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Association between cigarette smoking and release of tumour necrosis factor α and its soluble receptors by peripheral blood mononuclear cells in patients with rheumatoid arthritis

J. R. Glossop[1,2], P. T. Dawes[1] and D. L. Mattey[1,2]

Objective. **To investigate the relationship between cigarette smoking and release of TNF-α and its soluble receptors (sTNFRI and sTNFRII) by peripheral blood mononuclear cells (PBMCs) from RA patients.**
Methods. **We studied 71 RA patients with established disease (mean duration 10.6 yr). Smoking history was established by questionnaire. T lymphocytes and monocytes were isolated from peripheral blood and incubated with or without stimulation (phytohaemagglutinin and lipopolysaccharide, respectively). Release of TNF-α and sTNFR into culture medium was measured by enzyme-linked immunosorbent assay.**
Results. **TNF-α release by stimulated T lymphocytes was significantly higher in patients with a history of smoking than in those who had never smoked (1416.0 *vs* 767.4 pg/ml, $P = 0.04$), and showed a relationship with smoking duration and intensity (P for trend ≤ 0.009). Monocyte TNF-α release was not associated with smoking status. Release of sTNFR showed no clear relationships with extent of smoking, although release by stimulated T lymphocytes was higher in past smokers than in those who had never smoked ($P \leq 0.03$). The ratio of TNF-α/sTNFR released from T lymphocytes was higher in past and current smokers, and was associated with extent of smoking. No relationship was found between smoking and plasma TNF-α levels, but levels of both receptors were higher in past smokers.**
Conclusion. **In RA patients who smoke there is an alteration in the ratio of TNF-α/sTNFR released by stimulated T cells that might favour increased TNF-α activity. The increased TNF-α/sTNFR ratio is associated with extent of smoking, and remains elevated after smoking cessation.**

KEY WORDS: Smoking, Tumour necrosis factor, Tumour necrosis factor receptors, Mononuclear cells, Rheumatoid arthritis.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the synovial joints, ultimately leading to joint destruction and permanent disability. Its precise aetiology remains to be elucidated, although both environmental and genetic components are believed to influence the development of disease. Of the former, cigarette smoking appears to be of importance in both disease susceptibility and severity.

A study of disease-discordant twins by Silman *et al.* [1] identified an association between ever smoking and RA in both monozygotic and dizygotic twin pairs. The results of other studies also indicate that smoking might be a susceptibility factor in RA [2–7], and that it is associated with more severe disease [7–14]. Most of these studies have reported an association between smoking and the incidence of rheumatoid factor (RF), suggesting that the effects of smoking in RA might be mediated via RF production. More recently, an interaction between smoking and carriage of the HLA-DRB1 shared epitope has been reported to increase the risk of developing RF positive RA [15].

The mechanisms responsible for the influence of smoking in RA are not clear, although it seems reasonable to implicate toxic compounds in cigarette smoke, such as nicotine and reactive oxygen species. This might explain, at least in part, the finding of altered immune cell functions in heavy smokers [16–18]. The consequence of such changes may include effects on cytokine secretion, since TNF-α production by peripheral blood mononuclear cells (PBMCs) is elevated in smokers [19, 20]. Lei *et al.* [20] further showed that nicotine was capable of inducing increased TNF-α release. However, the effect of nicotine does not appear to be simply inductive since nicotine at higher concentrations inhibited TNF-α secretion. Similarly, Madretsma *et al.* [21] reported that nicotine, at levels equivalent to those in the plasma of smokers, inhibited TNF-α production in non-adherent mononuclear cells.

TNF-α is considered to be a central cytokine in RA inflammation [22], so evidence that components of cigarette smoke may influence the release of TNF-α implicates smoking as an important factor in the inflammatory process. The actions of TNF-α are mediated through binding to two different cell surface receptors, TNF receptor (TNFR) I and TNFRII [23, 24]. Both are transmembrane glycoproteins with a conserved cysteine-rich extracellular domain that facilitates ligand binding. The two receptors can promote distinct TNF-α -induced cellular responses, although both are able to induce the NF-κB and apoptotic

[1]Staffordshire Rheumatology Centre, University Hospital of North Staffordshire and [2]Institute of Science and Technology in Medicine, Keele University, Keele, UK.

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Correspondence to: D. L. Mattey, Staffordshire Rheumatology Centre, Haywood Hospital, High Lane, Burslem, Stoke-on-Trent, Staffordshire ST6 7AG, UK. E-mail: Derek.Mattey@uhns.nhs.uk

pathways [25–29]. Both TNFRI and TNFRII can be shed from cells as soluble proteins, derived from the receptor extracellular domains [30–34] by the actions of TNF-α converting enzyme (TACE) or serine proteases such as elastase [35, 36]. Ligand binding capacity is retained in this form and they can act as natural inhibitors of TNF-α. A recent study from our laboratory suggested that the level of soluble TNFR (sTNFR) released by T cells from RA patients is associated with polymorphism (T676G) in the TNFRII gene [37].

In this study we investigated the release of TNF-α and sTNFR from PBMCs of a group of RA patients who were past or current smokers and compared the data with data from patients who had never smoked.

Methods

Patients

A group of 71 patients with established RA were studied (Table 1). The patients were all of Caucasian origin and resident in North Staffordshire, UK. They satisfied the 1987 American College of Rheumatology criteria for RA [38]. Each patient was receiving anti-inflammatory and/or anti-rheumatic therapy. The majority of patients ($>95\%$) was being treated with one or more disease-modifying anti-rheumatic drugs, whilst a small minority of individuals ($<5\%$) received steroids and cytotoxic drugs such as azathioprine or cyclophosphamide. No patients were being treated with anti-TNF-α agents. The study was approved by the North Staffordshire local research ethics committee, and all patients gave written informed consent to participate.

Determination of smoking history

A history of current and past smoking was obtained from a questionnaire completed by each patient. Patients were initially classified by whether they had ever or never smoked. Ever smokers were those that had smoked at least one cigarette a day for 1 yr or more. All patients who had ever smoked had started smoking before the onset of RA. Those who had ever smoked were further categorized into past and current smokers. All past smokers had stopped smoking at least 1 yr before entry into the study. The extent of smoking was quantified in pack years. One pack year is equivalent to 20 cigarettes per day for 1 yr. The demographic and clinical characteristics of the smoking and non-smoking groups are shown in Table 1. Consistent with other studies, patients with a smoking history were more likely to be male [odds ratio (OR) 2.8, 95% confidence interval (CI) 1.0–7.9, $P=0.049$], and positive for RF (OR 2.9, 95% CI 1.1–7.9). There was also a trend towards increased frequency of nodular disease in smokers. No significant differences were seen between groups with regard to age of onset, disease duration, Health Assessment Questionnaire (HAQ) score, erosive disease and Larsen score, although past smokers were older than current smokers ($P=0.048$).

TABLE 1. Characteristics of RA patients according to smoking status

	Never smoked ($n=25$)	Past smoker ($n=29$)	Current smoker ($n=17$)
Male/female (n)	6/19	15/14	7/10
Age, mean ± S.D. (yr)	53.0 (11.8)	54.3 (7.9)	48.7 (10.8)
Age at onset, mean ± S.D. (yr)	43.0 (10.6)	42.5 (10.0)	39.3 (10.7)
Disease duration, mean ± S.D. (yr)	10.0 (8.3)	11.7 (6.3)	9.4 (4.5)
RF-positive	40%	67.9%	64.7%
Nodule-positive	16%	27.6%	41.2%
Erosions	91.7%	96.0%	88.2%
HAQ score	1.24	1.45	1.43
Larsen score	78.7	87.9	78.0

Cell isolation and culture

Fresh peripheral blood samples (22 ml) were collected during the morning from each patient. T lymphocytes were isolated from 4 ml blood by negative selection using a modified density gradient centrifugation technique that uses novel tetrameric antibody complexes (RosetteSep; Stemcell Technologies, Vancouver, BC, Canada). Monocytes were isolated form 12 ml blood using a standard density gradient centrifugation method (NycoPrep^{TM} 1.068; Nycomed Pharma AS Diagnostics, Oslo, Norway). Isolated T lymphocytes (2×10^5 cells/200 μl) and monocytes (10^5 cells/200 μl) were cultured in RPMI 1640 synthetic culture medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% autologous serum, in 96-well cell culture plates. T lymphocyte and monocyte cultures were incubated, with or without phytohaemagglutinin (PHA; 10 μg/ml) or lipopolysaccharide (LPS; 0.01 μg/ml), respectively, at 37°C in a 5% CO_2 humidified air environment. Cell supernatants were harvested after 24 (monocytes) and 48 h (T lymphocytes) and were stored at –20°C until required for analysis of TNF-α and sTNFR release.

Enzyme-linked immunosorbent assays (ELISAs)

The release of TNF-α and sTNFR into the culture medium by T lymphocytes and monocytes was quantified using the respective Duoset ELISA Development Kit as directed by the manufacturer (R&D Systems Europe, Abingdon, UK). For determination of stimulated TNF-α release, T lymphocyte and monocyte samples were diluted 1:3 and 1:10, respectively, whilst all unstimulated samples were diluted 1:2. To measure the release of both soluble TNFRI and TNFRII, all T-lymphocyte samples were diluted 1:3 and all monocyte samples were diluted 1:4. Levels of TNF-α, sTNFRI and sTNFRII were also measured in plasma collected at the same time as the mononuclear cells. Plasma samples were run undiluted, diluted 1:10 and diluted 1:20 respectively. All samples were run in duplicate with the appropriate standards on 96-well microplates, and measured at a wavelength of 450 nm.

Statistical analysis

Mononuclear cell release of TNF-α and sTNFRs was compared between smoking groups (never, past and current) by Kruskal–Wallis one-way analysis of variance (ANOVA) for TNF-α levels, or analysis of covariance (ANCOVA) with adjustment for age (where appropriate) for sTNFR levels. The extent of smoking was also stratified into a number of categories according to years smoked (duration) or pack years (intensity) (i.e. 0, 1–14, 15–29, $\geq$30), and general linear model (GLM) implementation of ANOVA or ANCOVA was carried out to examine any relationship with TNF-α or sTNFR release. In all analyses, correction for multiple comparisons was carried out by the Bonferroni (comparison with control) method. The relationships between cellular release of TNF-α and each soluble TNFR and their correlation with age and disease duration were assessed by Spearman's rank correlation (non-parametric data) or Pearson's correlation (normally distributed data) where appropriate. All data were analysed using the Number Cruncher Statistical Software package for Windows (NCSS 2000; NCSS Statistical Software, Kaysville, UT, USA).

Results

Smoking and mononuclear cell release of TNF-α

Of the 71 patients, 17 (23.9%) were current smokers, 29 (40.8%) were past smokers and the remaining 25 (35.2%) had never smoked. The extent of smoking in past and current smokers was similar, with no significant difference in the mean number of pack years (27.2 *vs* 26.5, respectively), although the current smokers tended to have smoked for longer (25.1 *vs* 32.8 yr).

Release of TNF-α by unstimulated T lymphocytes was undetectable (< 15 pg/ml) in 44/69 (63.8%) patients, but detectable in cells from all patients after stimulation. In the case of monocytes, TNF-α release was detected in unstimulated cells from the majority of patients (77.6%) and in stimulated cells from all patients. The amount of TNF-α released from mononuclear cell populations showed no relationship with age of patient or the duration of disease.

Kruskal–Wallis ANOVA with correction for multiple comparisons revealed that stimulated T lymphocytes from past smokers released significantly more TNF-α than did cells from patients who had never smoked (1393.4 *vs* 767.4 pg/ml, $P = 0.02$; Table 2). Comparison of the difference between current smokers and those who had never smoked did not achieve significance ($P = 0.3$), although levels of TNF-α released by current smokers and past smokers were very similar. Overall, stimulated T lymphocytes from patients who had ever smoked (past + current) released significantly more TNF-α than those from patients who had never smoked (1416.0 *vs* 767.4 pg/ml, $P = 0.04$). Increases in TNF-α release were also observed in unstimulated T cells from past and current smokers, although these were not significant. No associations were found between monocyte TNF-α release and smoking status.

Smoking and mononuclear cell release of soluble TNF receptors

Release of soluble TNFRI and TNFRII was detected in all T-lymphocyte and monocyte cultures. Stimulation with PHA or LPS had no discernible effect on sTNFRI release, whilst the release of sTNFRII increased approximately 2-fold for both cell types. The release of both soluble receptors was correlated with the release of TNF-α in stimulated T lymphocytes (TNF-α and sTNFRI, $r_S = 0.237$, $P = 0.050$; TNF-α and sTNFRII, $r_S = 0.643$, $P < 0.0001$). No correlations were observed in unstimulated cells, nor were any correlations observed between the release of TNF-α and soluble receptors in monocytes (data not shown). The levels of sTNFRI released by both unstimulated and stimulated mononuclear cells showed significant correlations with age of the patient ($r \geq 0.39$, $P \leq 0.001$). In the case of sTNFRII, only the levels released by unstimulated and stimulated T lymphocytes were correlated with age ($r \geq 0.26$, $P \leq 0.03$). No associations were found with disease duration after adjustment for age.

Differences between groups defined by smoking status were determined by ANCOVA with correction for age where appropriate. Significant increases were found in the release of both sTNFR from stimulated T lymphocytes from past smokers compared with cells from patients who had never smoked ($P \leq 0.03$; Table 3). Release of soluble TNFR by cells from current smokers showed no differences from the levels released by those who had never smoked.

In monocytes, no differences were found between groups after correction for multiple testing, although combining the past and current smoking groups (ever smoking) revealed a difference in sTNFRII release between ever and never smoking for unstimulated and stimulated monocytes ($P = 0.04$ and 0.02, respectively).

Influence of smoking on the ratio of TNF-α/sTNFR released by mononuclear cells

Although a history of smoking was associated with an increased capacity of T lymphocytes to release TNF-α and sTNFR after stimulation, there appeared to be a relatively greater increase in TNF-α release. We were therefore interested to see whether

Table 2. Mononuclear cell release of TNF-α in RA patients stratified by smoking status

Source and smoking status	*n*	TNF-α release (pg/ml)
Unstimulated T lymphocytes		
Never	25	27.6 (62.7)
Past	27	68.3 (201.9)
Current	17	84.9 (168.1)
Stimulated T lymphocytes[a]		
Never	25	767.4 (628.7)
Past	27	1393.4 (1056.7)
Current	17	1451.8 (1630.5)
Unstimulated monocytes		
Never	23	1038.5 (2213.7)
Past	28	728.5 (1936.5)
Current	16	998.6 (1863.7)
Stimulated monocytes		
Never	23	4908.9 (4598.7)
Past	28	4807.3 (4674.3)
Current	16	5853.4 (6103.3)

Values are mean (S.D.). Kruskal–Wallis ANOVA demonstrated a significant difference between the groups for release of TNF-α from stimulated T lymphocytes ([a]$P = 0.02$). After correction for multiple testing (Kruskal–Wallis *Z*-test), the release of TNF-α from stimulated T cells of past smokers was significantly higher than that for never smokers. There was no significant difference between past and current smokers, and combining these groups revealed a significant difference between ever and never smoking ($P = 0.04$, Mann–Whitney *U*-test).

Table 3. Mononuclear cell release of sTNFRI and sTNFRII in RA patients stratified by smoking status

Source and smoking status	*n*	sTNFRI release (pg/ml)	sTNFRII release (pg/ml)
Unstimulated T-lymphocytes			
Never	25	145.5 (58.0)	449.0 (145.3)
Past	27	182.6 (70.3)	648.4 (296.9)
Current	17	133.0 (53.4)	457.3 (239.3)
Stimulated T-lymphocytes[a]			
Never	25	151.2 (58.9)	834.4 (257.9)
Past	27	188.1 (72.7)	1084.8 (342.0)
Current	17	139.6 (53.7)	838.2 (406.7)
Unstimulated monocytes			
Never	23	182.3 (58.3)	1013.7 (260.7)
Past	27	217.9 (69.5)	1219.7 (374.2)
Current	16	175.8 (57.7)	1126.9 (416.4)
Stimulated monocytes			
Never	23	187.3 (54.3)	1665.9 (604.6)
Past	27	222.1 (64.5)	2119.4 (882.0)
Current	16	184.9 (56.9)	2036.6 (723.2)

Values are mean (S.D.). There was a significant difference between the groups for release of sTNFRI from stimulated T lymphocytes ([a]$P = 0.03$, ANCOVA with adjustment for age). There was also a significant difference between the groups for release of sTNFRII from stimulated T lymphocytes ([a]$P = 0.02$, ANCOVA adjusted for age). After correction for multiple testing, the release of sTNFRI and sTNFRII from the stimulated T cells of past smokers was significantly higher than in those who had never smoked. In monocytes, no differences were found between groups. However, combining the past and current smoking groups (ever smoking) revealed a significant difference in sTNFRII release between ever and never smoking for unstimulated and stimulated monocytes ($P = 0.04$ and 0.02, respectively).

TABLE 4. Levels of TNF-α and TNFR released by stimulated T lymphocytes from patients stratified by smoking duration and intensity

	n	TNF-α (pg/ml)	TNFRI (pg/ml)	TNFRII (pg/ml)	TNF/TNFRI ratio	TNF/TNFRII ratio
Duration (yr)						
0	25	767.4 (628.7)	151.2 (58.9)	834.4 (257.9)	5.83 (5.31)	0.86 (0.53)
1–14	10	820.7 (763.2)	174.5 (68.0)	1034.3 (446.0)	4.50 (3.54)	0.74 (0.56)
15–29	15	1391.4 (1270.0)	156.1 (63.7)	931.5 (333.9)	7.37 (4.50)	1.32 (0.83)
≥30	19	1748.7 (1445.4)	177.1 (74.7)	1011.8 (400.0)	10.61 (9.66)	1.59 (0.91)
Intensity (pack years)						
0	25	767.4 (628.7)	151.2 (58.9)	834.4 (257.9)	5.83 (5.31)	0.86 (0.53)
1–14	16	1294.7 (1350.3)	181.6 (74.9)	1051.9 (422.1)	5.68 (5.17)	1.09 (0.95)
15–29	12	1282.5 (896.0)	159.3 (48.4)	907.0 (380.8)	8.14 (6.60)	1.29 (0.67)
≥30	16	1637.4 (1509.7)	164.7 (73.1)	989.1 (356.9)	10.43 (9.25)	1.53 (0.90)

Values are mean (S.D.). Analyses were by GLM implementation of ANOVA or ANCOVA. TNF-α release increased with smoking duration (P for trend = 0.002) and intensity (P for trend = 0.009). Inclusion of age, disease duration and current smoking status as covariates made little or no difference to the significance of the associations. There were no significant trends in the relationship between sTNFR release and categories of smoking duration or intensity. Significant trends were seen in the relationship between TNF-α/TNFRI and TNF-α/TNFRII ratios and smoking duration (P for trend = 0.01 and 0.0004, respectively), as well as smoking intensity (P for trend = 0.03 and 0.008, respectively).

smoking status influenced the relationship between the amounts of TNF-α and sTNFR released by mononuclear cells. Examination of individual patient TNF-α/sTNFRI and TNF-α/sTNFRII ratios revealed that the ratio of TNF-α/sTNFRII released from stimulated T lymphocytes was significantly higher in both current and past smokers than in those who had never smoked (1.38 *vs* 0.86, $P = 0.03$ and 1.25 *vs* 0.86, $P = 0.04$, respectively). Similar non-significant trends were seen for the TNF-α/sTNFRI ratio (data not shown).

The ratios of TNF-α/sTNFRI and TNF-α/sTNFRII released by monocytes did not differ according to smoking status (data not shown).

Relationship between extent of smoking and cellular release of TNF-α and sTNFR

Information on the number of cigarettes and number of years smoked was available for 44 of the 46 patients who had ever smoked. To determine if there was any quantitative relationship between smoking and TNF-α release, we stratified the extent of smoking into a number of categories according to length of smoking history or number of pack years (Table 4). GLM ANOVA indicated that TNF-α production from stimulated T cells increased with length of smoking history (P for trend = 0.002) and number of pack years (P for trend = 0.009). The significance of these associations was altered little by the inclusion of age, disease duration or current smoking status as covariates. No relationships between the extent of smoking and TNF-α release by unstimulated T cells or monocytes were found (data not shown).

Investigation of the relationship between sTNFR release from stimulated T lymphocytes and categories of smoking duration or intensity revealed no clear trends (Table 4). Mean TNFRI and TNFRII release appeared to be greater in all smoking categories compared with never-smoking, but none were significantly different after adjustment for age and correction for multiple comparisons. No relationships were found between release of sTNFRI or RII from monocytes and categories of smoking duration or intensity (data not shown).

Examination of the TNF-α/sTNFR ratios released by stimulated T lymphocytes from patients stratified by smoking categories revealed significant trends in the relationship between the TNF-α/TNFRI and TNF-α/TNFRII ratios and smoking duration (P for trend = 0.01 and 0.0004, respectively), as well as smoking intensity (P for trend = 0.03 and 0.008, respectively) (Table 4). No relationships were found between TNF-α/sTNFR ratios and smoking categories for monocytes or unstimulated T lymphocytes (data not shown).

TABLE 5. Levels of TNF-α and soluble TNF receptors in plasma of RA patients stratified by smoking status

	Smoking status		
Plasma cytokine	Never ($n = 25$)	Past ($n = 29$)	Current ($n = 17$)
TNF-α (pg/ml)	257.0 (809.0)	328.3 (1231.6)	655.8 (1886.8)
sTNFRI (pg/ml)	1103.2 (411.8)	1489.7 (577.3)	976.1 (329.1)
sTNFRII (pg/ml)	4139.2 (1541.4)	5860.0 (2212.8)	4255.9 (1968.6)

Values are mean (S.D.). No significant differences were found between groups for plasma levels of TNF-α. ANCOVA (age and disease duration as covariates) with correction for multiple testing demonstrated that past smokers had significantly higher levels of sTNFRI and sTNFRII than current smokers and patients that had never smoked ($P \leq 0.01$).

Association between smoking and plasma levels of TNF-α and TNF receptors

No association was found between smoking status and plasma levels of TNF-α (Table 5). However, both sTNFR were found at higher levels in past smokers than in current smokers, or those who had never smoked ($P \leq 0.01$, ANCOVA, age and disease duration as covariates, with correction for multiple testing). Patients in all categories for smoking duration and intensity had higher mean plasma levels of both TNFRI and TNFRII than patients who had never smoked, but there were no significant differences between categories after adjustment for age and correction for multiple comparisons (data not shown).

Influence of RF status on the association of smoking with TNF-α and sTNFR release

Since the effects of smoking may be linked to RF production, analyses were also conducted to assess the influence of RF status on the associations observed with smoking. No significant differences in mononuclear cell release of TNF-α and sTNFR were observed between RF positive and RF-negative smokers (data not shown). The association of smoking with the increased TNF-α/sTNFRII ratio released from T lymphocytes was also independent of RF status.

Discussion

We report that TNF-α release by stimulated T lymphocytes from peripheral blood was greater in RA patients with a history of

smoking than in those who had never smoked. This suggests that the effects of smoking, either directly or indirectly, may include the priming of circulating T lymphocytes for enhanced TNF-α release when appropriately challenged. Other studies in healthy individuals have shown that TNF-α release by PBMCs is increased in smokers [19, 20], although T lymphocyte and monocyte populations were not investigated separately. In our study the effects of smoking on TNF-α release appeared to be restricted to T lymphocytes, since no significant difference in TNF-α release by monocytes was observed between smokers and non-smokers. This was unexpected since monocytes/macrophages are the major cellular source of TNF-α. The lack of association between smoking and plasma TNF-α levels in this study probably reflects the absence of an association between smoking and monocyte TNF-α release. However, T lymphocytes are also an important source of TNF-α when activated, and can produce significant amounts of this cytokine. The reason for the apparent cell-specificity in the association of TNF-α release with smoking is not clear, but suggests possible differences in the response of T lymphocytes and monocytes to components of cigarette smoke.

A number of studies have investigated the effect of nicotine on cytokine production in whole mononuclear cell populations from peripheral blood of healthy subjects. Nicotine at low concentrations was shown to stimulate TNF-α secretion [20], but at levels equivalent to that in the plasma of smokers it inhibited TNF-α and IL-2 production [21]. Another study investigating the effects of nicotine patch treatment found diminished production of IL-10 as well as TNF-α and IL-2 production by PHA-stimulated non-adherent mononuclear cells [39]. The cholinergic/nicotinic anti-inflammatory pathway recently described by Wang *et al.* may explain such findings [40]. Wang *et al.* demonstrated that activation of α7 nicotinic acetylcholine receptors (nAChRs) on macrophages by cholinergic agonists such as acetylcholine and nicotine reduces the release of TNF-α and other proinflammatory cytokines induced by LPS. The presence of α7 nicotinic receptors on lymphocytes has also been demonstrated [41]. One might have expected therefore that mononuclear cells isolated from current smokers would show diminished TNF-α release because of recent exposure to nicotine. However, our findings indicate that any inhibitory effect of recent nicotine exposure is not evident in isolated mononuclear cells from RA patients who are current smokers. The finding that TNF-α release from stimulated T lymphocytes is equally elevated in past as well as current smokers also argues against an effect due to recent nicotine exposure.

Clearly, nicotine is only one of many components of cigarette smoke that might influence cellular responses, and water-soluble extracts of cigarette smoke have been shown to stimulate TNF-α release from human blood monocytes [42]. It might have been anticipated that any effects of smoking would be greatest in current smokers where the toxic components of cigarette smoke are present. However, the levels of TNF-α released by stimulated T lymphocytes of past smokers were no different to those of current smokers, and up to 15 yr after smoking cessation they were significantly greater than those released by non-smokers (JR Glossop and DL Mattey unpublished observations). Our data indicate that the length of smoking history is more important than whether or not a patient is currently smoking. This suggests that increased TNF-α release from stimulated T cells may be a prolonged, chronic response that may last for many years after smoking cessation. This could be a result of long-term biological changes which might occur through alterations in T-cell phenotype and/or changes in the proportions of particular T-cell subsets.

Smoking also appears to influence the release of both TNF receptors from stimulated T lymphocytes, although, in contrast to TNF-α release, there was no apparent relationship between the extent of smoking and the level of sTNFR release. Again there was an indication that the effects of smoking may last long after smoking cessation, since the highest levels released were found in past smokers. The highest circulating levels were also found in past smokers. Current smokers in this study did not show an increase in sTNFR levels, although in other studies on young healthy diabetics and healthy smokers current smoking has been associated with increases in plasma TNFR levels [43, 44].

The lack of a clear relationship between sTNFR levels and the extent of smoking suggests the possibility of some other factor having a confounding affect on receptor levels in past smokers with RA. For example, some individuals may have given up smoking because they associated it with worsening disease, while those who continued to smoke might have done so because it did not apparently influence their condition. Other factors that might determine whether a patient continues to smoke include the development of comorbid disease (e.g. heart disease) or particular genetic polymorphisms. The latter could play a part, for example, in determining smoking persistence and/or levels of sTNFR. We have shown previously that the level of sTNFR released by T cells from RA patients is associated with polymorphism (T676G) in the TNFRII gene [37]. However, multiple regression analysis on data from this study indicated that the association of TNFR release with past smoking was independent of TNFRII genotype (JR Glossop and DL Mattey unpublished observations).

Of particular interest was the finding that the ratio of TNF-α/sTNFR released from stimulated T lymphocytes was increased in smokers. There were also significant relationships between the extent of smoking and the TNF-α/sTNFR ratios. This reflects an increase in TNF-α release with increased extent of smoking, relative to little or no change in TNFR release. This alteration in the balance between TNF-α and TNF receptor release appears to be retained after smoking cessation. We speculate that this imbalance would be likely to promote increased TNF-α activity and may better reflect the influence of smoking on the TNF-α axis. Further studies are needed to determine whether smoking causes differential synthesis and/or turnover of TNF-α and its receptors, or whether smoking has differential effects on cleavage of TNF-α and its receptors through effects on TACE and other possible sheddases. A recent report by Shao *et al.* [45] implicates TACE in some of the cellular responses to smoking. These authors found, in a human airway epithelial cell line, that the effects of cigarette smoke on transforming growth factor-α shedding, epidermal growth factor receptor phosphorylation and mucin expression were prevented by a TACE inhibitor and by specific knockdown of TACE with small interferins RNA. The study suggested that oxygen free radicals were responsible for the activation of TACE by cigarette smoke.

In conclusion, our results indicate that there is an association between smoking and the release of TNF-α and soluble TNFR by PBMCs from RA patients. In particular, stimulated T lymphocytes from patients with a history of smoking released significantly greater levels of TNF-α than did cells from patients who had never smoked, and this was related to the extent of smoking. The data suggest that smoking causes an imbalance in the ratio of TNF-α/sTNFR released by stimulated T cells that might favour increased TNF-α activity. Our findings may be of particular relevance to preliminary reports that RA patients who smoke are more likely to receive a biological agent [46], and that smoking is associated with a lower response rate to anti-TNF therapy [47].

Rheumatology	Key messages
	• In RA patients who smoke there is an increase in the ratio of TNF-α/sTNFR released by stimulated T lymphocytes. • The increased TNF-α/sTNFR ratio is associated with extent of smoking, and remains elevated after smoking cessation.

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