Journal of Autoimmunity 98 (2019) 95–102

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Crosstalk between tumor necrosis factor-alpha signaling and aryl hydrocarbon receptor signaling in nuclear factor –kappa B activation: A possible molecular mechanism underlying the reduced efficacy of TNF-inhibitors in rheumatoid arthritis by smoking

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ARTICLE INFO

Keywords:
TNFα
Smoking
AhR
NF-κB
TNF-inhibitor
Rheumatoid arthritis

ABSTRACT

Objectives: To examine the influence of smoking on biologics treatment against different therapeutic targets, such as TNFα, IL-6, and T cell, in rheumatoid arthritis (RA) and elucidate the underlying molecular mechanism.
Methods: The association between drug-discontinuation due to poor therapeutic response and smoking status was analyzed individually in biologics against different therapeutic targets by a multivariable logistic regression analysis using the "NinJa" Registry, one of the largest cohorts of Japanese RA patients. *In vitro* enhancement of TNFα-induced NF-κB activation and subsequent proinflammatory cytokine production by cigarette chemical components was examined by RT-PCR, qPCR, ELISA, and western blotting using an immortalized rheumatoid synovial cell line, MH7A.
Results: The rate of drug-discontinuation due to poor therapeutic response was higher in the current smoking group than in the never- or ever-smoking groups (the odds ratio of current/never smoking: 2.189, 95%CI; 1.305–3.672,P = 0.003; current/ever: 1.580, 95%CI; 0.879–2.839,P = 0.126) in the TNF inhibitor (TNFi) treatment group. However, this tendency was not observed in either the IL-6 or T cell inhibitor treatment groups. Cigarette smoke chemical components, such as benzo[α]pyrene, known as aryl hydrocarbon receptor (AhR) ligands, themselves activated NF-κB and induced proinflammatory cytokines, IL-1β and IL-6. Furthermore, they also significantly enhanced TNFα-induced NF-κB activation and proinflammatory cytokine production. This enhancement was dominantly inhibited by Bay 11-7082, an NF-κB inhibitor.
Conclusions: These results suggest a crosstalk between TNFα signaling and AhR signaling in NF-κB activation which may constitute one of the molecular mechanisms underlying the higher incidence of drug-discontinuation in RA patients undergoing TNFi treatment with smoking habits.

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https://doi.org/10.1016/j.jaut.2018.12.004
Received 13 November 2018; Received in revised form 14 December 2018; Accepted 16 December 2018
Available online 25 December 2018
0896-8411/

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized by persistent polysynovitis and subsequent bone/joint destruction [1]. Although its etiology remains unknown, cumulative evidence has revealed that lymphocytes and proinflammatory cytokines play a pivotal role in RA pathogenesis [2,3]. In recent years, molecular targeted therapies using biologics such as monoclonal antibodies and soluble receptors against the pathogenic proinflammatory cytokines and lymphocytes including TNFα, IL-6, IL-1, T cells and B cells, have been widely used and resulted in significantly successful treatment of RA [4–7]. However, some patients are still forced to discontinue biologics treatment due to adverse effects, poor therapeutic response, and high cost [8–10]. Therapeutic discontinuation is a critical issue, considering that RA is a progressive disease with irreversible articular damage. To avoid therapeutic discontinuation of biologics in advance, both the causes and the underlying mechanisms need to be determined.

Cumulative evidence suggests that some genetic and environmental factors affect the efficacy of anti-rheumatic drugs, including biologics [11–15]. Among these, smoking is considered a crucial environmental factor and there have been a substantial number of supporting reports [16–20]. However, the molecular mechanism underlying this observation is yet to be completely clarified.

In the present study, to investigate the influence of smoking on biologics-treatment in RA, the association between biologics-discontinuation due to poor therapeutic response and smoking habits was analyzed using data from the “NinJa” Registry [21]. In addition, the underlying molecular mechanisms were examined using an immortalized rheumatoid synovial cell line, MH7A [22].

2. Materials and methods

2.1. Patients/the “NinJa” registry

The present study was carried out in compliance with the Helsinki Declaration and approved by the institutional ethical review board (NHO Sagamihara Hospital, #2013-9). We obtained written informed consent from each participant. The “NinJa” Registry established in 2002 is one of the largest registries of Japanese RA patients, which included clinical data of 11,940 patients in 2012. We used the “NinJa” data from 2007 to 2012. All registered patients were diagnosed RA with the standard diagnostic criteria. The contents of the “NinJa” are indicated in Supplementary Box 1. The data for each patient continuously renewed annually. As the “NinJa” is an open prospective cohort, approximately 85% of the registered patients were followed continually. Using the “NinJa” registry, we can determine the trends of treatments and outcomes in Japanese RA patients both cross-sectionally and longitudinally. Smoking status was assessed using a questionnaire (60.1% of the total patients answered) and the patients were divided into three groups as follows: current-, ever-, and never-smoking.

2.2. Reagents

RPMI 1640 and HEPES from Gibco, Life Technologies, Carlsbad, CA; penicillin/streptomycin from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; Fetal bovine serum (FBS) from Hyclone Laboratories, GE Healthcare Japan, Tokyo, Japan; Dimethyl sulfoxide (DMSO), benzo[α]pyrene (BP) and α−naphthoflavone (α−NF) from Sigma-Aldrich Japan, Tokyo; recombinant human TNFα and IL-6 from R&D Systems, Minneapolis, Minnesota, USA, and theTNFα inhibitor, adalimumab was from Abbvie Inc., North Chicago, Illinois, USA.

2.3. Cells

MH7A is an immortalized cell-line established by stably transfecting rheumatoid fibroblast-like synoviocytes with SV40 T antigen gene [22]. The cells were cultured in RPMI1640 supplemented with 10%heat-inactivated FBS.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)/quantitative real time PCR (qPCR)

MH7A cells were cultured for 18 h, transferred to culture medium with 0.1%FBS and cultured for 3 h. The cells were then re-cultured with or without the stimulants for 24 h. The cDNA was synthesized from 1 μg of mRNA extracted from the cultured cells using SuperScript III First Strand Synthesis System of the RT-PCR kit (Invirogen, Carlsbad,CA,USA) according to the manufacturer's protocol. RT-PCR was performed using primers specific for IL-1β (forward primer: 5′-CCTTCATCTTTGAAGAAGAA-3′, reverse primer: 5′-GGCAGACTCAAATTCCAGCT-3′), IL-6 (forward primer: 5′-TCTTCAGAACGAATTGACAA-3′, reverse primer: 5′-GTGCCTGCAGCTTCGTCAGC-3′), AhR (forward primer: 5′-ACTCCACTTCAGCCACCATC-3′, reverse primer: 5′-TGGGACTCGGCACAATAAAG-3′), CYP1A1 (forward primer: 5′-TTCGCTACCTACCCAACCCT-3′, reverse primer: 5′-AGCAGGATAGCCAGGAAGAG-3′) and GAPDH (forward primer: 5′-GCACCACCAACTGCTTAGCACC-3′, reverse primer: 5′-GTCTGAGTGTGGCAGGGACTC-3′). PCR was performed using AmpliTaq Gold® 360 PCR Master Mix (Applied Biosystems/Thermo Fisher Scientific Inc., Tokyo, Japan). TaqMan® qPCR was performed using the Stratagene Mx3005P sequence detection system (Agilent Technologies, Waldbronn,Germany) and TaqMan® Universal Master Mix II (Applied Biosystems/ThermoFisher Scientific Inc,Tokyo, Japan). These experiments were performed according to the manufacturer's protocol. To confirm amplification specificity, the PCR products were subjected to melting curve analysis. The data were analyzed with MxPro Ver.4.10 software (Agilent Technologies, Waldbronn, Germany).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentrations in MH7A cell culture supernatants were measured using ELISA-kits for IL-6 and IL-1β (Human IL-6 Immunoassay, D6050; Human IL-1β Immunoassay, DLB50, R&D Systems, Minneapolis, MN).

2.6. Western blotting analysis for p65 and Ser^{536}-phosphorylated p65 subunits of NF-κB

To assess NF-κB activation, levels of both p65 and Ser^{536}-phosphorylated p65 (p-p65) subunits of NF-κB were analyzed by western blotting. Whole proteins were isolated from MH7A cells 30min of the culture with or without the stimulants, followed by separation of the nuclear fraction from the cytosolic fraction using the Nuclear Extraction Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocol. Each protein (7 μg) from both the cytosol and nucleus was separated on SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membrane (Bio-Rad com, Hercules, CA). Both p65 and p-p65 were detected by probing with anti-p65 and anti-p-p65 antibodies respectively (anti-NF-κB p65 antibody, #8242; anti-NF-κBp-p65 antibody, #3033; Cell Signaling Technology, Japan KK, Tokyo, Japan). GAPDH and Lamin B1 were used as internal controls for cytosol and nuclear proteins, respectively. They were detected using anti-GAPDH and anti-Lamin B1 antibodies respectively. Anti-rabbit IgG-HRP antibody was used as the secondary antibody (anti-GAPDH, #5714; anti-Lamin B, #13435; anti-rabbit IgG-HRP antibodies, #7074; Cell Signaling Technology, Japan KK, Tokyo, Japan). The antigen-antibody complexes were visualized using the chemiluminescence substrate, ECL Select Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) as recommended by the manufacturer. Band intensities were measured semiquantitatively on a chemiluminescence image analyzer, Amersham Imager 680 (FUJIFILM, Tokyo,Japan).

Table 1
Baseline characteristics of three groups of biologics-treated RA patients with different smoking status (current, ever and never smoking).

Smoking Status			Current smoker	Ever smoker	Never
Total patients No.			168	250	1225
Age			57.2 ± 12.6	62.6 ± 11.9	61.3 ± 13.4
Disease duration			12.0 ± 7.1	14.3 ± 9.5	16.3 ± 10.0
Female (%)			58.4	52.4	93.9
RF positivity (%)			77.0	78.5	75.3
ACPA positivity (%)			81.0	87.6	85.6
Stages	I	(%)	15.8	8.5	9.7
	II		40.6	30.4	24.2
	III		20.0	28.7	23.5
	IV		23.6	32.4	42.6
Disease activity (DAS28ESR)			3.0 ± 1.5	3.1 ± 1.4	3.2 ± 1.3
csDMARDs (%)			98.2	98.4	97.3
MTX (mg/W)			8.6 ± 2.9	8.0 ± 3.2	8.1 ± 2.8
Steroid (mg/day)			4.7 ± 3.0	4.2 ± 2.7	3.6 ± 2.2

All data were obtained from RA patients treated with biologics and answered the questionnaire for smoking habits in the "NinJa" Registry. The data for "Age", "Disease duration", "Disease activity", "MTX" (dose of MTX), "Steroid" (dose of corticosteroids) are presented as mean ± S.D. The data for "Female", "RF positive", "ACPA positive", "Stages", and concurrent use of "cs DMARDs" are presented as percentages of the total number.
RF: rheumatoid factor; ACPA: anti-citrullinated peptide antibodies; DAS28ESR:disease activity score 28 ESR; MTX: mean ± S.D. of dose of methotrexate; Steroid: mean ± S.D. of dose of corticosteroids calculated as doses of prednisolone. csDMARDs: conventional synthetic disease modifying anti-rheumatic drugs.

2.7. Inhibition assay using signaling pathway-specific inhibitors

MH7A cells were cultured for 16 h, then pretreated with 10 μM of various inhibitors (Bay11-7082, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; SB203580, Adipo-Gen Life Sciences, Liestal, Switzerland; U0126, Promega KK, Tokyo, Japan) for 30min. Cells were then stimulated with 1 μM BP, and 10 ng/ml TNFα for 24 h. After 24 h incubation, the culture-supernatants were collected and subjected to ELISA.

2.8. Statistics

To assess the effect of smoking on the failure of TNFi treatment, we conducted a multivariable logistic regression analysis with adjustment for patients' baseline (age, sex, Disease Activity Score 28-erythrocyte sedimentation rate (DAS28-ESR), methotrexate (MTX) dose, prednisolone (PSL) dose, anti-cyclic citrullinated peptide antibody (ACPA), and the disease duration). This multivariable logistic regression model contained non-linear restricted-cubic-spline to allow non-linear effects of the above numerical covariates that were modeled in this multivariable logistic regression model using restricted-cubic-spline. Furthermore, we examined whether the influence of smoking on the failure of TNFi treatment differs from that on the failure of other biologics (IL-6i and Tci). We used the multivariable logistic regression model involving the cross-product term between smoking status and the category of biologics treatments (TNFi, IL-6i, and Tci) among patients treated with these biologics. The covariate adjustments were conducted in a manner similar to the multivariable logistic regression model described above. Because some patients had been treated with multiple biologics and evaluated for the effect of each drug, we utilized Huber-White robust sandwich estimator of variance-covariance matrix to account for dependence in repeated measures within a single patient. Missing data were imputed with the help of multiple imputation method using "areg.impute" function in "rms" package of R software1. All statistical analyses were performed with a 2-sided test at the 5% significance level using R software version 3.4.3 (https://www.r-project.org/foundation/) with the "rms" package.

3. Results

3.1. Causes of therapeutic discontinuation of biologics in the "NinJa" registry

Of the total patients (11,940), 3187(26.7%) had been treated with one or more biologics. At the time, six biologics (IFX: infliximab, ETN: etanercept, ADA: adalimumab, GLM: golimumab, TCZ: tocilizumab and ABT: abatacept) were approved and available in Japan. 584(18.3%), 1321(41.4%), 397(12.5%), 73(2.3%), 589(18.5%), and 223(7.0%) patients had been treated with IFX, ETN, ADA, GLM, TCZ and ABT respectively. The total numbers and percentages of therapeutic discontinuation of biologics were 1015(42.7%), 162(27.5%) and 65(29.1%) for TNFi (IFX, ETN, ADA and GLM), IL-6i (TCZ) and Tci (ABT) respectively. The causes of therapeutic discontinuation were analyzed according to four categories; poor therapeutic response (Failure), adverse events (AE), remission, and others. As shown in Supplementary Table 1, drug-discontinuation due to poor therapeutic response (Failure) in the TNFi treatment group (21.0%) seemed to be higher than that observed in either the IL-6i (10.7%) or the Tci (16.6%) treatment groups.

3.2. Association between smoking status and biologics-discontinuation due to poor therapeutic response in the TNFi treatment group and comparison with that in the other biologics (IL-6i, Tci) treatment groups

Smoking status was assessed using a questionnaire and patients were divided into three groups: current- (C), ever- (E), and never-smoking (N). The baseline characteristics of the patients are shown in Table 1. Female patients were dominant in the (N) group compared to the other two groups. The percentage of early disease-stage patients in the (C) group tended to be higher compared to that in the other two groups. No other obvious difference was seen. To assess the influence of smoking status on TNFi-discontinuation due to poor therapeutic response, we conducted a multivariable logistic regression analysis with adjustment for patients' baseline (age, sex, DAS28-ESR, MTX dose, PSL dose, ACPA, disease duration). The odds ratios of (C)/(N) and (C)/(E) were 2.189 (95%CI:1.305–3.672, P = 0.003) and 1.580 (95%CI:0.879–2.839, P = 0.126) respectively. TNFi-discontinuation due to poor therapeutic response in the (C) group was higher than that observed in the other two groups. Furthermore, this influence tended to increase depending on the smoking status (C) > (E) > (N) (Fig. 1A).

Next, to examine whether the influence of smoking on biologics treatment was different among individual biologics with different therapeutic targets, the multivariable logistic regression model including a cross-product term between smoking status and biologics treatment with different targets was used as described in the section of Statistics. As shown in Fig. 1B, the association between smoking status and drug-discontinuation due to poor therapeutic response for the individual biologics differed depend on their therapeutic targets. In the

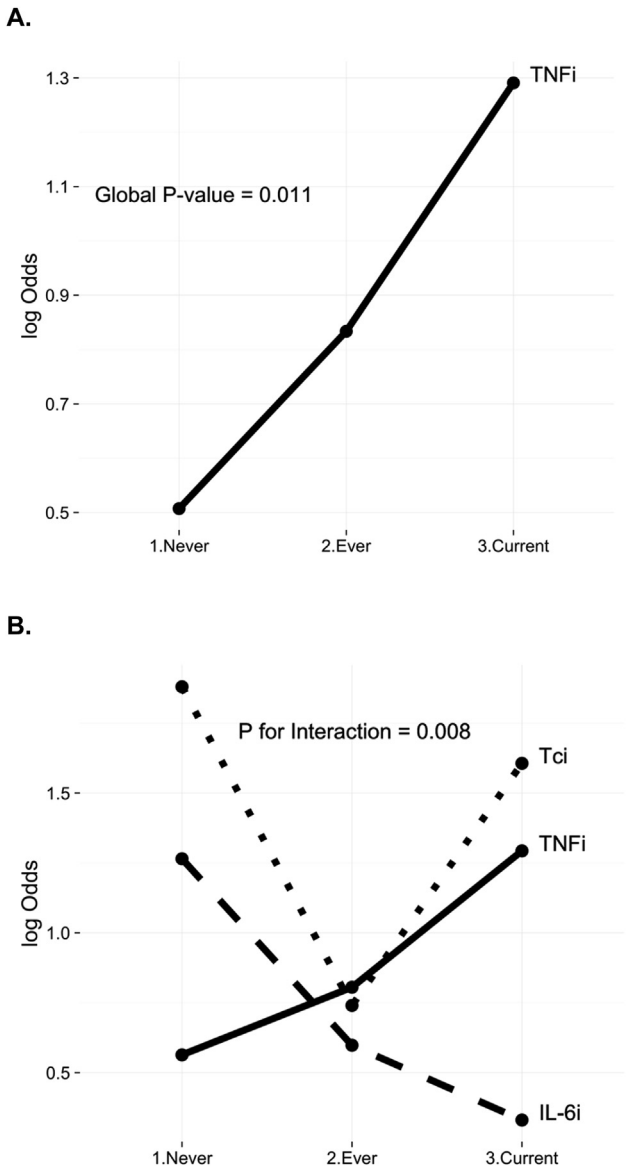

Fig. 1. Association between smoking status and discontinuation of biologics due to poor therapeutic response in the TNFi treatment group and comparison with other biologics (IL-6i or Tci) treatment groups. The data are indicated as log Odds. P value for interaction was calculated using a multivariable logistic regression model including a cross-product term between smoking status and the category of the prescribed biologic (TNFi, IL-6i, or Tci). P value for interaction < 0.05 indicates that the effect of smoking on the therapeutic failure of each biologic treatment is significantly different from each other biologics.
A: TNFi, B: Comparison among TNFi, IL-6i, and Tci.
No. of patients:

	Total	Never	Ever	Current
TNFi	1002	740	148	114
IL-6i	217	155	44	18
Tci	104	66	22	16

TNFi:TNF inhibitor; IL-6i:IL-6 inhibitor; Tci:T cell inhibitor; Never: never-smoking group; Ever: ever-smoking group; Current:current-smoking group.

TNFi treatment group, as described above, drug-discontinuation due to poor therapeutic response in the (C) group was significantly higher than the (N) group and this influence tended to be dependent on the smoking status ((C) > (E) > (N)). In the IL-6i treatment group, an opposite correlation to that in the TNFi treatment group was noted. In the Tci treatment group, no obvious correlation was observed. These results suggest that the efficacy of TNFi was reduced in RA patients with smoking habits but not in the IL-6i and Tci treatment groups, suggesting the existence of special mechanisms for the reduced efficacy of TNFi by smoking.

3.3. A chemical component of cigarette smoke, benzo[α]pyrene (BP) enhances TNFα-induced proinflammatory cytokine (IL-6, IL-1β) production at both mRNA and protein levels

Cigarette smoke contains several ligands specific to aryl hydrocarbon receptor (AhR), such as benzo[α]pyrene (BP), 3-methyl cholanthrene (3-MC), 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), which can activate NF-κB and induce proinflammatory cytokine production [27–30]. We speculated that smoke might enhance TNFα-induced NF-κB activation and subsequent proinflammatory cytokine production, owing to which, patients with current smoking show higher incidences of therapeutic failure upon TNFi treatment.

As shown in Supplementary Fig. 1, the RT-PCR assay demonstrated that BP enhanced TNFα-induced IL-1β and IL-6 mRNA expression. This enhancement was significantly diminished by αNF, an AhR antagonist. The expression of AhR seemed to be insignificantly influenced. These results were confirmed with a qPCR assay. As shown in Fig. 2 A, BP significantly enhanced TNFα-induced IL-6 and IL-1β production and this enhancement was significantly inhibited by αNP. In addition, as shown in Fig. 2 B, the ELISA results demonstrated that BP itself induced IL-1β and IL-6 production and significantly enhanced the TNFα-induced production of these proinflammatory cytokines. Another cigarette smoke component, 3-MC, also showed similar enhancement (data not shown). Thus, some cigarette smoke chemical components themselves induce proinflammatory cytokines and enhance TNFα-induced proinflammatory cytokine production at both mRNA and protein levels through AhR signaling.

3.4. Inhibitory effect of the TNFi, adalimumab (ADA) on TNFα-induced IL-6 production is reduced in the presence of BP

As shown in Fig. 3, one of the TNFi, ADA, inhibited TNFα-induced IL-6 production in a dose-dependent manner. However, in the presence of BP, the inhibitory effects of ADA were significantly reduced. This may reflect on the poor therapeutic response in TNFi-treated RA patients with current smoking. Similar results were obtained with another TNFi, IFX (data not shown).

3.5. BP enhances TNFα-induced NF-κB activation

Proinflammatory cytokines, such as IL-1β and IL-6, are induced by TNFα mainly through the NF-κB signaling pathway [2]. We thus examined whether BP could enhance TNFα-induced NF-κB activation. NF-κB activity is mainly regulated through phosphorylation of inhibitor of NF-κB (IκB) by inhibitor of NF-κB (IκB) kinase (IKK) and IKK also simultaneously phosphorylates subunits of NF-κB, including p65 [27–29]. Therefore, MH7A cells were stimulated by TNFα, BP alone or their combination and in order to assess the activation of NF-κB, Ser^{536}-phosphorylated p65 (p-p65) along with total p65 was semi-quantitatively measured in both cytoplasmic and nuclear fraction-proteins extracted from cultured MH7A cells by western blotting. The western blotting pattern is shown in Fig. 4A and the quantities of both p65 and p-p65 in the cytosol and nuclei were determined by the ratios of band-intensity of both p65 and p-p65 to GAPDH and Lamin respectively. As shown in Fig. 4B, in the nuclei, p65 was increased by stimulation with TNFα and this increase was enhanced by BP, although BP itself did not obviously increase it (a). Such enhancement was more obviously observed in p-p65 (b). In addition, both the ratio (Nuclei/Cytoplasmic) of

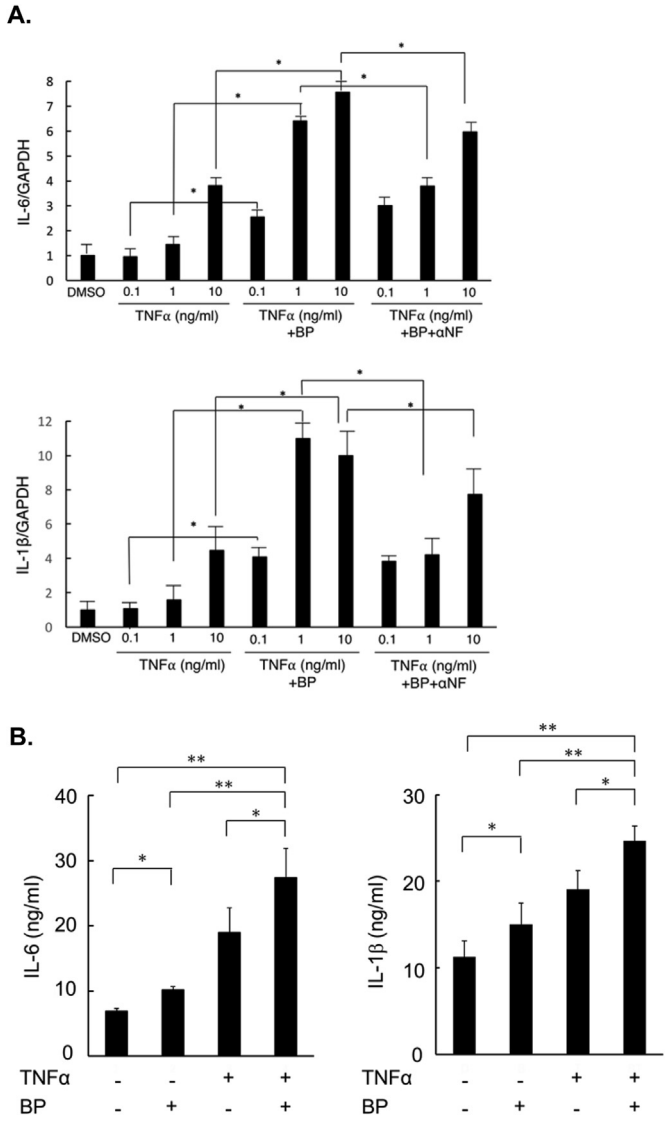

Fig. 2. A chemical component of cigarette smoke, BP enhances TNFα-induced proinflammatory cytokine production at both mRNA and protein levels. (A): MH7A cells were cultured with 5 μM BP, TNFα (0.1, 1.0 or 10 ng/mL) or both in the presence or absence of α-NF (5 μM) for 24 h. IL-6 and IL-1β mRNA expression levels were assessed by quantitative real-time PCR (qPCR) as described in the section of Methods. GAPDH was used as an internal control. The data are shown as the relative expression of IL-6 or IL-1β to GAPDH and indicated as mean ± S.E. of three independent experiments. Statistical significance was assessed by Student's *t*-test. * P < 0.05. (B): Culture supernatants were collected and IL-6 and IL-1β were measured by ELISA. The data are indicated as mean ± S.E. of three independent experiments and analyzed statistically using Student's *t*-test. * P < 0.05, ** P < 0.01.

p65 and that of p-p65 were increased by TNFα stimulation and were enhanced in the presence of BP (c), (d). These results suggested that BP could enhance TNFα-induced NF-κB activation synergistically rather than additively.

3.6. An NF-κB inhibitor, Bay11-7082 dominantly inhibits the enhancement of IL-6 production upon co-stimulation with TNFα and BP

It is well-known that multiple signaling pathways are involved in TNFα−induced inflammation [2]. These include three main signaling pathways, NF-κB, extracellular signal-regulated kinase (ERK), and mitogen-activated protein kinase p38 (p38). To examine which signaling pathway is dominantly involved in the enhancement of TNFα-induced

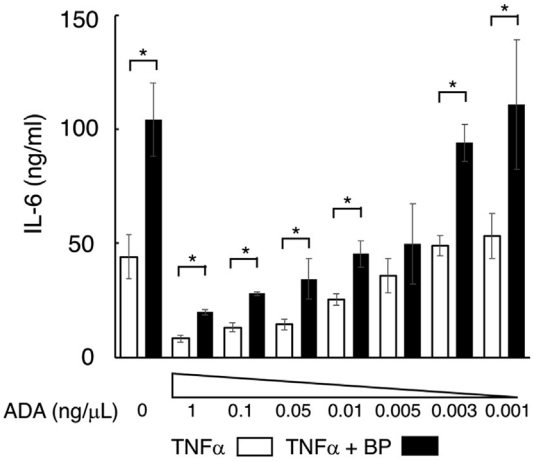

Fig. 3. Inhibitory effect of TNFi, Adalimumab (ADA) on TNFα-induced IL-6 production is reduced in the presence of BP. MH7A cells were cultured with or without Adalimumab (ADA) at various concentration (1 ng/mL, 0.1 ng/mL, 0.05 ng/mL, 0.01 ng/mL, 0.005 ng/mL, 0.003 ng/mL, 0.001 ng/mL), and stimulated with BP (1 μM), TNFα (10 ng/mL) or both for 24 h. Culture supernatants were collected and IL-6 was measured by ELISA. The data are indicated as mean ± S.E. of three independent experiments and analyzed statistically by Student's *t*-test. *P < 0.05.

proinflammatory cytokine production by cigarette chemical components, individual inhibitors for three signaling pathways, Bay11-7082 for NF-κB, SB203580 for p38 or U0126 for ERK, were added to the culture of MH7A cells stimulated with TNFα, BP alone or combination. As shown in Fig. 5, each inhibitor significantly inhibited the enhancement of TNFα-induced IL-6 production by BP, however Bay11-7082 showed the strongest inhibition among these inhibitors, suggesting that the enhancement of TNFα-induced IL-6 production by BP involves multiple signaling pathways and that the NF-κB signaling pathway is dominant among them.

4. Discussion

In the first part, we examined the association between biologics-discontinuation due to poor therapeutic response and smoking status for biologics against three different therapeutic targets, i.e. TNFi, IL-6i, and Tci [30–34]. The frequency of therapeutic discontinuation indicated by the odds was increased according to the smoking status in the TNFi treatment group. On the other hand, in the IL-6i treatment group, the odds conversely correlated with the smoking status and no obvious correlation was observed in the Tci treatment group. Thus, smoking affects the efficacy of biologics differently depending on the therapeutic targets, and shows therapeutic resistance more dominantly in the TNFi treatment group. Although there are a substantial number of similar supportive reports on TNFi treatment [18,35–37], it is not clear why only some patients in the current smoking group show TNFi-discontinuation due to poor therapeutic response. However, there are some possible explanations of which one is the impact of the intensity of smoking. Mattey et al. demonstrated that poor therapeutic response to TNFi is linked to the pack-year history of smoking [17]. Unfortunately, smoking status in this study was assessed by self-reported smoking history (current-,ever-, and never smoking) of individual patients. Next, some genetic backgrounds may also have an impact. In fact, it has been suggested that polymorphisms of associated genes such as TNF Receptor Superfamily Members (*TNFRSF1A* and *TNFRSF1B*) [38], TNFα induced protein 3 (TNFAIP3) [39], and steroid hormone related genes [40], affect the therapeutic response to TNFi.

On the other hand, very little is known regarding the IL-6i and Tci treatments [41]. Interestingly, we observed a contrasting effect of smoking in IL-6i treatment. Although we do not know the exact reasons,

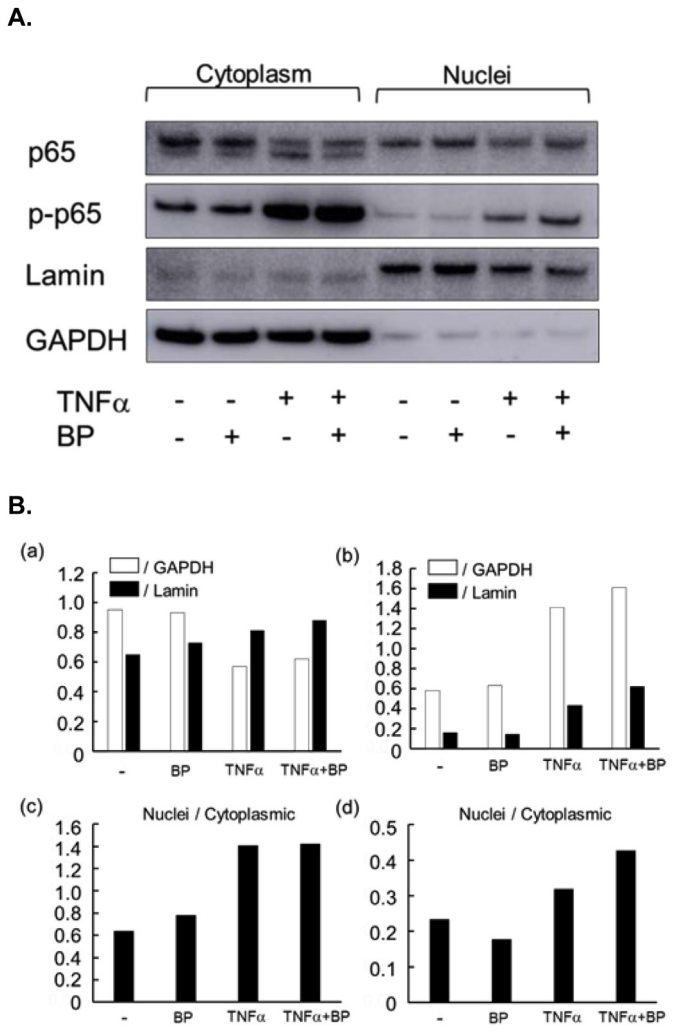

Fig. 4. BP enhances TNFα-induced NF-κB activation. (A): To assess NF-κB activation, the levels of both p65 and phosphorylated (Ser ^{536}) p65 (p-p65) subunits of NF-κB were analyzed separately in the cytoplasmic or nucleic fractions by western blotting as described in the section of Methods. GAPDH and Lamin B1 were used as internal controls for cytoplasmic and nuclear proteins respectively. (B): Band intensities were measured semiquantitatively by the chemiluminescence image analyzer as described in Methods. Experiments were conducted thrice and the data representative of three independent experiments with similar results are shown. The relative expression of p-65 and p-p-65 proteins to GAPDH or Lamin B, used as internal controls for cytoplasmic and nuclear proteins respectively. (a): p65, (b):p-p65, (c):the ratio of nuclear p65 to cytoplasmic p65 proteins, (d): the ratio of nuclear p-p65 to cytoplasmic p-p65 proteins.

there is a supportive report that no negative effect of smoking was observed in IL-6i treatment [42].

AhR, known as a dioxin receptor, has been originally studied extensively in toxicology [43], there is increasing evidence suggesting that AhR signaling plays an important role in regulation of immune response [44]. It has been reported that smoke induces both Th17 cells and T regulatory (Treg) cells through the AhR signaling pathway depending on the pathological conditions [45]. Both Th17 and Treg cells are well-known subtypes of T helper cells and play contradictory roles in the pathology of autoimmune inflammatory diseases [46,47]. Th17 cells play a pathogenic role whereas Treg cells a protective role in RA. Therefore, the influence of smoking on the efficacy of Tci might be complicated.

So far, our findings suggest that smoking affects the efficacy of biologics differently based on their therapeutic targets. We confirmed

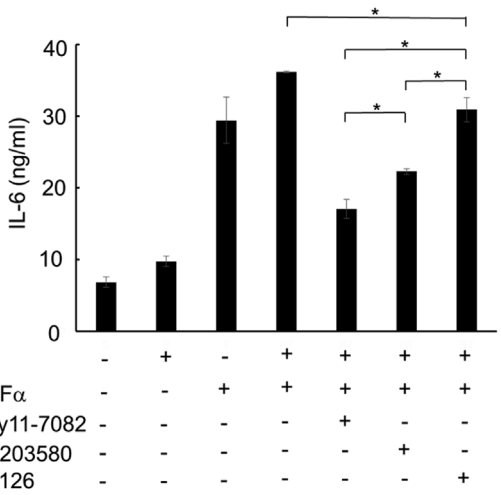

Fig. 5. An NF-κB inhibitor, Bay11-7082 dominantly inhibits the enhancement of IL-6 production upon co-stimulation with TNFα and BP. MH7A cells were pretreated with 10 μM of Bay11-7082, 10 μM of SB203580 or 10 μM of U0126 for 30 min, followed by stimulation with 1 μM of BP, 10 ng/mL of TNFα, or both for 24 h. The culture supernatants were collected and IL-6 levels were determined by ELISA. The data are indicated as mean ± S.E. of three independent experiments and analyzed statistically using Student's *t*-test. * $P < 0.05$.

an obvious negative effect on TNFi treatment as reported previously [18,35–37].

In the second part of the study, we investigated the molecular mechanism underlying the reduced efficacy of TNFi by smoking. Smoking is a well-known and crucial environmental risk factor for RA: cigarette smoke contains several chemical components, such as 3-MC, BP, and TCDD, which act as AhR ligands. Recently, it has been reported that RA patient-derived synoviocytes and MH7A cells produce proinflammatory cytokines upon exposure to cigarette condensates or the above AhR ligands present in smoke [23,24]. Interestingly, osteoarthritis patient-derived synoviocytes do not produce these proinflammatory cytokines even upon stimulation with smoke-derived AhR ligands [25]. In addition, this induction of proinflammatory cytokines results in activation of NF-κB [25].

In the present study, we confirmed that MH7A cells produce proinflammatory cytokines, such as IL-1β and IL-6, at both mRNA and protein levels by stimulation with some smoke-derived chemical components such as BP and 3-MC, through AhR signaling. Moreover, they significantly enhanced TNFα-induced proinflammatory cytokine production and this enhancement was significantly diminished by α-NF, an AhR antagonist. Taken together, we speculate that there is a crosstalk or an interaction between TNFα signaling and AhR signaling pathways involved in the induction of proinflammatory cytokines.

Proinflammatory cytokine production through AhR signaling results in the activation of NF-κB [25]. It is also well-known that proinflammatory cytokine production by TNFα mainly occurs through NF-κB signaling [2]. We also demonstrated that TNFα-induced NF-κB activation was significantly enhanced by BP. In addition, enhancement of TNFα-induced IL-6 production by BP was most dominantly reduced by the NF-κB inhibitor, Bay11-7082. These results suggest that the enhancement of TNFα-induced IL-6 or IL-1β production by BP mainly occurs through the NF-κB signaling. All together, these results suggest a crosstalk between TNFα signaling and AhR signaling in NF-κB activation and subsequent proinflammatory cytokine production. Although we suggested a crosstalk with TNFα signaling and AhR signaling for the first time, a crosstalk between LPS or Toll-like receptor 4 (TLR4) signaling and AhR signaling pathways has been reported in human dendritic cells [48].

Several considerable mechanisms regarding NF-κB activation by

AhR ligands have been also reported. First, AhR physically interacts with NF-κB to form heterodimers such as AhR/RelA and AhR/RelB, and induces NF-κB responsive gene activation [49,50]. Second, AhR-dependent novel NF-κB sites were reported in the promoter region of IL-1β gene [25]. Third, AhR expression is upregulated by TNFα [26]. However, we did not observe any significant upregulation of AhR expression by TNFα stimulation. On the other hand, AhR-deficient mice exhibited more severe inflammation symptoms following exposure to LPS or cigarette smoke extracts compared to the wild type controls. Thus, AhR may function bi-directionally as either a pro- or anti-inflammatory regulator depending on the situations (variable stimuli, cell types, and diseases) [51,52]. To elucidate this, further precise molecular analysis is required.

5. Conclusions

The findings of present study suggest that smoking affects the efficacy of biologics differently depending upon the individual therapeutic targets and shows a negative effect most notably in TNFi treatment. Thus, RA patients with smoking habits should be strongly recommended to stop smoking in order to avoid drug-discontinuation due to poor therapeutic response, particularly during the TNFi treatment. We also demonstrated that smoke could enhance TNFα-induced NF-κB activation and subsequent proinflammatory cytokine production through AhR signaling, suggesting a crosstalk between TNFα signaling and AhR signaling pathways in NF-κB activation. This might partly explain the high incidence of discontinuation of TNFi due to poor therapeutic response in RA patients with smoking habits. Further studies to elucidate the precise molecular mechanisms will provide us new insights into the pathogenesis and the development of new therapeutic targets in RA.

Funding

This study was supported mainly by the Grant-in-Aid for Scientific Research (C) of Japan Society for the Promotion of Science (No.15K09544) and partly by the grant from Japan National Hospital Organization (H28-NHO (Immunological disorder diseases)-02).

Statements

The present study was carried out in compliance with the Helsinki Declaration and approved by the institutional ethical review board (NHO Sagamihara Hospital, #2013-9). Written informed consent was obtained from each participant. It is duly confirmed that this work is original and has not been published elsewhere. Moreover, it is not currently under consideration for publication elsewhere.

Competing interest

The authors have declared no conflict of interest exists.

Authors contributions

T.N. performed and analyzed most experiments and drafted the manuscript. K.K. performed key experiments and analyzed the clinical data. D.K. and A.S. performed statistical analysis. T.M., M.Y., E. K-T., S.T., Y.H., Y.Y., K.T., S.T., S.O., and J.H., analyzed the clinical data. T.O., H.M., A.M.,T.S.,E.O., Y.O., C.U., and Y.T. performed or contributed to specific experiments. Y.S. conceptualized the study, designed and supervised the experiments, analyzed data, and wrote the manuscript.

Conflict of interests

The authors have no declared conflict of interests.

Acknowledgement and affiliations

We thank the members of the “NinJa” Study Group for recruiting the patients.

We would like to thank Editage (www.editage.jp) for English language editing.

Abbreviations

TNFα	tumor necrosis factor alpha
IL-6	interleukin 6
IL-1β	interleukin 1beta
AhR	aryl hydrocarbon receptor
NF-κB	nuclear factor-kappa B
IκB	inhibitor of NF-κB
IKK	inhibitor of NF-κB (IκB) kinase
Treg	regulatory T cells
ELISA	Enzyme linked immunosorbent assay
RT-PCR	reverse transcription polymerase chain reaction
qPCR	quantitative real-time reverse transcription polymerase chain reaction
CYP1A1	Cytochrome P4501A1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ERK	extracellular signal-regulated kinase, p38: mitogen-activated protein kinase p38

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2018.12.004.

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