Increased Expression of Granzyme B and Transforming Growth Factor-β in Intralesional Plasma of Oral Lichenoid Reactions: A Preliminary Study

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Research Article

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Abstract

Background: Oral lichenoid reactions are intractable inflammatory diseases of oral mucosa. The cytokine profiles of intralesional blood remain unclear. We aim at revealing the intralesional cytokine profiles and providing some actual and stable intralesional cytokine biomarkers to evaluate the severity and therapeutic effects of oral lichenoid reactions.

Methods: Paired intralesional and peripheral plasma from 26 patients with oral lichenoid reactions were collected. The concentration of 15 cytokines of granzyme B, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL17A, TNF-α, IFN-α, IFN-β, IFN-γ, TGF-β1, TGF-β2 and TGF-β3 was measured by Luminex assays. REU score was used for evaluating the severity of the disease.

Results: Eleven cytokines including IL-10, IFN-α, IL-6, IL-17A, granzyme B, TGF-β1, TGF-β2, TGF-β3, IL-2, TNF-α, IL-12p70 were detected within the reliable working range. IL-10 was detected in less intralesional samples (19/26) than peripheral samples (26/26, p=0.01). The cytokine concentrations from intralesional plasma were significantly elevated in granzyme B (median 108.94 *vs.* 16.00), TGF-β1 (mean 30448.92 *vs.* 10199.04), TGF-β2 (mean 1659.73 *vs.* 1308.49) and TGF-β3 (mean 914.33 *vs.* 573.13) than that in peripheral plasma (p=0.001, p□0.001, p□0.001 and p□0.001, respectively). The concentration of IL-12p70 in peripheral plasma was positively correlated with REU score (coefficient of correlation=0.463, p=0.02).

Conclusions: The concentration of granzyme B and TGF-β are more abundant in intralesional microenvironment than in peripheral plasma of oral lichenoid reactions. IL-12p70 may be a potential molecular biomarker for evaluating the severity of oral lichenoid reactions. Cohort study of large population is required.

Introduction

Oral lichenoid reactions (OLRs) are considered to represent a spectrum of diseases which involve the interface of oral mucosa. OLRs can be classified as oral lichen planus (OLP) and its sister disease, oral lichenoid lesion (OLL), which include oral lichenoid contact reactions, oral lichenoid drug eruptions and oral lichenoid reactions of graft-versus-host disease etc. [1]. The global prevalence of OLP is 0.89% among the general population [2]. The exact etiology of OLRs is not fully elucidated [3]. A common feature of these diseases is that T cells are activated in the microenvironment of lesions [4].

Cytokines are proteins with pleiotropism and redundancy. Among the numerous cytokines, T cell-associated cytokine profiles have been widely investigated with peripheral blood or saliva samples in the field of OLRs. However, different samples may express altered levels of cytokine. For instance, tumor necrosis factor-α (TNF-α), which is extensively studied in OLRs, was increased in lesions and saliva of OLP compared to health controls [5, 6], while in peripheral blood mononuclear cells (PBMCs) from OLP patients, the concentration of TNF-α was decreased [7]. In our previous study, a complicated Th1/Th2-related cytokine profile was found from the saliva of OLL patients [8], indicating a complex regulatory network in the pathogenesis of OLRs.

Cytokines exert a transient immunomodulatory function at high local concentrations in autocrine or paracrine fashions [9-11]. They are also elevated in peripheral blood in endocrine fashion in some inflammatory diseases [12]. However, in most oral mucosal diseases, cytokines do not work in endocrine fashion [13]. Therefore, it seems inaccurate to monitor the severity of OLRs through the cytokines from peripheral blood. Meanwhile, the level of salivary cytokines is greatly affected by factors such as oral microorganism, collection time and method, making it unstable for reflecting the severity of OLRs.

Recently, it was reported that the cytokines from intralesional blood contain disease-specific biomarkers and have diagnostic and prognostic functions [14]. In this study, we will collect intralesional blood samples to detect the OLRs-related cytokine profiles, aiming at revealing some actual and stable cytokine biomarkers to monitor the development of OLRs.

Methods

Study design

This study was independently reviewed and approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (approval ID: SH9H-2020-TK376-1, approved on May 25th, 2020) and conducted according to the Declaration of Helsinki and the additional requirements. This study was undertaken with the understanding and written informed consent of each participant. We conducted this preliminary study in patients with OLRs from department of oral medicine, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. We included patients whose diagnoses were OLRs according to the modified WHO criteria [15]. Patients who used topical or systemic corticosteroids within 3 months were excluded. We also excluded patients with graft-versus-host disease. Intralesional and peripheral blood samples from 26 patients were collected for study.

Collection of intralesional and peripheral blood

Fluconazole 50 mg daily as well as 1% sodium bicarbonate solution was used for approximately 2 weeks prior to blood collection. After local anesthesia, 5 mm incision was made in the lesion at a depth of approximately 3 mm. The intralesional blood was collected at the site of lesion (buccal mucosa or tongue), and collected immediately into an EDTA-embedded Eppendorf tube, followed by centrifugation at 6000 rpm for 8 minutes. The peripheral blood was collected and treated with a same protocol. The supernatant plasma was then collected and stored at -80 ℃.

Cytokine concentration analysis

According to our previous study [8], the cytokine profile is this study consisted of totally 15 selected cytokines, which could be divided into four subgroups. The first subgroup included T helper (Th)1 cytokines: IL-2, TNF-α, IFN-α, IFN-β, IFN-γ and IL-12p70. The second subgroup included Th2 cytokines: IL-4, IL-5, IL-6 and IL-10. The third subgroup included Th17 cytokines: IL17A. The other cytokines, which

included transforming growth factor (TGF)-β1, TGF-β2, TGF-β3 and granzyme B, were divided as the fourth subgroup.

The cytokine profile was detected and measured with Human XL Cytokine Luminex Performance Panel Premixed Kit (R&D Systems, Minneapolis, USA, Cat. No FCSTM18) and Bio-Plex Pro™ TGF-β 3-plex Assay (Bio-Rad, California, USA, Cat. No 171W4001M) according to the manufacturers' protocols. Briefly, 50 μL of diluted resuspended beads were embedded into each well in a 96-well plate to capture antibodies. Then, 50 μL of standard substance and samples were added. Diluted biotin labeled antibody complex, streptavidin labeled PE and detergent were added accordingly into each well, and fluorescence intensity readings for the standards and samples were detected on a Luminex 200 sorting and detection platform (Luminex Inc., Texas, USA). Fluorescence data were furtherly converted into the corresponding cytokine concentrations through the R&D Analyst software.

From the standard curve, the reliable working range of each cytokine could be illustrated and the detection rate could be calculated. When the detection rate of a given cytokine in any of the intralesional or peripheral plasma group was over 60%, the detection rates could be compared. When the detection rate of a given cytokine in both intralesional and peripheral plasma groups were over 80%, the concentrations could be numerically compared [16]. Each sample was detected once due to the limited quantity of the samples.

Evaluation of severity

The REU score system was used to evaluate the severity of OLRs according to previously publication [17]. In brief, oral cavity was divided into totally 10 sites: labial mucosa, right buccal mucosa, left buccal mucosa, dorsal tongue, ventral tongue, floor of mouth, hard palate mucosa, soft palate/tonsillar pillars, maxillary gingiva, mandibular gingiva. In this system, R represents reticular/hyperkeratotic lesions and each site scored from 0 to 1; E and U represent erosive/erythematous and ulcerative areas, respectively, and both scored in each site from 0 to 3. REU score was calculated through the formula that REU score=$1\times\Sigma R+1.5\times\Sigma E+2\times\Sigma U$.

Statistical Analysis

The results of continuous variables were presented in the form of means ± standard deviations (SD) or median (interquartile range, IQR). Independent t-test and Mann-Whitney U test were performed where data were of normal distribution and non-normal distribution, respectively. Chi-square or Fisher exact probability test was used for calculating the differences of detection rates. Spearman's correlation test was used to analyze the correlations between REU scores and cytokine concentrations, and results were presented as coefficient of correlation (R). The REU score was not applicable in 1 subject where series mean of replacing missing values was used. Data analysis was performed using SPSS 24.0 software (Armonk, New York, USA). Graph Prism 7 (Graph Pad Software, CA, USA) was used for graphic figures. Possibility (p) value less than 0.05 was considered statistically significant.

Results

Participant characteristics

In total, 26 participants with OLRs were enrolled in our study, in which 14 (53.85%) were diagnosed as OLP and 12 (46.15%) were OLL. The number of female participants was larger than that of male participants (17 females and 9 males). The average age was 49.27±13.77 years. Detailed information on lesion site, systemic diseases, drugs used within 3 months and REU score of each participant was described in Table 1.

Detection rate of cytokines

The cytokines whose detection rates in both intralesional and peripheral plasma were more than 80% included granzyme B, IL-2, IL-12p70, TNF-α, TGF-β1, TGF-β2 and TGF-β3. Concentrations of these 7 cytokines would be further analyzed. For IFN-α, IL-6, IL-10 and IL-17A, the detection rates were compared (Table 2). For IFN-β, IFN-γ, IL-4 and IL-5, the detection rate in either group was less than 60%, which could not be analyzed qualitatively or quantitatively.

IL-10 was detected within the reliable working range in 19 intralesional samples (19/26, 73.08%) and all the 26 peripheral samples (26/26, 100%, p=0.010). IFN-α was detected in 23 intralesional sample (23/26, 88.46%) compared to 20 peripheral samples (20/26, 76.92%, p=0.465). IL-6 was detected in 19 intralesional samples (19/26, 73.08%) compared to 21 peripheral samples (21/26, 80.77%, p=0.510). The detection rate of IL-17A was slightly lower in intralesional samples (16/26, 61.54%) compared to that in peripheral samples (19/26, 73.08%, p=0.375). These data indicated that IL-10 was expressed in more peripheral plasma samples than intralesional plasma (Figure 1).

Comparisons of cytokine concentrations

In the participants with OLRs, the cytokine concentrations in intralesional plasma are significantly elevated in granzyme B, TGF-β1, TGF-β2 and TGF-β3 than that in peripheral plasma (p=0.001, p□0.001, p□ 0.001 and p□0.001, respectively, Table 3, Figure 2A-D). The cytokine concentrations of IL-2 and TNF-α in intralesional plasma are significantly lower than those in peripheral plasma (p=0.009 and p=0.048, respectively, Table 3, Figure 2E-F). For IL-12p70 and, no significant difference was found (p=0.697, Figure 2G).

Correlation of REU score and cytokine concentrations

In the participants with OLL, the concentration of IL-12p70 in peripheral plasma was positively correlated with REU score (coefficient of correlation=0.463, p=0.02, Table 4). No significant correlation between REU score and cytokine concentration was found in intralesional plasma of IL-12p70 or other detected cytokines.

Discussion

In this study, participants with OLRs were enrolled instead of OLP alone. Up to now, the diagnostic criteria to distinguish OLP from OLL are still lacking [18]. OLL has frequently been used to refer to indicate an uncertain diagnosis of OLP [19]. As a typical spectrum of interface oral mucositis, OLRs could, to a large extent, reflect the features of microenvironment of OLP.

There are few studies on the regional immune characteristics of oral mucosa in recent years. OLRs are intractable inflammatory diseases mainly caused by immune deficiency or dysregulation of oral immune system [20]. Cytokines are reliable biomarker for monitor the severity of inflammatory diseases [21, 22]. In this study, unlike many previous studies on cytokine profiles from PBMCs between diseased and healthy subjects, we detected cytokine concentrations of intralesional and peripheral plasma from the participants, aiming at providing a more regional immune profiles of OLRs and revealing a reliable biomarker for monitoring the severity of OLRs on the molecular level.

We find the concentration of granzyme B as well as TGF-β is higher in intralesional plasma of OLRs than those in peripheral plasma. It is reported that increased expression of granzyme B in OLP lesions are found compared to cutaneous lichen planus, which is consistent with our findings [23]. The concentration of TGF-β in OLRs is controversial. In our previous study, salivary TGF-β is not significantly different between OLP and healthy control [8], while Zenouz *et al*. reveals a significant decrease in the serum levels of TGF-β from OLP patients [24]. These findings suggest that lesional granzyme B can be a potential biomarker for diagnosis of OLRs, and further studies are warranted for evaluating whether lesional granzyme B or TGF-β can be used to monitor the disease activity and therapeutic effects of OLRs.

Like granzyme B, TNF-α is another important cytokine that involves in killing target cells. In our study, concentration of TNF-α is decreased in intralesional plasma of OLL, which may due to the compensatory effect of granzyme B. Besides, It is reported that different detecting methods may lead to different results. Most studies observed elevated levels of TNF-α in the serum of patients with OLL through ELISA [25, 26], while when bead-based flow cytometry was used, no statistical difference was found in the serum levels of TNF-α between patients with OLP and health control [8, 27]. Further comparisons among various methods for cytokine detection are required.

In this study, the concentration of IL-12p70 in peripheral plasma is positively correlated with REU score, and the concentration of IL-12p70 in intralesional plasma is not statistically different from that in peripheral plasma. Considering that intralesional plasma is always collected with invasive examination of oral mucosa, and that executing invasive oral examination regularly is unrealistic, IL-12p70 in peripheral plasma may be appropriate for monitoring OLRs. Higher levels of IL-12 are found in systemic lupus erythematosus patients compared to controls, and p40 subunit serum levels were found to be positively correlated with the severity of the disease, which is in consistent with our result. Ohno *et al*. reported that PBMCs from OLP patients produced higher amounts of IL-12 than those from healthy controls [28]. Janardhanam *et al*. reported the salivary epithelial cells from OLP subjects express increased IL-12 than from health control [29]. These findings suggest IL-12 as a potential diagnostic biomarker for OLP.

However, the function of salivary IL-12 in monitoring the severity of the disease needs to be proved by further study.

There exist some limitations in this preliminary study. The concentration of some cytokine is below the lower limitation. Neither detection rate of IFN-γ or IL-4, which represent Th1/Th2 cytokines, meets the criteria for data analysis. As the Th1/Th2 imbalance is controversial [30, 31], further studied using methods with more sensitivity are needed in future. Besides, in the process of intralesional blood collection, the volume of the collected blood from few patients cannot meet the need for Luminex assay. A standard collection protocol of intralesional blood is essential for future study. In addition, due to ethic problems, we do not collect the intralesional plasma from healthy control subjects as well as from participants after treatment, which restricted us from comparing our data with these control groups.

Conclusions

In this preliminary study, the concentration of granzyme B and TGF-β are more abundant in intralesional microenvironment of OLRs than in peripheral plasma. IL-12p70 may be a potential molecular biomarker for evaluating the severity of OLRs. Cohort study of large population should be considered to furtherly confirm our results.

List Of Abbreviations

IFN, interferon

IL, interleukin.

IQR, interquartile range.

OLL, oral lichenoid lesion.

OLP, oral lichen planus.

OLRs, oral lichenoid reactions.

PBMCs, peripheral blood mononuclear cells.

SD, standard deviations.

TGF, transforming growth factor.

TNF, tumor necrosis factor.

Declarations

Ethics approval and consent to participate

This study was independently reviewed and approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (approval ID: SH9H-2020-TK376-1, approved on May 25th, 2020) and conducted according to the Declaration of Helsinki and the additional requirements. This study was undertaken with the understanding and written informed consent of each participant.

Consent for publication

Not applicable

Available of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KS, Methodology, Data Curation, Validation, Writing Original Draft, Formal Analysis, Funding, Resources. LP, Methodology, Data Curation, Writing Review & Editing. YWD, Methodology, Resources. JC, Methodology, Resources. GHD, Resources. CS, Methodology. JC, Methodology, Resources. XH, Investigation. YW, Conceptualization, Resources, Funding. GT, Conceptualization, Visualization, Funding. All authors read and approved the final manuscript.

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Tables

Table 1. Description of clinical characteristics of the participants with OLRs.

No.	Gender	Age (years)	Lesion Site	Systemic Disease	Drugs Used within 3 Months	REU score	Diagnosis
1	F	69	dorsum of tongue	osteoporosis, rheumatoid arthritis	vitamin D3, calcium, glucosamine hydrochloride	8	OLP
2	F	56	buccal mucosa	hypertension, hyperlipidemia	telmisartan, amlodipine aspartate, fenofibrate	5	OLL
3	F	45	buccal mucosa	denied	denied	5	OLP
4	M	34	buccal mucosa	denied	denied	2	OLP
5	F	58	buccal mucosa	hypertension	amlodipine, vitamins	2	OLL
6	F	62	buccal mucosa	hypertension, oral leukoplakia	valsartan, concentrated tinidazole gargles	3	OLP
7	M	39	buccal mucosa	denied	denied	4	OLL
8	F	49	buccal mucosa	denied	cefdinir	3	OLL
9	M	32	dorsum of tongue	denied	denied	7.5	OLL
10	F	61	buccal mucosa	carotid plaque, chronic gastritis, facial paralysis	rosuvastatin calcium, omeprazole, artificial bezoar metronidazole, mecobalamin	15	OLP
11	F	64	buccal mucosa	denied	denied	11.5	OLL
12	F	39	buccal mucosa	malignant teratoma	denied	6.5	OLP
13	M	65	buccal mucosa	diabetes mellitus, hypertension, cerebral insufficiency of blood supply	metformin hydrochloride, irbesartan and hydrochlorothiazide, TCM for promoting blood circulation (Yin Dan Xin Nao Tong capsule)	13	OLL
14	F	67	buccal mucosa	denied	denied	5.5	OLP
15	M	30	buccal	denied	denied	8	OLP

			mucosa				
16	F	25	buccal mucosa	NA	NA	NA	OLP
17	F	44	buccal mucosa	denied	denied	3	OLP
18	M	25	ventral tongue	denied	denied	7	OLP
19	M	58	buccal mucosa	diabetes mellitus, lacunar infarction, hypertension	metformin hydrochloride, atorvastatin calcium, valsartan	10	OLL
20	M	46	buccal mucosa	denied	denied	4.5	OLL
21	F	56	buccal mucosa	diabetes mellitus, psoriasis	metformin hydrochloride	10	OLP
22	F	55	buccal mucosa	lumbar disc protrusion	mecobalamin	10	OLP
23	M	55	ventral tongue	denied	TCM for clearing heat and toxic material (Bi Bai Ke caspule)	4.5	OLL
24	F	48	buccal mucosa	chronic gastritis	vitamin U belladonna and aluminium capsules, cefdinir	6	OLL
25	F	32	buccal mucosa	denied	denied	4	OLP
26	F	67	buccal mucosa	denied	vitamins	7	OLL

OLRs, oral lichenoid reactions; OLL, oral lichenoid lesion; F, female; M, male; TCM, traditional Chinese medicine; NA, not applicable.

Table 2. Detection rate of each cytokine

Cytokines	Range (pg/ml)	No. of samples		P
		Intralesional	Peripheral	
Granzyme B*	3.46-29884	25/26	24/26	1.000
IFN-α*	1.54-38240	23/26	20/26	0.465
IFN-β*	1.27-29364	1/26	5/26	0.191
IFN-γ	2.94-42396	0/26	12/26	<0.001
IL-2*	1.22-31590	26/26	26/26	-
IL-4	0.41-9564	4/26	14/26	0.004
IL-5*	2.49-47546	0/26	4/26	0.110
IL-6	3.84-71032	19/26	21/26	0.510
IL-10*	18.42-203564	19/26	26/26	0.010
IL-12p70*	7.57-155916	23/26	25/26	0.610
IL-17A	2.84-80204	16/26	19/26	0.375
TNF-α*	4.53-106272	23/26	23/26	1.000
TGF-β1	5.50-334970	26/26	26/26	-
TGF-β2	12.40-388090	26/26	26/26	-
TGF-β3*	4.05-370010	25/26	26/26	1.000

Chi-square was used. *, Fisher exact possibility test was used. The concentrations of granzyme B, IL-2, IL-12p70, TNF-α, TGF-β1, TGF-β2 and TGF-β3 could be further analyzed due to the high detection rate. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor.

Table 3. Comparisons of cytokine concentrations of intralesional and peripheral plasma of OLRs samples.

Cytokines	Concentrations (pg/ml)		P
	Intralesional	Peripheral	
Granzyme B	108.94 (57.80,404.94)	26.00 (20.25,58.98)	<0.001
IL-2	2.84 (2.04,3.26)	3.45 (2.49-5.53)	0.019
IL-12p70	10.59 (8.41,12.93)	10.59 (8.41,11.76)	0.697
TNF-α	7.66 (6.82,9.42)	10.34 (7.66,12.24)	0.048
TGF-β1	30448.92±19210.18	10199.04±5303.33	<0.001
TGF-β2	1659.73±319.49	1308.49±166.85	<0.001
TGF-β3	914.33±232.90	573.13±208.32	<0.001

Data are presented as mean ± standard deviations or median (25% interquartile, 75% interquartile) where appropriate. OLRs, oral lichenoid reactions; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor.

Table 4. Correlations between REU score and cytokines.

Cytokines	Intralesional		Peripheral	
	R	p	R	p
Granzyme B	0.017	0.936	-0.102	0.634
IFN-α	0.012	0.955	0.165	0.499
IL-2	0.072	0.725	0.093	0.652
IL-4	0.943	0.057	-0.08	0.785
IL-6	-0.116	0.637	0.015	0.947
IL-10	-0.105	0.669	0.028	0.891
IL-12p70	0.339	0.113	0.463	0.02*
IL-17A	0.041	0.879	0.031	0.899
TNF-α	-0.035	0.876	0.046	0.836
TGF-β1	-0.254	0.21	0.077	0.707
TGF-β2	-0.117	0.57	0.357	0.073
TGF-β3	-0.254	0.22	0.175	0.392

R, coefficient of correlation. *, p<0.05. IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor.

Figures

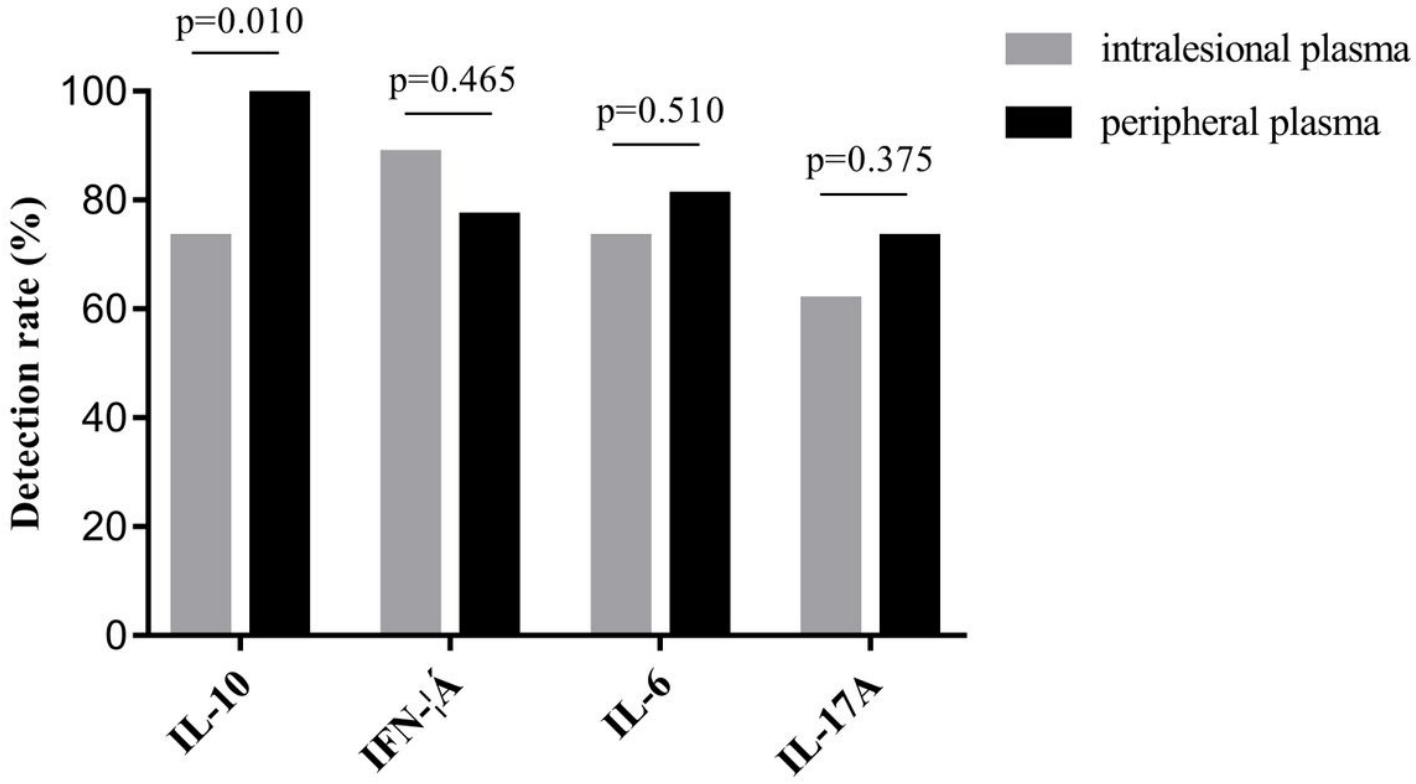
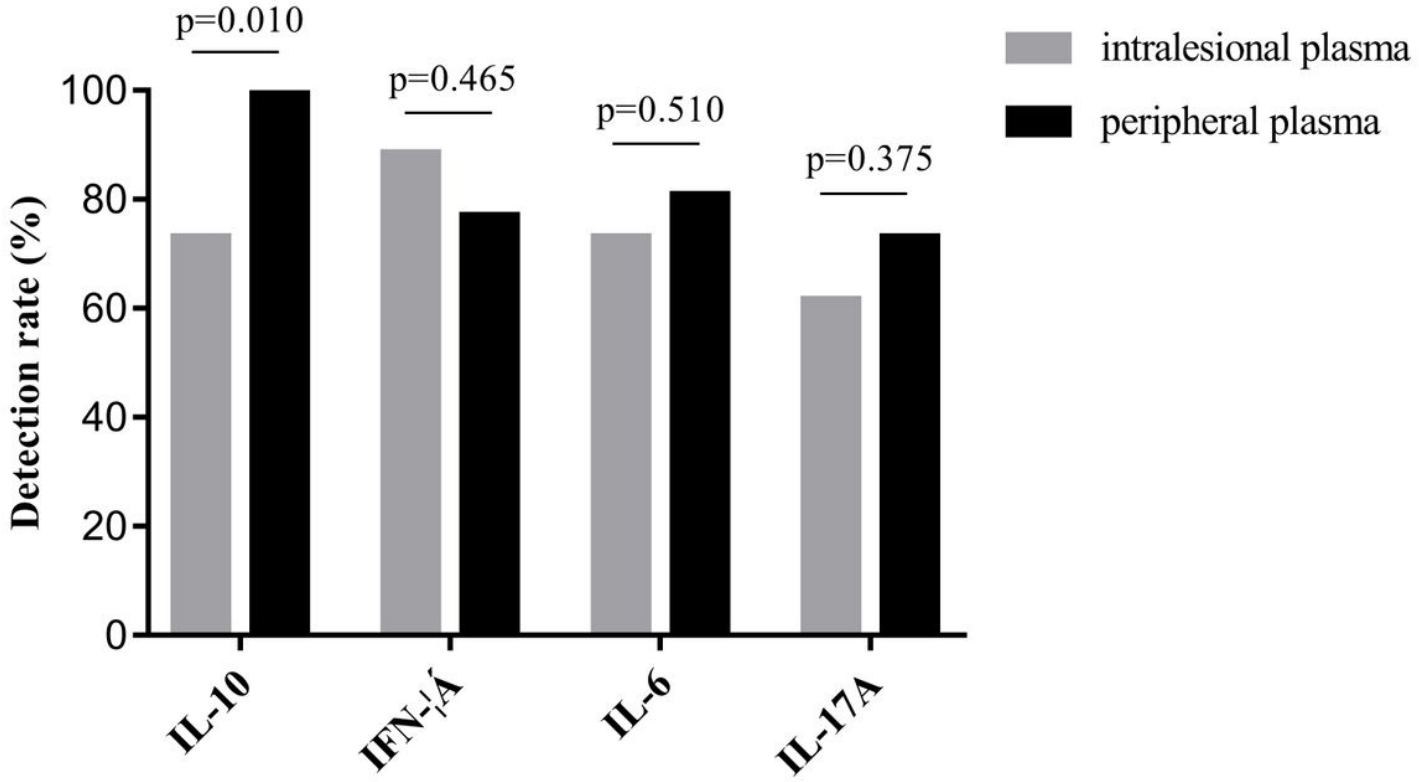


Figure 1

Comparisons of detection rate for cytokines of IFN-α, IL-6, IL-10 and IL-17A. Detection rate of these cytokines meets the criteria for qualitative analysis. IFN, interferon; IL, interleukin.



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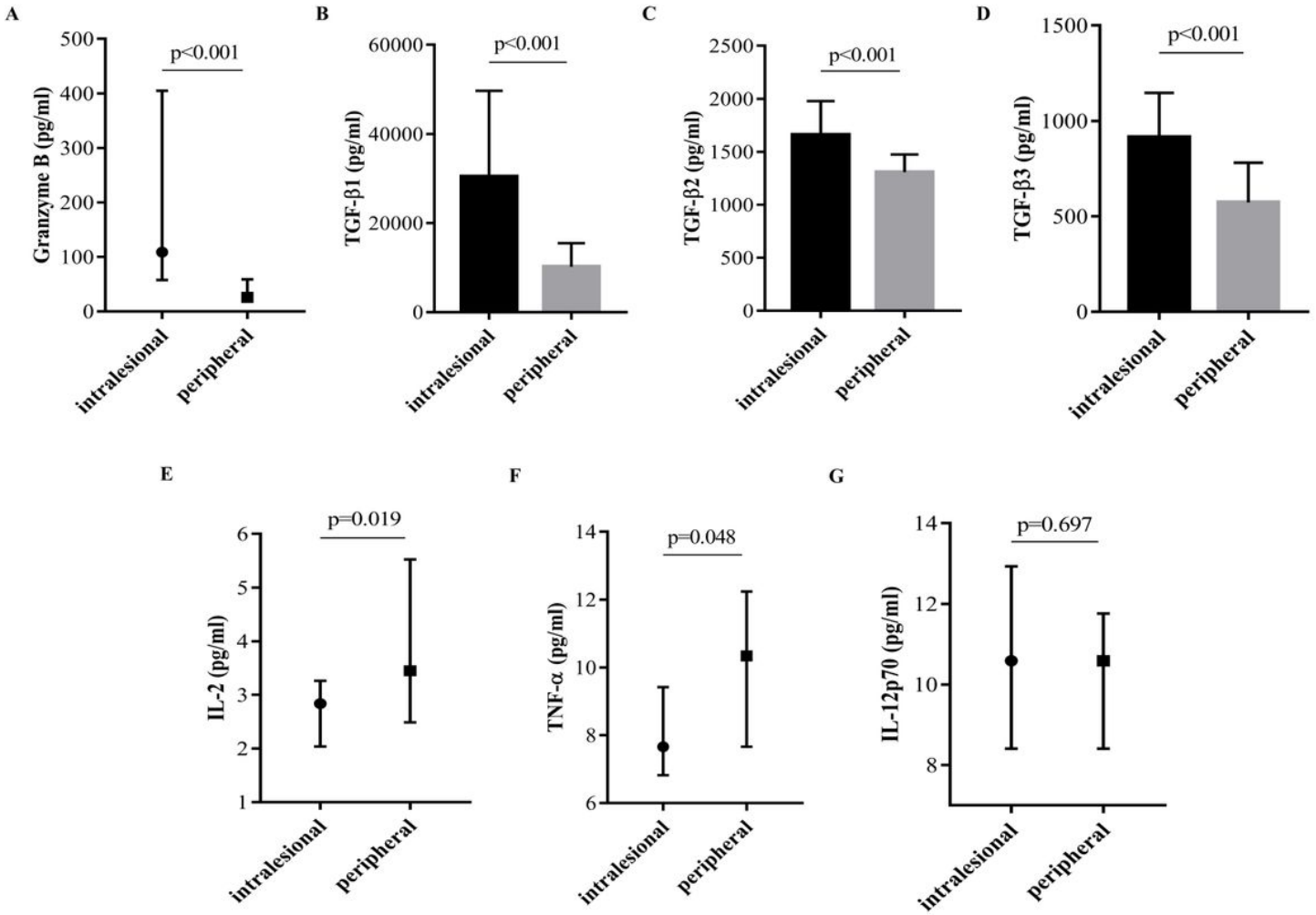
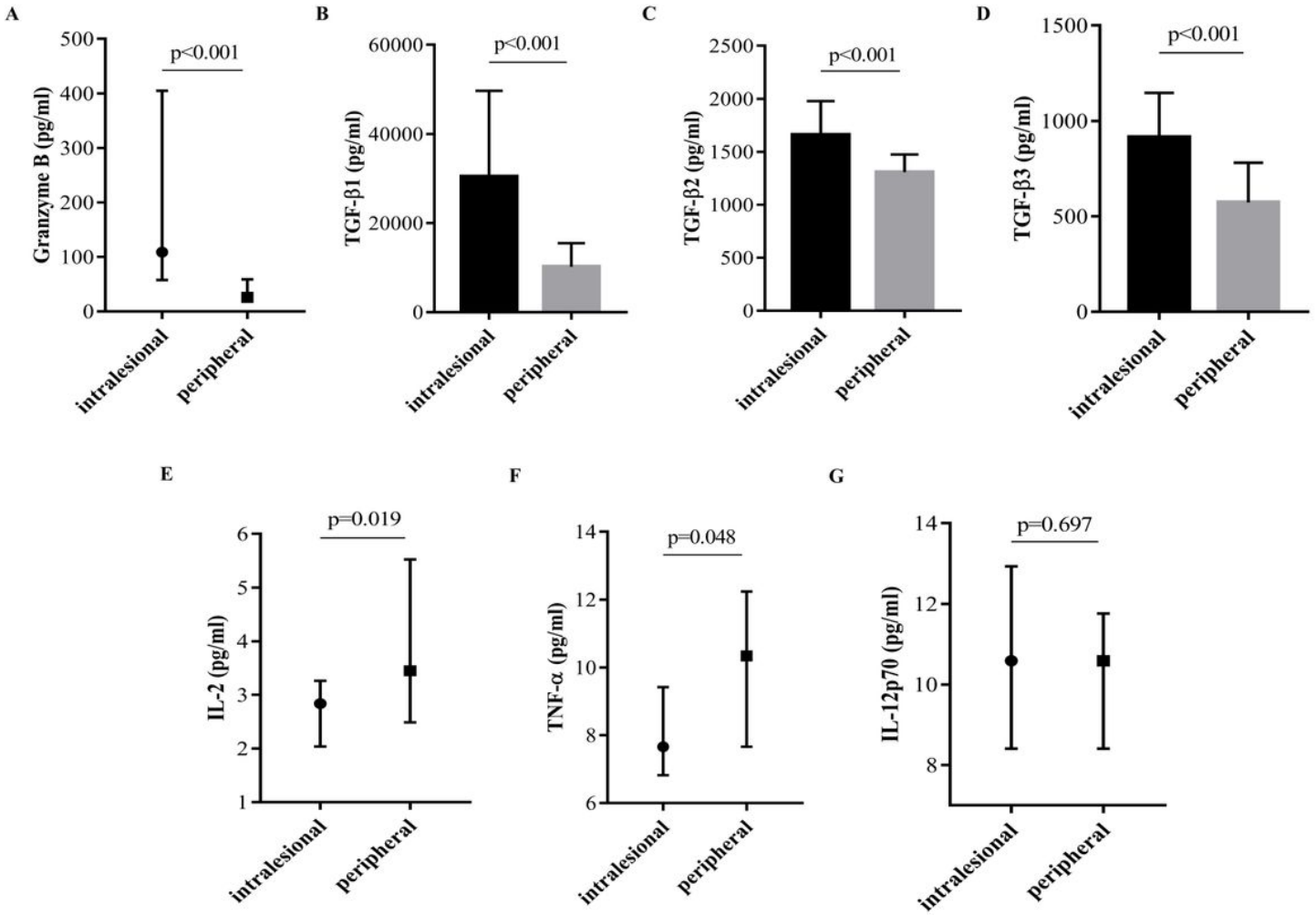


Figure 2

Comparisons of cytokine concentrations for granzyme B, IL-2, IL-12p70, TNF-α, TGF-β1, TGF-β2 and TGF-β3. Detection rate in both groups were over 80%. Data were analyzed by t test or Mann-Whitney test. IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor.



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