on the basis of the present data but will require extended studies with strengthened clinical focus. Accordingly, further studies will be required to elucidate the clinical significance of isolated IL-10 positive scores.

Conversely, the re-treatment group patient who showed strong upregulation of IFN-γ during follow-up testing reflects a different situation. A likely explanation is that in this case revision may have triggered the release of sulphur compounds from the canal. Alternatively, these patients may have developed another independent endodontic lesion in the meantime.

The present data show that an immunological laboratory test may serve as a diagnostic tool for patients with chronic necrotic dental pulp tissue conditions. Follow-up testing may yield objective criteria for the success of endodontic treatment. As expected from its well-described proinflammatory action, positive IFN-γ confers a higher predictive value. Still, the predictive significance of positive IL-10 is underscored by its higher frequency in the patient group and by the fact that root canal re-treatment and extraction lead to IL-10 reduction in most cases. The data reveal that the novel laboratory tests presented here may provide additional criteria for the prognosis of endodontic treatment, supporting clinical judgement and well-established imaging techniques.

In addition to our data implicating the TNFA -308 G/A polymorphism as a predisposing factor for failure of endodontic treatment or apical periodontitis, earlier studies have shown a contribution of this polymorphism to a wide range of chronic inflammatory disorders, such as diabetes mellitus (25), inflammatory bowel disease (26), atherosclerosis and stroke (27) as well as periodontitis (28) and periimplantitis (29). Other studies show a significant association of TNFA -308 G/A with local inflammatory processes, activating osteoclasts and synovial fibroblasts through induction of RANKL expression, which in turn increases bone resorption (30). At the same time, TNF-α increases periodontal tissue destruction by inducing matrix metalloproteinases (31). It seems likely that patients with increased inflammatory predisposition respond to sulphur compounds as potentially immunogenic stimuli with increased local inflammation. Importantly however, TNFA -308 G/A occurs also in the control group, albeit less frequently, making clear that this polymorphism does not represent the only relevant risk factor.

Increased serum TNF-α levels in our patient group further support the clinical significance of this cytokine. Intriguingly, TNFA -308 G/A is reportedly associated with increased TNF-α transcription (32). Considering the relatively small average differences

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in serum levels between patients and controls however, serum TNF-α does not represent a valid marker to identify endodontic patients. Also serum MBL and IP-10 proved unsuitable for endodontic diagnostic discrimination, even though the latter showed a trend to higher levels in our patient group. It is important to note that normal serum IP-10 does not preclude systemic immune activation based on a local T-cell mediated immune process. IP-10 is significantly elevated in systemic infectious TH1-mediated diseases such as chronic active hepatitis or concurrent HIV/tuberculosis infection (33) but not in other inflammatory diseases, such as acute peptic ulcer (34), atopic dermatitis or Parkinson's disease (35). The fact that TH1 lymphocytes contribute to organ pathologies and systemic inflammation in these IP-10 negative diseases emphasizes that normal serum IP-10 does not exclude activation of TH1 lymphocytes. Diagnostic sensitivity of serum IP-10 is most likely not sufficient to reliably detect local inflammatory processes.

Taken together, this retrospective study shows that sulphur compound specific T cells are significantly increased in patients with clinical or radiologic signs of endodontic treatment failure. The data argue that endodontic re-treatment following a standardized protocol effectively normalizes immune parameters, presumably by reducing sulphur compounds in the root canal system. Our novel *ex-vivo* sulphur compound stimulation assay may therefore serve as a tool to complement established clinical and radiological diagnostic measures for monitoring endodontic treatment outcome. Furthermore, TNFA -308 G/A is associated with a higher risk for endodontic treatment failure, predisposing for local inflammatory processes.

This study points out systemic and prognostic immune parameters that are relevant for endodontic proinflammatory processes. Further studies will be necessary to confirm the reported association between sulphur compounds and systemic inflammatory processes and to verify the diagnostic value of our novel laboratory parameters.

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