Interleukin-6 expression in inflamed and non-inflamed temporal arteries from patients with giant cell arteritis

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ABSTRACT

Objectives. *To evaluate whether interleukin-6 expression in the temporal arteries could be a more sensitive marker of active inflammation compared to the presence of an inflammatory infiltrate.*

Methods. *Sixty-three formalin-fixed, paraffin-embedded temporal artery biopsies performed between 2009 and 2012 from 32 patients with biopsy-proven giant cell arteritis, 8 patients with a negative biopsy but with a final diagnosis of giant cell arteritis, and 23 controls (patients with an initial clinical suspicion of giant cell arteritis in whom an alternative diagnosis subsequently was made) were examined. Biopsy specimens showing a transmural inflammatory infiltrate were considered positive for giant cell arteritis. Immunochemistry was performed to detect interleukin-6 in the temporal artery specimens. Slides of temporal artery biopsies were independently assessed by five readers. Interleukin-6 expression was graded as 0 (absent), 1 (mild), 2 (moderate) and 3 (marked). We considered anti-IL-6 staining positive if staining was of grade 2 or 3.*

Results. *Temporal artery biopsies specimens from patients with biopsy-proven giant cell arteritis, biopsy-negative giant cell arteritis and controls were positive for anti-interleukin-6 staining in 59%, 13% and 48% of cases, respectively.*

Conclusion. *Interleukin-6 expression does not increase the sensitivity of temporal artery biopsy in patients with giant cell arteritis who have morphologically uninflamed arteries.*

Introduction

Giant cell arteritis (GCA) is the most common vasculitis in individuals aged 50 years or older (1). The diagnostic gold standard of GCA remains temporal artery biopsy (TAB), which typically shows a transmural granulomatous inflammatory infiltrate consisting of lymphocytes, macrophages and sometimes giant cells (2). Less frequently, inflammation restricted to the adventitial or periadventitial tissue without medial involvement is observed, although it is less specific for GCA (2, 3). However, TAB can be negative (in about 23–27% of cases) (4, 5) in patients with an established diagnosis of GCA for a number of reasons. First, because temporal artery inflammation is segmental, the particular vessel segment sampled may not show inflammatory features, especially if the length of the biopsy specimen is too short (6, 7). Second, glucocorticoid treatment may reduce the sensitivity of TAB (4, 5). Finally, the temporal arteries may be truly spared by the inflammatory process, especially in patients with large-vessel involvement in whom TAB findings are negative in a substantial number (42–48%) of cases (8, 9).

Because TAB is important in confirming the diagnosis of GCA, attempts have been made to increase its sensitivity. In this regard, Muratore *et al*. investigated the histopathologic findings of patients with GCA with a negative TAB and compared them to those without arteritis to establish whether there were any morphological features that could be associated with GCA in the absence of an inflammatory infiltrate (10). However, no histologic changes of the temporal artery wall were found to be specific to GCA when no inflammatory infiltrate was evident.

A different approach was resorted to by Lally *et al*., who wondered whether inflammatory molecules not routinely assessed in TAB specimens might discriminate patients with GCA with positive (*i.e*. morphologically inflamed) temporal arteries from those with uninflamed arteries (11). Specifically, they

looked at the temporal artery expression of phosphorilated ezrin/radixin/moesin (pERM), a downstream target and surrogate of rho kinase. Rho kinase is an intracellular GTPase that regulates several cell processes including IL-17 response, which is known to be activated in GCA (12). High pERM staining was found in 86% of patients with GCA (79% of those with a positive and 94% of those with a negative TAB), but only 44% of unaffected controls, suggesting that rho kinase activity is increased in the temporal arteries of GCA patients irrespective of the presence of standard inflammatory changes.

Interleukin-6 (IL-6) is a key proinflammatory molecule, whose role in GCA is underscored by several lines of evidence. In GCA, IL-6 is synthesised both by vascular macrophages and circulating monocytes (13). Serum levels of IL-6 are elevated in active disease (14), correlate with disease activity (15) better than the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (16), and decline following the institution of glucocorticoid therapy (17), while IL-6 arterial wall production has been linked to higher glucocorticoid requirements and longer duration of therapy (18). The relevance of IL-6 to the pathogenesis of GCA has been confirmed by the clinical efficacy of IL-6 blockade in GCA with the monoclonal antibody tocilizumab (19).

Recently, O'Neill *et al*. have shown that temporal artery explants from patients with GCA spontaneously release significant amounts of IL-6 regardless of the presence of an inflammatory infiltrate at TAB; in contrast, IL-6 release was negligible from temporal arteries taken from subjects without GCA (20). Taken together, these findings may suggest that IL-6 expression in the temporal arteries could be a more sensitive marker of active inflammation compared to the presence of an inflammatory infiltrate. In this study, we aimed to test this hypothesis by evaluating IL-6 expression in temporal artery specimens from GCA patients with morphologically inflamed and uninflamed temporal arteries, respectively.

Patients and methods

All patients with suspected GCA seen by Rheumatologists or referred by medical practitioners and community-based specialists to the Rheumatology Unit of Santa Maria Nuova Hospital of Reggio Emilia, Italy are routinely assessed by Rheumatologists and undergo a standard work-up including temporal artery ultrasonography and biopsy. Santa Maria Nuova Hospital is the only referral center for a population of 462,860 people living in Reggio Emilia area.

For the purpose of our study, 63 formalin-fixed, paraffin-embedded (FFPE) TABs performed between 2009 and 2012 from 32 patients with biopsy-proven GCA, 8 patients with a negative TAB but with a final diagnosis of GCA, and 23 controls (patients with an initial clinical suspicion of GCA in whom an alternative diagnosis subsequently was made) were retrieved. The length of the TAB specimen after fixation was at least 0.5 cm in all cases. Our Pathology Department applied during the study period the same protocol for microscopic examination of TAB. The artery was transversely divided in several pieces of 3-4 millimeters in length, which were routinely fixed in formalin and embedded in paraffin. Four-μm-thick sections were cut from the paraffin blocks and were stained with haematoxylin and eosin. Biopsy specimens showing transmural inflammatory infiltrate consisting of lymphocytes and macrophages, with or without multinucleated giant cells, were considered positive for GCA (2). Methods have been described in detail elsewhere (21). Briefly, a detailed clinical history and a complete physical examination was performed in all patients by a trained rheumatologist at diagnosis and at each follow-up visit. For patients with negative TAB, the final diagnosis was made at the end of the follow-up period by consensus by two rheumatologists (F.M. and C.S.), who retrospectively evaluated all the medical records from symptoms' onset to the end of the study follow-up period (30rd June 2014), last visit or death. Patients with a final diagnosis of GCA were not required to fulfill the 1990 American College of Rheumatology (ACR) criteria, but they had to be older than 50 years (22). A Pathologist with an expertise in immunostaining (IT) who had no access to the clinical data or knowledge of the previous pathology report performed the immunochemistry to detect IL-6 in the temporal artery specimens. Immunohistochemistry was performed on 4μm formalin-fixed, paraffin-embedded tissue sections with a 1:400 dilution of rabbit polyclonal anti-human IL-6 antibody (NOVUS Biologicals Littleton, Co.) for 60' at 37°. The tissue sections were pre-treated with heat antigen retrieval in EDTA buffer for 64'. The immunohistochemical staining was developed on platform Ventana Bench-Mark XT using the OptiView DAB Detection Kit (Ventana-Roche, Tucson, AZ, US) and amplified with Amplification Kit (Ventana-Roche, Tucson, AZ). Each section used for immunohistochemical analysis was counterstained with haematoxylin and examined for GCA positivity by standard light microscopy by a pathologist (A.C.) with expertise in vasculitides who had no access to the clinical data or knowledge of the previous pathology report. Furthermore, additional sections were cut from all the 31 negative TABs (8 TAB-negative GCA and 23 controls) and stained with haematoxylin and eosin in order to avoid missing skip lesions.

With regard to the subjects studied, seven out of 8 (88%) TAB-negative GCA patients satisfied the 1990 ACR classification criteria, and 4/8 (50%) had evidence of large-vessel involvement (LVI) at colour-duplex sonography (CDS) of the arteries above the aorta. Two of these 4 patients underwent F-18 fluorodeoxyglucose (FDG) positron emission tomography/computerised tomography (PET/CT) that confirmed LVI secondary to GCA in both cases. Other 3/8 (38%) TAB-negative GCA patients had the characteristic halo sign at temporal artery CDS. The only TAB-negative GCA patient not satisfying the 1990 ACR classification criteria had both the characteristic halo sign at temporal artery CDS and evidence of LVI at CDS of the arteries above the aorta. Only one of the 23 controls (4%)

satisfied the 1990 ACR classification criteria for GCA. CDS of the arteries above the aorta was performed in 19/23 controls, and F-18 FDG PET/CT in the remaining 4/23, without evidence of LVI in any case. After a median (IQR) follow-up period of 24.3 (3.3, 35.3) months, final diagnosis for these 23 controls were: 8 polymyalgia rheumatica; 4 non-arteritic anterior ischaemic optic neuropathy; 2 ANCA-associated vasculitis; 3 rheumatoid arthritis; 3 fibromyalgia; 1 fever of unknown origin; 2 osteoarthritis. At the histopathologic analysis, all the 32 TAB-positive GCA evaluated showed the classic histological pattern of GCA (transmural inflammation). The analysis of the additional sections cut from the 31 negative TABs (8 TAB-negative GCA and 23 controls) did not show inflammation.

Statistical analysis was performed using SPSS statistical package, version 18.0. Continuous data were described as mean±SD or median and interquartile range, and categorical variables as percentages. Differences between patients were analysed using the non-parametric Mann-Whitney U-tests for continuous variables and the chi-square test (or Fisher's exact test whenever indicated) for categorical variables. *p*-values less than 0.05 were considered significant.

The study was approved by the Ethics Committee of Reggio Emilia Hospital, and informed consent was obtained from all patients or their relatives.

Results

Slides of TAB specimens were independently assessed by five readers. IL-6 expression was graded as 0 (absent), 1 (mild), 2 (moderate) and 3 (marked). Inter-reader differences were resolved by consensus. Representative images of the grades of staining intensity are shown in Figure 1.

We considered anti-IL-6 staining positive if staining was of grade 2 or 3 since grade 1 was faint, sometimes difficult to differentiate from background, and showed the least degree of agreement between readers. TAB specimens from patients with biopsy-proven GCA, biopsy-negative GCA and controls were positive for anti-IL-6 staining in 59%, 13% and 48% of cases, respectively (Table I), the difference between biopsy-proven and biopsy-negative GCA patients being significant.

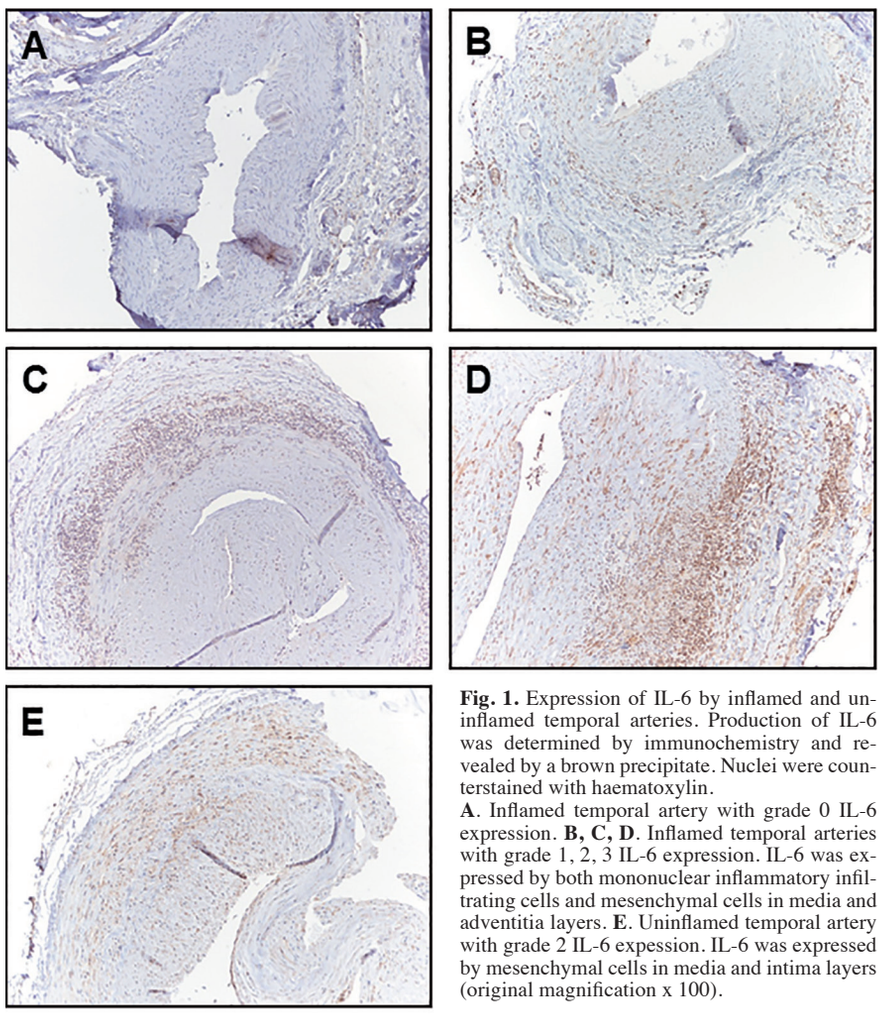

Fig. 1. Expression of IL-6 by inflamed and uninflamed temporal arteries. Production of IL-6 was determined by immunochemistry and revealed by a brown precipitate. Nuclei were counterstained with haematoxylin.
A. Inflamed temporal artery with grade 0 IL-6 expression. **B, C, D**. Inflamed temporal arteries with grade 1, 2, 3 IL-6 expression. IL-6 was expressed by both mononuclear inflammatory infiltrating cells and mesenchymal cells in media and adventitia layers. **E**. Uninflamed temporal artery with grade 2 IL-6 expression. IL-6 was expressed by mesenchymal cells in media and intima layers (original magnification x 100).

In non-inflamed TABs, IL6 was mainly expressed in media and intima layers, while in inflamed TABs IL6 was mainly expressed in media and adventitia layers and in inflammatory infiltrating cells.

No correlations were found between IL-6 expression and the following parameters: age, gender, presence of temporal artery abnormalities at physical inspection or of a halo sign at CDS, cranial symptoms including headache and claudication, constitutional manifestations including fever, polymyalgia rheumatica (PMR), nor with histopathological features such as neoangiogenesis, degree of inflammatory infiltrate, and thrombosis. However, there was a significant correlation between IL-6 expression and blindness (p=0.011). Blindness was recorded in 2 patients with biopsy-proven GCA and 4 controls (all with a final diagnosis of non-arteritic ischaemic optic neuropathy) (Table II).

We stratified control patients for the presence/absence of PMR. No significant differences for the expression of IL-6 were observed between patients with and without PMR (5/8 - 62.5% *vs*. 6/15 - 40%, p=0.400). No significant differences were also observed for the expression of IL-6 between patients with isolated PMR and TAB+ GCA (62.5% *vs*. 59%, p=1.000).

Discussion

We devised this study on the basis of the hypothesis that IL-6 arterial wall staining might be more sensitive than the presence of an inflammatory infiltrate for the diagnosis of GCA. However, the study findings do not support our original hypothesis, since IL-6 was expressed only in a minority of patients

Table I. Demographic, clinical, and laboratory characteristics of TAB+ GCA patients, TAB- GCA patients, and unaffected controls*.

	GCA TAB+ n=32 (A)	GCA TAB- n=8 (B)	Controls n=23 (C)	*p*-value A *vs*. B	*p*-value A *vs*. C	*p*-value B *vs*. C
Age at TAB, mean ± SD, years	72.4 ± 6.9	71.6 ± 11.3	73 ± 9.2	1.000	0.505	0.912
Female gender	25/32 (78%)	8/8 (100%)	18/23 (78%)	0.309	0.990	0.291
Time from symptom onset to TAB, median (IQR), weeks	11.5 (7, 17)	18 (6, 51.5)	10 (6, 42.5)	0.571	0.904	0.605
Fulfillment of ACR criteria for GCA	32/32 (100%)	7/8 (88%)	1/23 (4%)	0.200	<0.0001	<0.0001
Patients taking prednisone prior to TAB	25/32 (78%)	4/8 (50%)	15/23 (65%)	0.182	0.289	0.676
Prednisone dose at TAB, mean ± SD, mg/day	30.8 ± 18.9	14.4 ± 8.8	18.3 ± 13.6	0.124	0.040	0.736
Prednisone treatment duration prior to TAB, median (IQR), days	15.5 (8, 33.5)	94 (5.5, 272.5)	30 (9.5, 217.5)	1.000	0.432	1.000
Any cranial symptoms§	30/32 (94%)	7/8 (88%)	15/23 (65%)	0.498	0.011	0.379
Systemic signs/symptoms#	23/32 (72%)	7/8 (88%)	10/23 (44%)	0.653	0.034	0.045
Permanent visual loss	2/32 (6%)	0/8 (0%)	4/23 (17%)	1.000	0.223	0.550
ESR at TAB, mean ± SD, mm/hour	76.7 ± 24.6	74.8 ± 28.1	45.6 ± 38.8	0.654	0.002	0.048
CRP at TAB, mean ± SD, mg/dl	8.1 ± 4.3	6.6 ± 6.6	3.2 ± 5.1	0.250	<0.001	0.082
TAB positive for any grade of anti-IL-6 staining	30/32 (94%)	4/8 (50%)	20/23 (87%)	0.010	0.639	0.053
TAB positive for grade 2-3 anti-IL-6 staining	19/32 (59%)	1/8 (13%)	11/23 (48%)	0.044	0.396	0.108

*Values are the number of positive patients/total number of patients for whom data were available (percentage), unless otherwise indicated.
TAB: temporal artery biopsy; SD: standard deviation; IQR: interquartile range; ACR: American College of Rheumatology; GCA: giant cell arteritis; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.
§At least 1 of the following: headache, scalp tenderness, jaw claudication, abnormalities of temporal arteries.
#At least 1 of the following: fatigue, anorexia, weight loss of at least 4 kg, or fever.

Table II. Comparisons of demographic, clinical, and histologic findings of patients with grade 2-3 and those with grade 0-1 IL-6 expression in TAB specimens*.

	Grade 2-3 anti-IL6 staining (n = 31)	Grade 0-1 anti-IL6 staining (n=32)	*p*-value
Female gender	24/31 (77.4%)	27/32 (84.4%)	0.536
Age at TAB, mean ± SD, years	72.0 ± 7.7	73.0 ± 8.9	0.397
Time from symptom onset to TAB, median (IQR), weeks	12 (6, 17)	11 (6, 35.5)	0.903
Fulfillment of ACR criteria for GCA	21/31 (67.7%)	19/32 (59.4%)	0.490
Patients taking prednisone prior to TAB	23/31 (74.2%)	21/32 (65.6%)	0.459
Prednisone dose at TAB, mean ± SD, mg/day	27.4 ± 17.4	22.4 ± 17.9	0.196
Prednisone treatment duration prior to TAB, median (IQR), days	16.5 (9, 56)	25 (8, 255)	0.882
Any cranial symptoms§	26/31 (83.9%)	26/32 (81.2%)	0.784
Headache	21/31 (67.7%)	20/32 (62.5%)	0.663
Abnormalities of temporal arteries	14/30 (46.7%)	12/29 (41.4%)	0.683
Systemic signs/symptoms#	17/31 (54.8%)	23/32 (71.9%)	0.160
Any visual symptoms°	9/31 (29%)	5/32 (15.6%)	0.201
Permanent visual loss	6/31 (19.4%)	0/32 (0%)	0.011
PMR	12/31 (38.7%)	19/32 (59.4%)	0.101
Temporal artery halo on CDS	17/26 (65.4%)	11/28 (39.3%)	0.055
Large-vessel involvement at imaging	3/23 (13%)	4/27 (14.8%)	1.000
ESR at TAB, mean ± SD, mm/hour	65.5 ± 35.1	64.6 ± 33.2	0.912
CRP at TAB, mean ± SD, mg/dl	6.2 ± 5.3	5.8 ± 5.6	0.670
Haemoglobin, mean ± SD, gm/dl	12.4 ± 1.7	11.8 ± 1.6	0.485
Platelets, mean ± SD, ($x10^3/\mu l$)	383.4 ± 102.0	342.5 ± 106.1	0.197
Calcification	2/31 (6.5%)	3/32 (9.4%)	1.000
Neoangiogenesis	12/29 (41.4%)	10/29 (34.5%)	0.588
Intimal hyperplasia	21/26 (80.8%)	21/29 (72.4%)	0.467

*Values are the number of positive patients/total number of patients for whom data were available (percentage), unless otherwise indicated.
TAB: temporal artery biopsy; SD: standard deviation; IQR: interquartile range; ACR: American College of Rheumatology; GCA: giant cell arteritis; PMR: polymyalgia rheumatica; CDS: colour duplex sonography; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.
§At least 1 of the following: headache, scalp tenderness, jaw claudication, abnormalities of temporal arteries.
#At least 1 of the following: fatigue, anorexia, weight loss of at least 4 kg, fever.
°At least 1 of the following: permanent visual loss, transient vision changes, diplopia.

with biopsy-negative GCA. In contrast, IL-6 was expressed in about half of patients with biopsy-proven GCA and control individuals. A limitation of our approach is that to detect IL-6 expression we used only immunohistochemistry, which is more a qualitative than a quantitative technique, without a control isotype-matched IgG to exclude non-specific IgG binding.

The high expression of IL-6 in the arterial wall of patients with morphologi-

cally inflamed temporal arteries is in agreement with previous data (18, 20). In this context, IL-6 expression seems to be due to infiltrating monocytes rather than lymphocytes (23). However, IL-6 gene (although not protein expression), has also been demonstrated in histologically uninflamed arteries from subjects in whom GCA was excluded (18). On a similar line, a study showed that cultured temporal artery explants from GCA patients and controls released high amounts of IL-6 (24), while other data demonstrated high IL-6 release from smooth cells derived from histologically normal temporal arteries (25). Finally, other authors have documented spontaneous IL-6 release over 24 hours from temporal artery explants of both biopsy-proven and biopsy-negative GCA patients (20).

It is possible that measuring IL-6 released from temporal artery explants is a more sensitive test than looking at its tissue expression. Our study was not designed to address this question, but preliminary, unpublished data from our group (SC) have shown that IL-6 is released in the supernatants from both morphologically inflamed and non-inflamed temporal artery specimens from GCA patients. Differently from previous studies which highlighted higher immunohistochemical scores for IL-6 in patients with a strong systemic inflammatory reaction (18), we did not find any correlation between IL-6 expression and clinical or laboratory parameters associated with GCA, except for blindness. The association between IL-6 expression and blindness may be spurious (*i.e.* due to a type 1 error) given the large number of parameters that we analysed, because we applied no correction for multiple parameters. In fact, opposite results were obtained from a larger patients' cohort (26). Alternatively, because IL-6 has been shown to be endowed with angiogenic properties (18, 27), it may be speculated that the high IL-6 expression in the arteries from subjects that went blind (regardless of whether blindness resulted from arteritic or non-arteritic anterior ischaemic optic neuropathy) could be a compensatory mechanism to the ischaemia leading to vision loss. Yet another hypothesis is that endothelial dysfunction, which has been linked to elevated serum IL-6 levels (28), could be involved in the pathogenesis of blindness.

Furthermore, in mouse models of anterior ischaemic optic neuropathy and central retinal artery occlusion IL-6 levels were elevated after induction of visual loss in plasma, optic nerve and retina, suggesting that this proinflammatory cytokine may play a role in the preservation of the tissue or in its destruction also in acute ischaemic events of the eye independently by the inflammatory or non-inflammatory nature of the event (29, 30).

Studies from different groups have shown that a high systemic inflammatory response, expressed as an ESR greater than 100 mm/1st hour, was associated with protection against the development of severe ischaemic complications in patients with GCA (31, 32). Since there was no correlation between IL-6 expression in the temporal arteries and clinical features in patients with GCA, it is possible that the clinical spectrum of the disease may be influence by the systemic levels of IL-6 rather than by the expression of IL-6 in the temporal artery. This may not be the case for IFN-gamma since both high transcription of IFN-gamma mRNA and IFN-gamma functional polymorphisms were found to be associated with clinical manifestations of severity, in particular with the development of visual ischaemic complications in patients with GCA (33, 34).

In conclusion, our study provides evidence that IL-6 expression does not increase the sensitivity of TAB in patients with morphologically uninflamed arteries. A search for further markers that may increase the sensitivity of TAB is warranted.

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