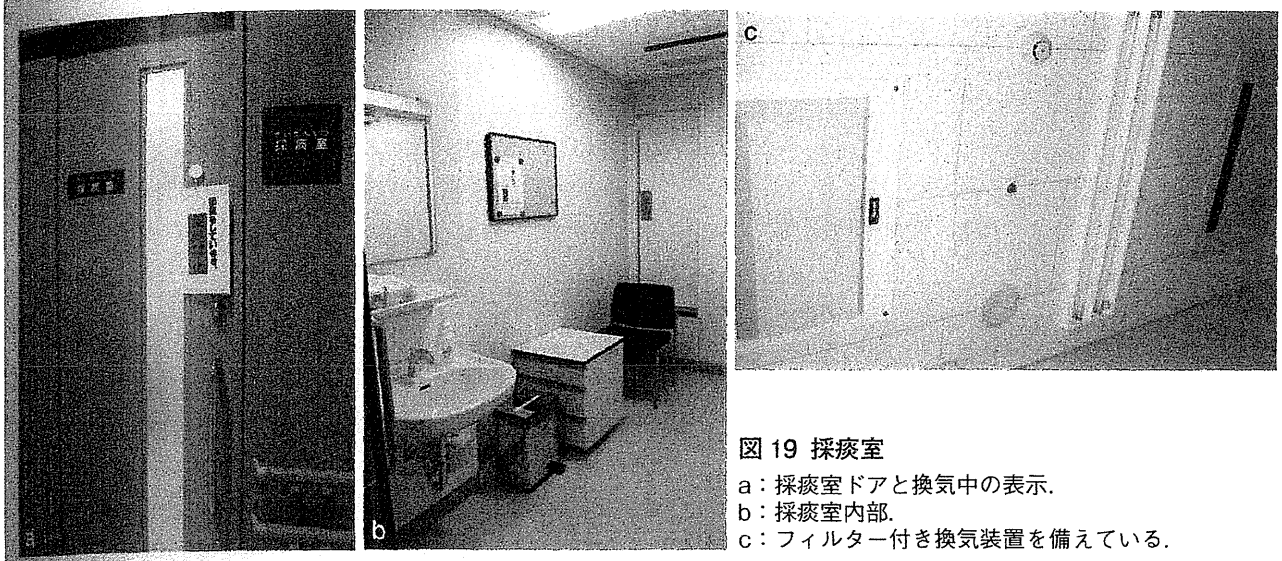

図 19 採痰室
a：採痰室ドアと換気中の表示.
b：採痰室内部.
c：フィルター付き換気装置を備えている.

医療面接のポイント

①患者の生活環境：結核は，地域流行の形をとるため，患者の住居，勤務地は，重要な情報である.

②既往歴：欧米と異なり，日本では内因性再燃と思われる高齢者の罹患率が高く，結核の既往の確認は重要である．膠原病などによる長期のステロイド服用や血液透析，コントロール不良の糖尿病，HIV 陽性，アルコール中毒などの有無も必ず聞く.

③生活習慣：若年者の場合，極端な食事制限や偏食，人の多い盛り場などへの出入りなどは，留意すべき点である.

診断・検査

a 胸部 X 線写真

散布性粒状影または結節影が特徴的である.

b 抗酸菌検査（喀痰塗抹培養・胃液培養・気管支鏡）

画像上陰影がみられれば，院内感染予防のため，採痰はなるべく個室や採痰室（**図 19**）で行う．痰が採れない場合には高張食塩水 40 mL を用いた超音波ネブライザーによる吸入誘発採痰を試みる．検体は培養同定検査，薬物感受性検査，核酸同定検査（結核菌，非定型抗酸菌の PCR 検査）に出す.

①塗抹検査はその日のうちに結果が出るので，湿性咳があれば，喀痰塗抹検査を緊急扱いにして結果を確認する．喀痰塗抹陽性であれば，原則入院治療の対象となる.

②塗抹陰性であるが，胸部 X 線写真や CT で結核の可能性が否定できないときは，喀痰（連続 3 日早朝痰）あるいは胃液検査（朝食止にて朝一番に採取）を繰り返すか，気管支鏡により，結核菌の検出を行う.

③従来，日本では小川培地による培養検査とナイアシンテストにより結核菌の同定を行うことが多かったが結果が出るまで 4 ～ 8 週かかるため，近年は，液体培地による短期間培養や PCR などの核酸増幅・同定検査などが普及してきている．薬剤感受性検査のために培養により菌のコロニーを得るのが望ましい.

c 胸部 CT 検査

いわゆる tree-in-bud 所見が特徴的である．病巣部の石灰化の有無や，胸膜への進展状況，肺門・縦隔リンパ節結核の有無などの検索に有用である.

d ツベルクリン反応

結核菌に対する遅延型過敏反応をみる検査である．強陽性の場合でも感染者とは限らないし，逆に陰性でも感染を否定できず（粟粒結核や進行した AIDS 合併結核の多くは陰性である）BCG 接種の影響も受けるため補助的な検査として考える.

e インターフェロンγ遊離試験（IFN-γテスト，クォンティフェロン）

結核菌に特異的な 2 種類の抗原（CFP-10 と ESAT-6）を患者血液に混ぜて培養し，T 細胞が分泌する IFN-γを ELISA で測る．この抗原は BCG 株にはないので，BCG 接種の影響を受けずに結核感染診断ができる．特に接触者健診の際に，潜在性結核感染症治療対象者の診断に有用とされる.

診断後の処置

a 外来での塗抹陽性

原則入院治療の対象となる．特に同居者に 14 歳以下の小児がいる場合や，集団生活をしている場合は，即日入院させる．結核病床がなくてもあわてずに，家の中でもマスクをしてもらい，外出を控えて家族ともなるべく接触を避けるようにして，結核病床をもつ施設と連絡をとり，ベッドを確保する.

b 入院中の患者の塗抹陽性

個室に移し，患者はマスク着用とし，室外に出ないように説明し（トイレも室内），医療従事者や家族の入室もマスク着用（**図 20**）として結核病棟への転院

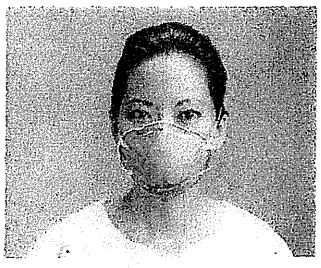

図20 微粒子用N95マスク

の手続きを進める.

c 喀痰塗抹陰性で培養陽性の場合

生活環境により外来でも投薬治療ができる.

d 届出

結核菌がたとえ少量でも検出されたら，感染症法の二類感染症として最寄りの保健所に直ちに届け出なければならない（感染症法第12条）．これをもとに，入院勧告（同第19条），医療の公費負担（入院—同第37条，結核医療—第37条の2），接触者の調査と健診（同第15条，第17条），結核登録票や家族訪問指導（同第53条2-15）等の措置がとられる．また従来，予防内服の対象となった潜在性結核感染症については，現行の感染症法下では無症状病原体保有者として届出をして治療する.

e 患者や家族への説明

長期の通院あるいは入院を要するので，患者や家族が正確な知識をもつことが重要である．結核の一般知識，患者の重症度，療養に必要な事項などを丁寧に説明する.

治療

結核菌の化学療法を，他の細菌感染症と同様に考えてはならない．結核菌は増殖が遅いが，病巣内の菌を完全に死滅させるために，感受性のある抗結核薬（イソニアジド〈INH〉，リファンピシン〈RFP〉，エタンブトール〈EB〉，ピラジナミド〈PZA〉，ストレプトマイシン〈SM〉・カナマイシン〈KM〉が基本）3～4剤を併用し，最低でも6か月間治療するのが原則である．化学療法の失敗は耐性菌の出現につながるので，責任重大であり，十分な種類・用量を規定の期間しっかり内服することが肝要である.

a 治療開始後の定期検査

2週に1回程度定期的に喀痰塗抹培養，胸部X線写真，CRP，赤沈を検査し治療効果を判定する．特に化学療法開始直後は慎重を要する．同時に肝機能，血算，血小板，皮疹，胃腸障害の有無をチェックし，副作用の出現に注意する．化学療法の副作用は死亡例もあるため，細心の注意を払う.

①食欲不振，倦怠，頭痛，悪心・嘔吐：肝機能障害（RFP，PZA）

②手足や口唇のしびれ：INH，EBによる神経障害

③視力低下：EBによる視神経障害

④発熱・発疹：RFP

⑤関節痛：PZAによる高尿酸血症

などは，医療面接上のポイントである.

6か月の治療後も2年間は経過観察期間とし，1年目は3か月に1度，2年目は6か月に1度の受診を促し，X線検査，検痰により再発をチェックする.

b 服薬指導

結核治療は，最低でも6か月を要するが，中途で脱落する患者が増えて問題になっている．治療の脱落は，再排菌による患者周囲への感染や，薬剤耐性結核の出現の原因となる．特にホームレスやアルコール依存症などの患者では，入院しても適応できずに自己退院してしまう場合や外来通院も脱落する場合が多い．1990年代初頭からニューヨークで行われて成果を収めた面前服薬指導（directly observed treatment, short course：DOTS，ヘルスワーカーが毎日患者の内服を直接確認するシステム）は，日本でも試みられ，感染症法の条文に継承され（第53条の14, 15），医療機関・保健所・地域の服薬支援者により進められている.

本書では結核については下記にも詳しく書かれているので参照されたい.

（▶「肺結核（症）」p.378）
（▶「4 胸壁結核」p.452）
（▶ Vol.4 肝・胆道・膵疾患「2 肝の結核症」p.317）

〔中田 光，田澤立之〕

◉文献

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Hansen病

概念

- *Mycobacterium leprae*（らい菌）による抗酸菌感染症である.
- 皮膚と末梢神経が主たる病変部位である.
- リファンピシンを含む多剤抗菌薬で治療する.
- 外見の変形や後遺症などのため，また法律などで偏見や差別，隔離政策，人権侵害などが起こった.

病因・病態・疫学

Mycobacterium leprae（らい菌）によって，皮膚と末梢神経（Schwann細胞）が主に侵される．呼吸器が主たる感染ルートで，幼小児期の感染歴が重要であるが，感染しても発症することはきわめてまれである.

PLOS ONE

Thioredoxin-1 Protects against Neutrophilic Inflammation and Emphysema Progression in a Mouse Model of Chronic Obstructive Pulmonary Disease Exacerbation

Naoya Tanabe[1], Yuma Hoshino[1]*, Satoshi Marumo[1], Hirofumi Kiyokawa[1], Susumu Sato[1], Daisuke Kinose[1], Kazuko Uno[2], Shigeo Muro[1], Toyohiro Hirai[1], Junji Yodoi[3,4], Michiaki Mishima[1]

1 Departments of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **2** Louis Pasteur Center for Medical Research, Kyoto, Japan, **3** Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto, Japan, **4** Center for Cell Signaling Research and Department of Bioinspired Science, Ewha Womans University, Seoul, Korea

Abstract

Background: Exacerbations of chronic obstructive pulmonary disease (COPD) are characterized by acute enhancement of airway neutrophilic inflammation under oxidative stress and can be involved in emphysema progression. However, pharmacotherapy against the neutrophilic inflammation and emphysema progression associated with exacerbation has not been established. Thioredoxin-1 has anti-oxidative and anti-inflammatory properties and it can ameliorate neutrophilic inflammation through anti-chemotactic effects and prevent cigarette smoke (CS)-induced emphysema. We aimed to determine whether thioredoxin-1 can suppress neutrophilic inflammation and emphysema progression in a mouse model of COPD exacerbation and if so, to reveal the underlying mechanisms.

Results: Mice were exposed to CS and then challenged with polyinosine-polycytidylic acid [poly(I:C)], an agonist for virus-induced innate immunity. Airway neutrophilic inflammation, oxidative stress and lung apoptosis were enhanced in smoke-sensitive C57Bl/6, but not in smoke-resistant NZW mice. Exposure to CS and poly(I:C) challenge accelerated emphysema progression in C57Bl/6 mice. Thioredoxin-1 suppressed neutrophilic inflammation and emphysema progression. Poly(I:C) caused early neutrophilic inflammation through keratinocyte-derived chemokine and granulocyte-macrophage colony-stimulating factor (GM-CSF) release in the lung exposed to CS. Late neutrophilic inflammation was caused by persistent GM-CSF release, which thioredoxin-1 ameliorated. Thioredoxin-1 enhanced pulmonary mRNA expression of MAP kinase phosphatase 1 (MKP-1), and the suppressive effects of thioredoxin-1 on prolonged GM-CSF release and late neutrophilic inflammation disappeared by inhibiting MKP-1.

Conclusion: Using a mouse model of COPD exacerbation, we demonstrated that thioredoxin-1 ameliorated neutrophilic inflammation by suppressing GM-CSF release, which prevented emphysema progression. Our findings deepen understanding of the mechanisms underlying the regulation of neutrophilic inflammation by thioredoxin-1 and indicate that thioredoxin-1 could have potential as a drug to counteract COPD exacerbation.

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* E-mail: yuma@kuhp.kyoto-u.ac.jp

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory condition involving oxidative stress and various types of inflammatory cells such as neutrophils and macrophages [1,2]. It is the fourth leading cause of death worldwide [1]. The degree of pulmonary emphysema, which is a major pathological change in COPD, correlates with lung function [3,4] and prognosis [5]. Cigarette smoke (CS) is the most important risk factor for emphysema [1], but emphysema can progress even after some patients with COPD stop smoking [6]. A pharmacological intervention to sufficiently regulate inflammation in COPD and to prevent emphysema progression has not yet been established. Indeed, chronic inflammation under conditions of oxidative stress is relatively resistant to corticosteroids that comprise the standard anti-inflammatory treatment [7,8].

Exacerbation of COPD, which is clinically defined as a sudden worsening of COPD symptoms, is characterized by acute enhancement of airway inflammation [9,10,11], oxidative stress [11] and proteolysis [12], and further amplification of neutrophilic inflammation is a prominent feature [9]. Exacerbation negatively

affects mortality [13] and lung function [14]. Moreover, we previously showed that emphysema progression involves exacerbations [15], the prevention and treatment of which are quite important for COPD management. Clinical trials have shown that systemic corticosteroid therapy can improve clinical status and lung function in the short term [16,17,18], However, our previous findings have suggested that current standard treatment regimens including systemic corticosteroids might not sufficiently suppress exacerbation-induced, long-term emphysema progression [15]. It remains unclear whether acute-on-chronic inflammation during exacerbation can be sufficiently regulated by systemic corticosteroid, although chronic inflammation in stable state of COPD has been previously shown to poorly respond to corticosteroids [8]. Thus, not only the effects and limitations of corticosteroids, but also the potential of alternative therapeutics in exacerbation of COPD should be investigated.

Thioredoxin-1 (TRX) is a ubiquitous, redox-acting, small protein of 105 amino acids with a conserved CXXC construct in its active site that exchanges dithiol to disulfide to maintain the redox status of other molecules [19,20,21]. In addition to this antioxidative effect, TRX has anti-inflammatory [22,23,24] and antiapoptotic properties [25]. TRX overexpression and recombinant TRX administration are effective in animal models of many diseases such as emphysema and acute respiratory distress syndrome [26,27,28]. TRX inhibits neutrophil chemotaxis induced by lipopolysaccharide [22] and CS [26] and thus it could be a candidate drug for treating COPD exacerbation characterized by airway neutrophilic inflammation and emphysema progression [9,15].

Viral infection is a major cause of COPD exacerbation [29,30]. Studies have shown that viral infection in mice exposed to CS enhances lung inflammation similar to that in humans [31,32,33], and this enhancement can be mimicked by administration of polyinosine-polycytidylic acid (poly(I:C)), a synthetic double stranded RNA that is an agonist for innate immunity to viral infection [32,33]. Moreover, these poly(I:C) challenges accelerate emphysema progression in CS-exposed mice. This model can be used for investigating the immune-pathological changes seen in human COPD exacerbations.

We postulated that recombinant TRX suppresses the excessive inflammatory response, especially neutrophilic inflammation, and subsequent emphysema progression induced by COPD exacerbation. We therefore evaluated the effects of TRX in the mouse model of COPD exacerbation and the underlying mechanisms involved.

Materials and Methods

The Animal Research Committee of Kyoto University approved the study protocols.

Animals and Exposure to Cigarette Smoke

Male C57Bl/6NCrSlc and NZW mice purchased from Japan SLC (Shizuoka, Japan) were housed in a temperature-controlled conventional room and freely supplied with laboratory chow and water for at least 3 weeks before being exposed to CS. Eleven-week-old mice were exposed to CS of 10 filter-cut standard cigarettes (Kentucky 3R4F reference cigarette, Cigarette Laboratory at the Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) for 50 minutes per day for 5 days per week for 22, 24, and 45 days using a nose-breathing exposure system (SG- 200; Shibata Scientific Technology Ltd., Tokyo, Japan) [26]. CS was prepared with a standard puff of 35 ml volume and 2 puffs per minute, and diluted to 3% with compressed air. Blood carboxy-hemoglobin levels were about 10% immediately after exposure and the concentration of total particulate matter in mainstream CS was 512.6 mg/m^3.

Poly(I:C) Challenge

Under light anesthesia with isoflurane, 1 mg/kg (body weight) of poly(I:C) (Sigma Aldrich, St. Louis, MO, USA) in 100 microliter of saline was administered by oropharyngeal aspiration [34]. Figure 1 summarized duration of exposure to CS and time course of poly(I:C) challenges for each experimental protocol. In single challenge experiments, poly(I:C) was administered 4 h after exposure to CS on day 22. Some groups of mice were exposed to CS to day 24 and sacrificed on day 25 (3 days after the poly(I:C) challenge, Figure 1A), while the remaining were sacrificed 6 hours after the challenge (Figure 1B). In repeated challenge experiments for lung morphometry, poly(I:C) was also administered 4 h after exposure to CS on days 22, 25, 29, 32, 36, 39, and 43. CS exposure was continued to day 45, and mice were sacrificed on day 46 (Figure 1C).

Treatment with Systemic Corticosteroid, TRX, Anti-granulocyte-macrophage Colony-stimulating Factor (GM-CSF) Antibody, and NSC

Dexamethasone (DEX; D2915, Sigma Aldrich) was intraperitoneally injected 1 h before poly(I:C) challenge at doses of 0.1, 0.3 and 1.0 mg/kg. At 1 h before and 3 h after poly(I:C) challenge, 4 mg/kg of recombinant human TRX (Redox Bioscience Inc., Kyoto, Japan) was intraperitoneally injected. To determine the effect of GM-CSF on airway neutrophil inflammation induced by CS combined with poly(I:C), rat anti-mouse GM-CSF antibody (R&D Systems, Abingdon, UK) was delivered to the lung by oropharyngeal aspiration 3 h after poly(I:C) challenge. To investigate the effects of inhibition of dual-specificity phosphatase 1 [MAP kinase phosphatase 1 (MKP-1)], 2 mg/kg of the cell-permeable, quinone-based, dual-specificity phosphatase inhibitor, NSC 95397 (#N1786, Sigma Aldrich) was intraperitoneally injected both 1.5 h before and 4 h after the poly(I:C) challenge.

Bronchoalveolar Lavage (BAL)

Three days after a single administration of poly(I:C), mice were anesthetized with 20 mg/kg of intraperitoneal pentobarbital. Lungs were lavaged through an intratracheal cannula twice with

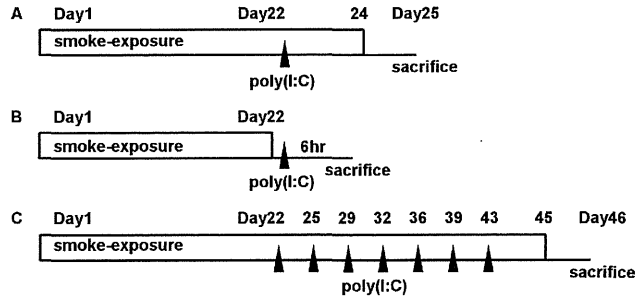

Figure 1. Time course of cigarette smoke exposure and poly(I:C) challenge. (A) Mice were exposed to cigarette smoke (CS) or air to day 24. Poly(I:C) or saline was challenged on day 22, and mice were sacrificed on day 25. (B) In CS-exposed mice, poly(I:C) was challenged on day 22. CS exposure was continued to day 22, and mice were sacrificed 6 hours after the challenge. (C) CS- or air-exposed C57Bl/6 mice were challenged with poly(I:C) or saline seven times (days 22, 25, 29, 32, 36, 39, and 43). CS or air exposure was continued to day 45, and mice were sacrificed on day 46.
doi:10.1371/journal.pone.0079016.g001

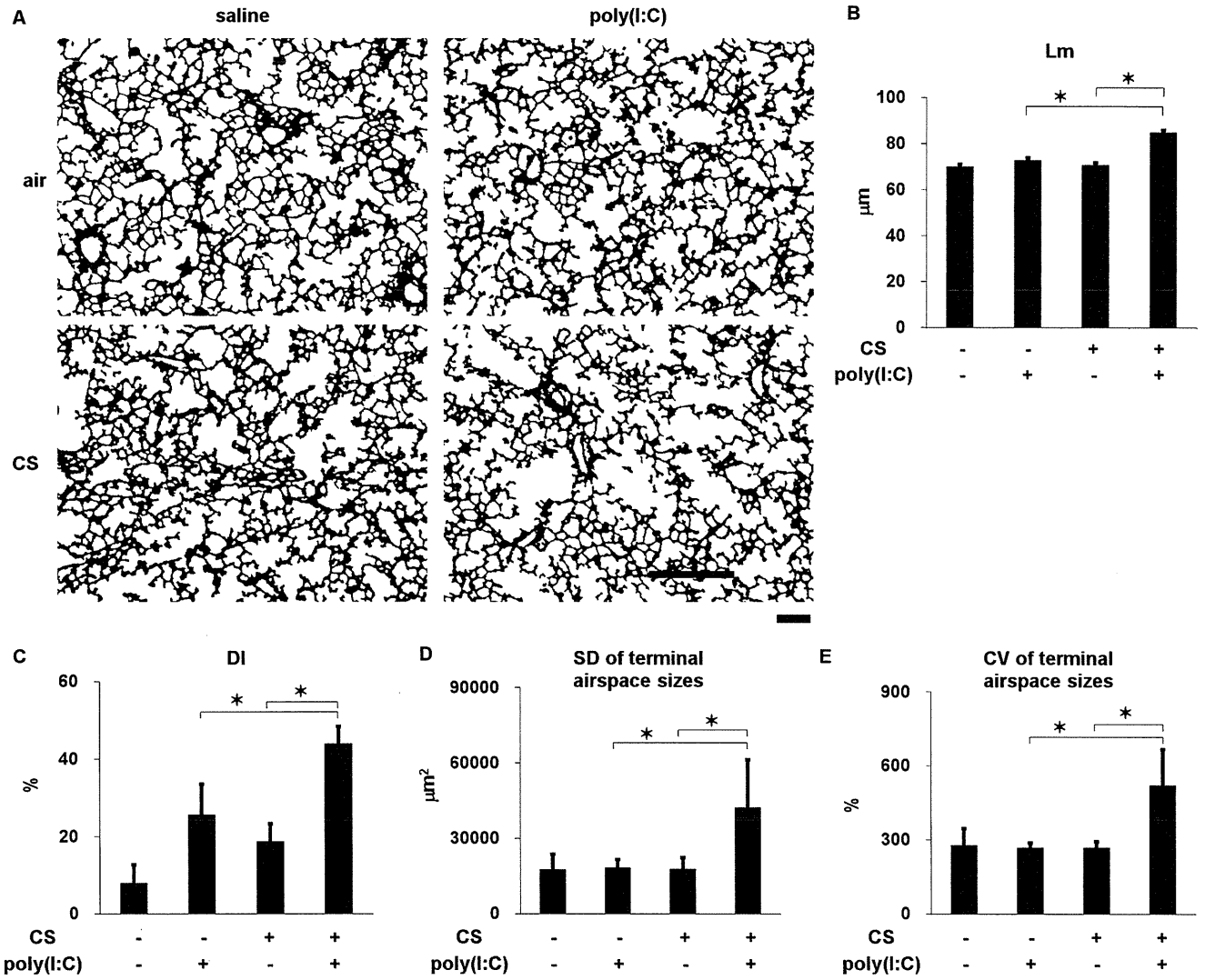

Figure 2. Lung morphometry in C57Bl/6 mice cigarette exposed to cigarette smoke or air and challenged with poly(I:C) or saline seven times. (A) Representative binary images of lung photomicrographs (×4). (B) Mean linear intercept (Lm). (C) Destructive index (DI). (D) Standard deviation (SD) and (E) coefficient of variation (CV) of terminal airspace sizes. Scale bar, 200 μm. Error bars represent SD (n = 5–6 per group); *p<0.05.
doi:10.1371/journal.pone.0079016.g002

1 mL of cold saline and then the inflammatory cell differential, inflammatory cytokines and oxidative stress in the airway were assessed in BAL fluid (BALF). Inflammatory cytokines were also measured 6 h after poly(I:C) challenge in another experiment. The BALF was centrifuged and inflammatory cell differential was determined (Shandon Scientific Ltd., Runcorn, Cheshire, UK). Supernatants were stored at −80°C. At least 400 cells were counted on each cytospin slide stained with Diff-Quik (Dade Behring, Inc., Deerfield, IL, USA) under a light microscope.

Protein Carbonyls and Inflammatory Cytokines in BALF

Protein carbonyl (a marker of oxidative stress) and inflammatory cytokines were measured using Protein Carbonyl Enzyme Immuno-Assay kits (BioCell Corporation Ltd., Papatoetoe, New Zealand) and Bioplex (Bio-Rad Laboratories, Richmond, CA, USA), respectively. Levels of GM-CSF were measured using Bioplex assay and ELISA kit (R&D Systems, Abingdon, UK).

Tissue Preparation

Right lungs were frozen in liquid nitrogen and stored for mRNA and protein analysis. Left lungs were inflated with 50% optimal cutting temperature fluid at 25 cm of H_2O pressure and frozen in cold isopentane for immunohistochemistry and morphometry. Frozen sections (7 μm thick) were cut using a Cryostat (Thermo Fisher Scientific, Tokyo, Japan).

RNA Isolation and Real-time PCR

Total RNA was isolated from lung homogenates using Trizol (Invitrogen, Carlsbad, CA, USA). Gene transcripts of MMP-9, MMP-12, GM-CSF (CSF2), MKP-1 (DUSP-1), MKP-3 (DUSP-3), and 18S as an endogenous control were quantified using the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with oligonucleotide PCR primer pairs and fluorogenic probes (TaqMan Gene Expression Assay; Applied Biosystems).

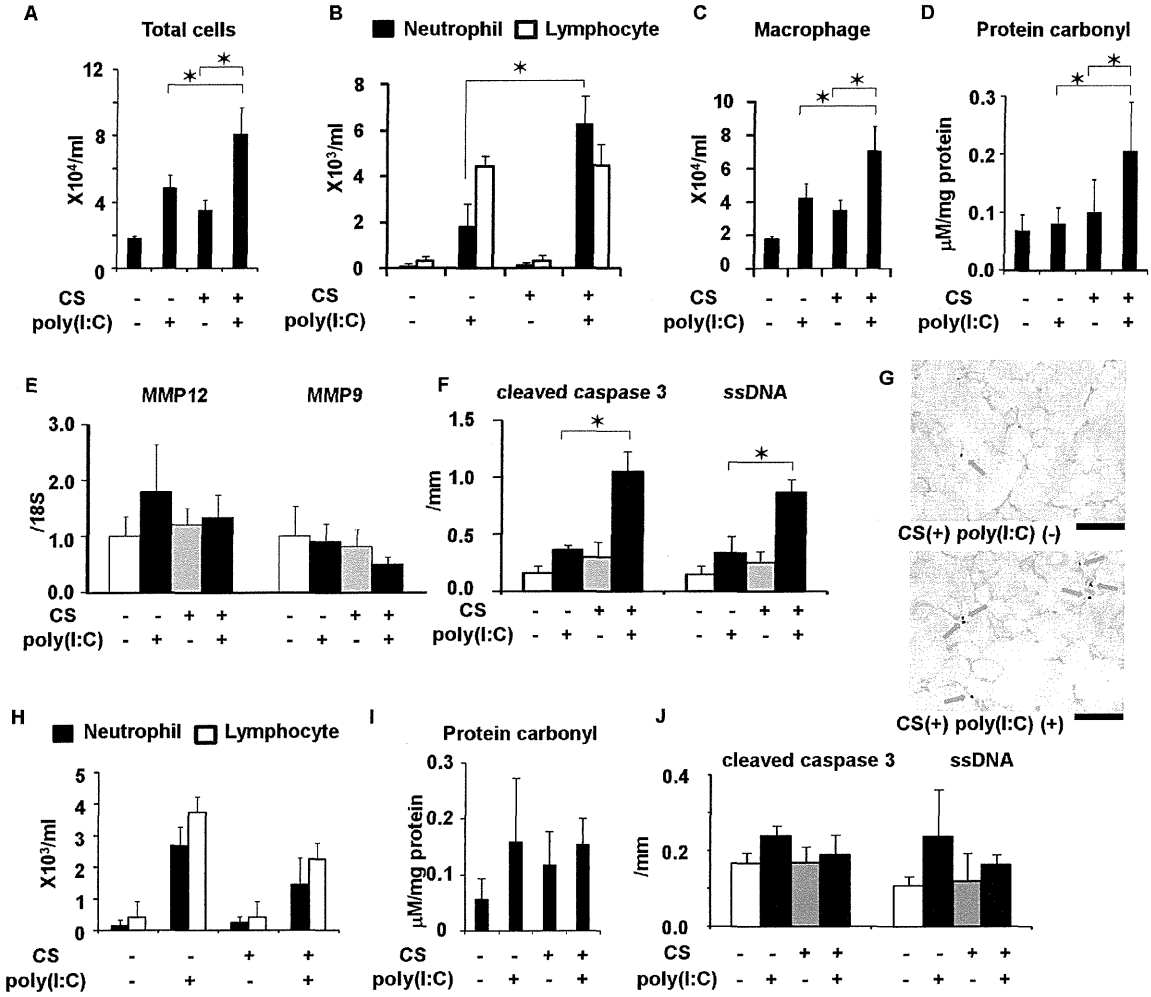

Figure 3. Comparison of impact of poly(I:C) between C57Bl/6 and NZW mice exposed to cigarette smoke. (A) Total cell counts, (B) neutrophil and lymphocyte counts, and (C) macrophage count, and (D) protein carbonyl in BALF. (E) mRNA expression of MMP-12 and MMP-9 in lung homogenates and (F) cleaved caspase 3- and ssDNA-positive cells in lung sections from C57Bl/6 mice exposed to cigarette smoke or air and challenged with poly(I:C) or saline once. (G) Representative images showing cleaved caspase 3-positive cells (arrow) in the lungs of C57BL/6 mice (×20). Scale bar, 100 μm. (H) Neutrophil and lymphocyte counts in BALF, (I) protein carbonyl in BALF, and (J) cleaved caspase 3- and ssDNA-positive cells in lung sections from NZW mice. Error bars represent standard deviation (SD) (n = 5–6 per group); *p<0.05.
doi:10.1371/journal.pone.0079016.g003

Microarrays

Total RNA samples were pooled for each experimental group and analyzed using the 3D-Gene™ Mouse Oligo chip 24 k (Toray Industries Inc., Tokyo, Japan) and then gene expression ratios of TRX-treated to non-treated mice were calculated. The expression array data are deposited in Gene Expression Omnibus under accession number GSE49450.

Immunohistochemistry

Frozen lung sections were incubated with anti-single-stranded DNA (ssDNA) antibody (1:2000 dilution; Dako North America Inc., Carpinteria, CA, USA) and anti-cleaved caspase-3 antibody (1:200 dilution; Cell Signaling, Danvers, MA, USA) [26,35]. Sections were stained using the Dako EnVision system (peroxidase/DAB; Dako, Kyoto, Japan). Immunoreactive cells are expressed as ratios of positive cell to the length of the alveolar septa.

Morphometry

Frozen lung sections were stained with Diff-Quik and assessed by investigators who were blinded to the status of the animals. The extent of emphysema was evaluated as mean linear intercept (Lm), destructive index (DI), and as the standard deviation (SD) and coefficient of variation (CV) of terminal airspace sizes as described [26,36]. Lm and DI were manually measured in at least 10 fields. The original microscope images were converted into binary images and each contiguous air space was automatically identified using custom software to calculate the SD and CV of terminal airspace sizes [36] (Figures S1 and S2).

Statistics

Results are expressed as means ± SD. Data were statistically analyzed using JMP 7 software (SAS Institute, Cary, NC). Groups were compared by analysis of variance followed by the Tukey-Kramer or Dunnett's *post hoc* test. $P<0.05$ was considered significant.

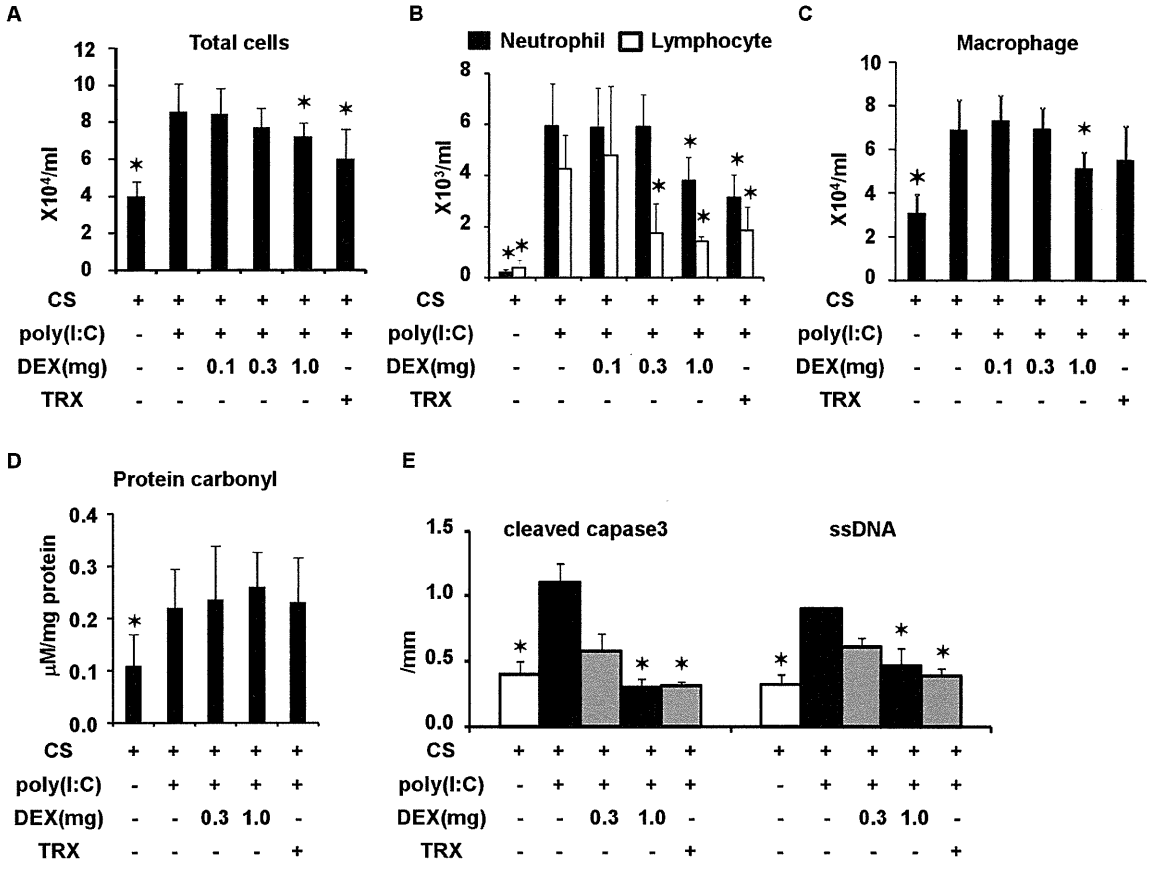

Figure 4. Effects of dexamethasone (DEX) at 0.1, 0.3, and 1 mg/kg and TRX in C57Bl/6 mice exposed to cigarette smoke and challenged with poly(I:C). (A) total cell counts, (B) neutrophil and lymphocyte counts, (C) macrophage count, and (D) protein carbonyl in BALF, and (E) cleaved caspase 3- and ssDNA-positive cells in lung sections. Error bars represent standard deviation (SD) (n = 4–6 per group; *p<0.05 compared with untreated mice exposed to cigarette smoke and poly(I:C).
doi:10.1371/journal.pone.0079016.g004

Results

Comparison of Poly(I:C) Impact in Mice with Different Susceptibilities to CS-induced Emphysema After Exposure to CS

To determine the effects of CS and poly(I:C) on the progression of emphysema, C57Bl/6 mice, which are susceptible to the development of CS-induced emphysema [37], were exposed to CS or air for forty-five days. Poly(I:C) or saline was administered into the lungs seven times (days 22, 25, 29, 32, 36, 39, and 43) (Figure 1C). The Lm, DI, SD and CV in the terminal airspace sizes were significantly increased in the mice exposed to CS and poly(I:C) (Figure 2 and Figure S1), indicating that this combination of agents contributed to airspace enlargement, the destruction of alveolar walls and increased spatial heterogeneity, which is a structural feature of progressive emphysema [38].

To identify components enhanced by CS and poly(I:C), C57Bl/6 mice were exposed to CS or air for three weeks and then administered with poly(I:C) or saline once (Figure 1A). The counts of total cells neutrophils, and macrophages, but not of lymphocytes, were significantly increased in BALF by CS and poly(I:C) (Figure 3A B, and C). The levels of protein carbonyl in BALF and the numbers of cleaved caspase 3- and ssDNA-positive cells (markers of apoptosis) in the lungs were also significantly increased (Figure 3D, F, and G), whereas MMP-9 and MMP-12 mRNA induction was not affected (Figure 3E). Poly(I:C) combined with CS did not increase protein carbonyl levels or total cell, neutrophil, and macrophage counts in BALF, or apoptotic cell markers in the lungs of NZW mice that are resistant to developing emphysema induced by CS [37] (Figure 3F, G and H). Therefore, we considered that these components were exacerbation-related, rather than general non-specific changes caused by viral infections and that they could feasibly be used to evaluate responses to therapy in this model.

Effects of TRX and Systemic Corticosteroids on Poly(I:C)-induced Changes in C57Bl/6 Mice Exposed to CS

After three weeks of exposure to CS, poly(I:C) was administered together with an intraperitoneal injection of TRX and various doses of DEX or saline. Duration of CS exposure and time course of poly(I:C) challenge was shown in Figure 1A and B. At 1 h before and 3 h after the poly(I:C) challenge, 4 mg/kg of TRX was intraperitoneally injected. DEX (0.1, 0.3 and 1.0 mg/kg) was intraperitoneally injected 1 h before the poly(I:C) challenge. Total counts of cells and neutrophils in BALF 3 days after the poly(I:C) challenge were significantly decreased by TRX, as well as by 1.0, but not ≤0.3 mg/kg of DEX (Figure 4A and B). Macrophages in BALF were significantly decreased by 1.0 mg/kg of DEX, but not by TRX (Figure 4C). Levels of protein carbonyl in BALF were not decreased by TRX or DEX at any dose (Figure 4D). Cleaved caspase-3-positive cells and ssDNA-positive cells were significantly reduced by TRX and 1.0 mg/kg of DEX (Figure 4E).

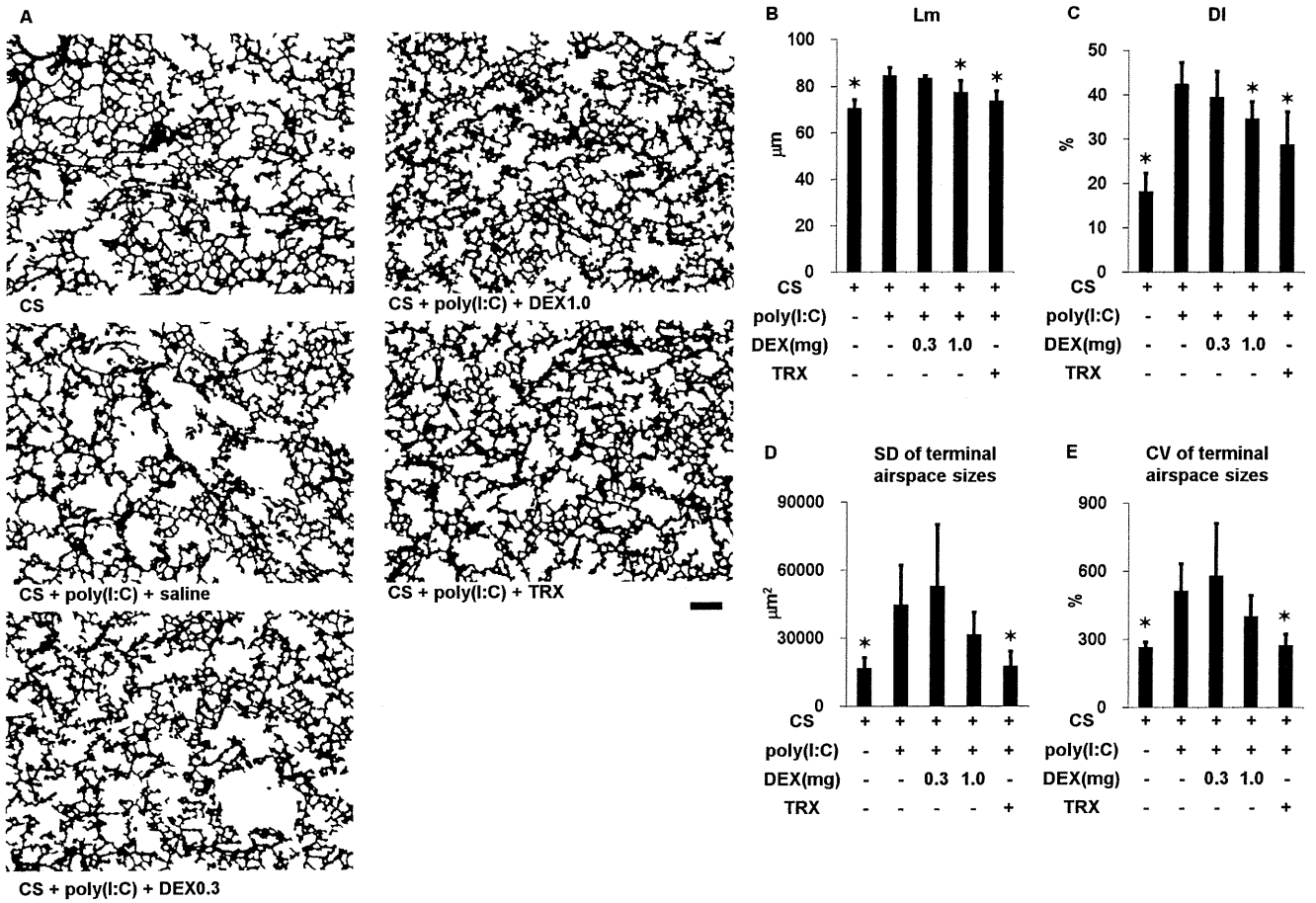

Figure 5. Lung morphometry in mice exposed to cigarette smoke and in those exposed to cigarette smoke, challenged with poly(I:C) and treated with dexamethasone (DEX) at 0.3 and 1 mg/kg, TRX or saline. (A) Representative binary images of lung photomicrographs (×4). (B) Mean linear intercept (Lm). (C) Destructive index (DI). (D) Standard deviation (SD) and (E) coefficient of variation (CV) of terminal airspace sizes. Scale bar, 200 μm. Error bars represent SD (n = 5–6 per group); *p<0.05 compared with mice exposed to cigarette smoke, challenged with poly(I:C) and treated with saline.
doi:10.1371/journal.pone.0079016.g005

Effects of TRX and Systemic Corticosteroids on Lung Morphometry in C57Bl/6 mice Exposed to CS and Poly(I:C)

We administered poly(I:C) seven times along with TRX, DEX (0.3 or 1.0 mg/kg) or saline in mice exposed to CS to determine lung morphometry. Duration of CS exposure and time course of poly(I:C) challenges was shown in Figure 1C. Challenge with poly(I:C) significantly increased the Lm, DI, and SD and CV of terminal airspace sizes (Figure 5 and Figure S2). The increases in Lm and DI were significantly prevented by TRX and by 1.0, but not by 0.3 mg/kg of DEX. TRX significantly ameliorated the increases in the SD and the CV of terminal airspace sizes, whereas DEX at all tested doses did not.

Anti-inflammatory Effect of TRX

We investigated how TRX regulates airway neutrophilic inflammation by measuring levels of inflammatory cytokines in BALF. Many cytokines, including neutrophil chemokines such as keratinocyte-derived chemokine (KC) and GM-CSF, were significantly increased at 6 h after poly(I:C) challenge (Table S1). Notably, the increase in GM-CSF was still detectable after 3 days, whereas that in KC spontaneously resolved (Figure 6A and B and Table S2). TRX ameliorated the sustained increase in GM-CSF 3 days after the challenge. Moreover, the significantly increased mRNA level of GM-CSF at 3 days after poly(I:C) challenge in lung homogenates of mice exposed to CS was ameliorated by TRX (Figure 6C). The neutrophil count in BALF in mice exposed to CS at 3 days after poly(I:C) challenge was significantly and similarly decreased by aspirated anti-GM-CSF antibody and TRX (Figure 6D).

Transcriptional Regulation by TRX in Mice Exposed to CS and Poly(I:C)

To identify a candidate molecule involved in anti-inflammatory effects of TRX, the expression profiles of pulmonary mRNA in mice exposed to CS and poly(I:C) and then treated or not with TRX were examined using microarrays. Among possible genes that were up- or down- regulated by TRX (data not shown), dual-specificity phosphatase 1, also called MAP kinase phosphatase 1 (MKP-1) was further investigated because TRX inhibits P38 MAP kinase in neutrophils [22] and MKP-1 negatively regulates inflammatory responses both *in vitro* and *in vivo* [39,40]. The results of real-time PCR showed that MKP-1 mRNA levels significantly increased in the lungs of mice exposed to CS at 3 days (Figure 7A), but not at 6 h (Figure 7B), after the poly(I:C) challenge and treatment with TRX compared with saline.

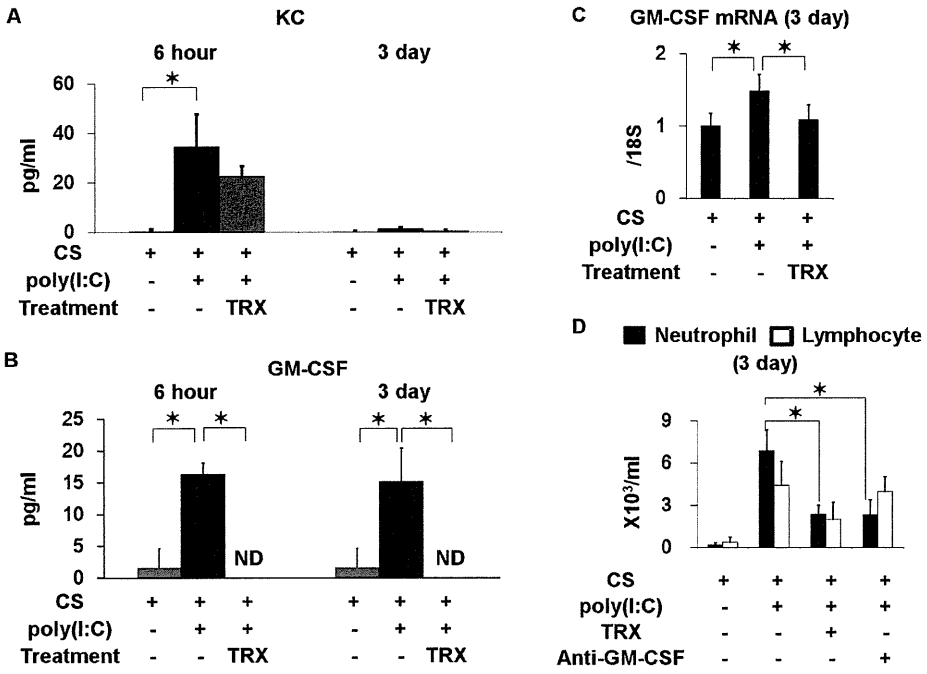

Figure 6. Effects of TRX on inflammatory cytokines in BALF from exposed to cigarette smoke and challenged with poly(I:C). (A) KC and (B) GM-CSF in BALF obtained 6 h and 3 days after poly(I:C) challenge. (C) Messenger RNA of GM-CSF in lung homogenates 3 days after poly(I:C) challenge in mice exposed to cigarette smoke treated with or without TRX. (D) Neutrophil and lymphocyte counts in BALF 3 days after poly(I:C) challenge in mice exposed to cigarette smoke treated with and without TRX or anti-GM-CSF antibody. Error bars represent standard deviation (SD) (A, B, and C, n = 3–4 per group; D and E, n = 5 per group); *p<0.05.
doi:10.1371/journal.pone.0079016.g006

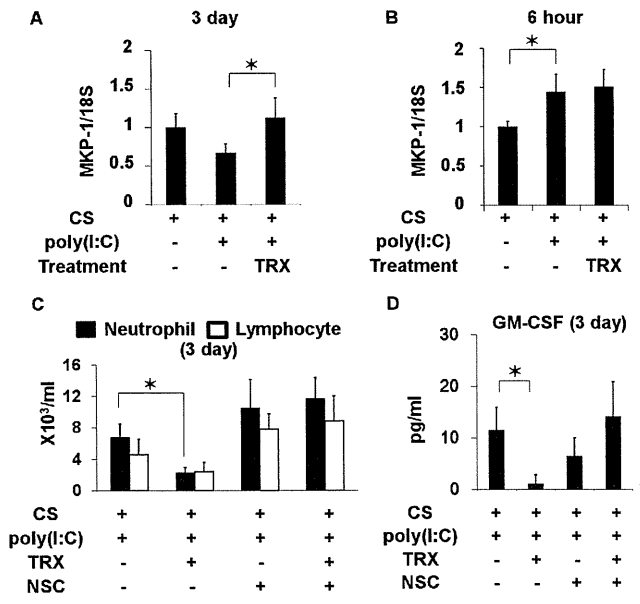

Figure 7. Pulmonary mRNA expression of MKP-1 in mice exposed to cigarette smoke then challenged with poly(I:C), and profiles of inflammatory cells and GM-CSF in BALF from mice treated with MKP-1 inhibitor. (A) MKP-1 mRNA in mice treated with TRX and saline at 3 days after poly(I:C) challenge. (B) MKP-1 mRNA at 6 h after poly(I:C) challenge. (C) Profiles of inflammatory cells and (D) GM-CSF levels in BALF at 3 days after poly(I:C) challenge from mice exposed to cigarette smoke and treated with or without TRX or cell-permeable, quinone-based, dual-specificity phosphatase inhibitor, NSC 95397. Error bars represent standard deviation (SD) (A and B, n = 3–4 per group; D and E, n = 5 per group); *p<0.05.
doi:10.1371/journal.pone.0079016.g007

We investigated whether MKP-1 up-regulation is associated with the suppressive effect of TRX on airway neutrophil inflammation and GM-CSF production by inhibiting MKP-1 using NSC 95397, which inhibits both MKP-1 and MKP-3 [41,42]. Unlike MKP-1, the extent of MKP-3 induction at both 6 h and 3 days after the poly(I:C) challenge in mice exposed to CS did not differ between TRX and saline treatment (Figure S3). TRX reduced BALF neutrophil counts and GM-CSF levels at 3 days after the poly(I:C) challenge in mice exposed to CS, but not in those treated with NSC95397 (Figure 7C and D).

Discussion

The present study showed that TRX has potential to counteract neutrophilic inflammation and emphysema progression in a mouse model of COPD exacerbation. Recombinant TRX suppressed the accelerated progression of emphysema in smoke-sensitive mice exposed to CS and repeatedly challenged with poly(I:C).

Our findings deepen understanding of the mechanism underlying the regulation of neutrophilic inflammation by TRX. Exaggerated airway neutrophilic inflammation was central to the accelerated progression of CS and poly(I:C)-induced emphysema, and neutrophilic inflammation comprised two phases (Figure 8). Poly(I:C)-induced production of neutrophilic chemokines such as KC and GM-CSF promoted neutrophil migration into the lung during the early phase, and then the sustained release of GM-CSF in the lung prolonged neutrophil survival [43] during the late phase, which led to persistent airway inflammation and pronounced parenchymal destruction. TRX can suppress neutrophilic inflammation, perhaps through directly inhibiting neutrophil infiltration into sites of inflammation [22]. Notably, we discovered that TRX suppresses prolonged GM-CSF release, indicating that

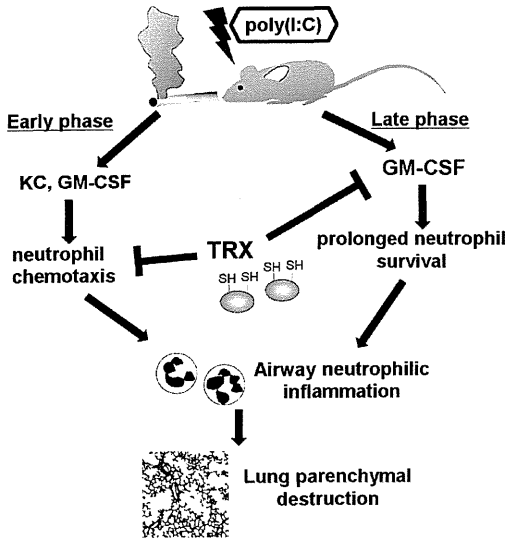

Figure 8. Estimated mechanism of dual regulation of poly(I:C)-induced neutrophilic inflammation by recombinant TRX in mouse lungs exposed to cigarette smoke. Poly(I:C)-induced neutrophilic inflammation consists of two phases. Poly(I:C) induces neutrophilic chemokines such as KC and GM-CSF that cause neutrophil migration into the lung during the early phase. Thereafter, sustained release of GM-CSF in the lung contributes to prolong neutrophil survival, resulting in persistent airway inflammation throughout the late phase. Thioredoxin-1 suppresses airway neutrophil inflammation through directly inhibiting neutrophil chemotaxis and reducing GM-CSF.
doi:10.1371/journal.pone.0079016.g008

recombinant TRX regulates neutrophilic inflammation via a dual mechanism.

We used the animal model established by Kang et al. [32] with slight modification. It has been shown that in this model, airway inflammation is greater and emphysema develops more rapidly than conventional mouse model of emphysema induced by CS exposure alone. This model is appropriate for exploring enhanced airway inflammation and accelerated emphysema progression, which are the main immunopathological changes in human COPD exacerbation [9,10,15]. Although the time course of poly(I:C) challenge and duration of smoke exposure slightly differed in the present, from the original study, similar inflammatory responses and progressive emphysema were detected (lung cell apoptosis and parenchymal destruction).

To identify "exacerbation-related changes", we compared smoke-sensitive C57Bl/6 mice (murine counterpart of patients with COPD) and smoke-resistant NZW mice (murine counterpart of asymptomatic smokers) assuming that changes induced by poly(I:C) in NZW mice were not related to exacerbation. Consequently, exposure to CS and poly(I:C) enhanced airway neutrophilic and macrophage inflammation and induced oxidative stress and lung apoptosis in smoke-sensitive, but not in smoke-resistant, mice. We considered that these findings were exacerbation-related changes that should be targeted with therapeutic interventions. It should be also noted that although various types of inflammatory cells such as neutrophil, macrophage, and T cells are associated with the pathogenesis of murine emphysema induced by CS alone [44], neutrophil and macrophage play an important role in amplifying airway inflammation in the present COPD exacerbation model.

TRX suppressed airway neutrophilic inflammation, lung apoptosis and the further progression of emphysema in mice exposed to CS and poly(I:C). Although TRX has anti-oxidant properties, these were not considered central in the present model because TRX did not improve the increase in oxidative stress assessed by carbonyl protein in BALF. This finding was consistent with previous reports concerning the limited anti-oxidant properties of exogenous TRX [45]. To reinforce this conclusion, other markers of oxidative stress such as F2-isoprostanes should be measured [46].

GM-CSF is a direct neutrophil chemotactic factor that increases neutrophil survival in the respiratory tract, and can be involved in CS-induced airway neutrophilic inflammation [43,47]. The present study showed that in mice exposed to CS and poly(I:C), the airway level of GM-CSF was increased at 6 h after poly(I:C) challenge and sustained until 3 days after the challenge, while the levels of other inflammatory cytokines especially associated with neutrophilic inflammation including KC, IL-6, RANTES, and TNF alpha were increased at 6 h after poly(I:C) challenge but spontaneously resolved at 3 days after the challenge. These results indicate that early phase of CS and poly(I:C)-induced neutrophilic inflammation could be prompted by many cytokines, but the enhanced inflammation could be sustained exclusively by prolonged GM-CSF release.

TRX ameliorated enhanced GM-CSF mRNA expression and protein production at 3 days after poly(I:C) challenge. The airway neutrophil inflammation at 3 days after the challenge was reduced as much by anti-GM-CSF antibody as by TRX in mice exposed to CS. These suggest that TRX regulates late phase of neutrophilic inflammation by suppressing prolonged GM-CSF release. The suppressive effects of TRX against the early increases in inflammatory cytokines such as IL-6, TNF alpha, and RANTES were also found, and this might have affected the reduction of GM-CSF and resolution of neutrophilic inflammation during the late phase.

To elucidate the signaling pathway associated with the suppression of GM-CSF release and regulation of neutrophilic inflammation by treatment with TRX, we focused on MKP-1 in the lung of mice treated with TRX based on the findings of an investigation into the suppressive effect of TRX on P38 MAP kinase in neutrophils [22]. Inflammatory cytokine release is regulated by MKP-1 in innate immune responses [39,40].

Pulmonary mRNA of MKP-1 was up-regulated at 6 h after poly(I:C) challenge in both mice exposed to CS and then treated with TRX or saline, but the extent of MKP-1 induction did not differ between the two groups. In contrast, 3 days after the challenge, more MKP-1 was expressed in the group treated with TRX than with saline. TRX reduced neutrophil counts and GM-CSF levels in BALF at 3 days after poly(I:C) challenge in mice exposed to CS, but this effect disappeared in mice exposed to CS and treated with the MKP-1 and MKP-3 inhibitor NSC95397 [41,42]. These findings suggest that MKP-1 might be involved in the suppression of GM-CSF release and late phase of neutrophilic inflammation by TRX.

In addition to mRNA expression, we examined MKP-1 protein levels in the lungs at 3 days after poly(I:C) challenge using Western blotting. However, MKP-1 protein levels did not significantly differ between mice treated with or without TRX (data not shown). This is a major limitation of the present study. Nevertheless, our findings are quite important, because they show for the first time an association between TRX, MKP-1, and inflammation. The findings also provide a hypothesis that MKP-1 induction by TRX is essential for suppressing persistent GM-CSF release and neutrophilic inflammation. This should be verified in future studies.

Effects of systemic corticosteroids on "exacerbation-related changes" such as airway neutrophilic inflammation and emphysema progression were also evaluated. In human, 30–40 mg/body (approximately 0.5–0.67 mg/kg) of prednisolone has been recommended for treatment of COPD exacerbations [1]. Given that 0.75 mg/kg of DEX is equivalent in anti-inflammatory activity to 5 mg/kg of prednisolone, 0.1 mg/kg of DEX in the present model could be relevant to the clinical dose currently applied to manage COPD exacerbation. Notably, airway neutrophilic inflammation and emphysema progression could be suppressed only when the dose of DEX was increased up to 1.0 mg/kg, which may reflect approximately 10 times of the standard dose in practice. These suggest that the current regimen of systemic corticosteroids cannot always prevent emphysema progression induced by exacerbation. Together with concern that high dose of systemic corticosteroid has risk of adverse effects, our results emphasize the importance of further investigation about the role of TRX as alternative therapeutics.

We found that many inflammatory cytokines such as IL-6, TNF alpha, and RANTES in BALF were increased and pulmonary mRNA of MKP-1 were up-regulated at 6 h after poly(I:C) challenge in CS-exposed mice. Since MKP-1 negatively regulates inflammatory cytokines such as IL-6 and TNF alpha [40,48], it is possible that the early up-regulation of MKP-1 acts as negative feedback regulator leading to the spontaneous reductions in IL-6, RANTES, and TNF alpha at 3 days after the challenge.

Some limitations are associated with this study. Poly(I:C) challenges proceeded before emphysema was established. The present model reflects exacerbations during the early, but not the moderate to severe stages of COPD. However, a distinct subgroup of patients with COPD can experience frequent exacerbations independently of disease severity [49]. We believe that the present model provides information about the immune-pathological changes that are qualitatively similar to those in COPD patients.

Our animal model of COPD exacerbation was established using poly(I:C), and not a virus infection and thus the influence of pharmacological intervention on viral clearance or the adaptive immune response in exacerbations could not be assessed.

In the present study, after identifying "exacerbation-related changes" by using CS- or air-exposed mice challenged with saline or poly(I:C), effects of TRX and DEX were evaluated only in CS-exposed mice, but not in air-exposed mice, because the main aim of the present study was to investigate effects of TRX against acute-on-chronic inflammation and lung parenchymal destruction during exacerbation, and because mice exposed to CS and then challenged with or without poly(I:C) were considered as murine counterpart of exacerbation or stable state of COPD, respectively. However, considering that inflammation under oxidative stress generally shows a poor response to corticosteroid [7,8], it is also an important issue whether effects of TRX and DEX against poly(I:C)-induced inflammation might differ between mice exposed to CS and air. This should be investigated in future studies.

In conclusion, airway neutrophilic inflammation and the progression of emphysema was suppressed by TRX and a relatively high dose, but not by a moderate dose of systemic corticosteroid in smoke-sensitive model mice exposed to poly(I:C) and CS. Our findings also suggest a novel mechanism of neutrophilic inflammation regulated by TRX. In addition to the inhibition of neutrophil chemotaxis, the suppression of prolonged GM-CSF release by TRX is involved in the resolution of late phase of poly(I:C)-induced neutrophilic inflammation. The present findings suggest that TRX has a dual regulatory effect on neutrophilic inflammation induced by poly(I:C) in the lungs of model mice exposed to CS and indicate that TRX has potential as a novel therapeutic agent for treating COPD exacerbation.

Supporting Information

Figure S1 Representative original images (Diff-Quik), binary images, and color map images that identify each terminal airspace in cigarette smoke- or air-exposed C57Bl/6 mice challenged with poly(I:C) or saline seven times (magnification ×4). Scale bar, 200 um.
(TIF)

Figure S2 Representative original images (Diff-Quik), binary images, and color map images that identify each terminal airspace in cigarette smoke-exposed poly(I:C)-challenged mice treated with different doses of dexamethasone (DEX; 0.3 and 1 mg/kg), TRX, and saline (magnification ×4). Scale bar, 200 um.
(TIF)

Figure S3 Pulmonary mRNA expressions of MKP-3 in cigarette smoke-exposed mice challenged with poly(I:C) once. (A) MKP-3 mRNA in mice treated with TRX and saline 6 hours after poly(I:C) challenge. (B) MKP-3 mRNA 3 days after the poly(I:C) challenge. Error bars represent standard deviation (SD) (n = 3–4 per group).
(TIF)

Table S1 Cytokine levels in bronchoalveolar lavage fluid of cigarette smoke-exposed mice treated with thioredoxin or saline 6 hours after poly(I:C) challenge.
(DOC)

Table S2 Cytokine levels in bronchoalveolar lavage fluid of cigarette smoke-exposed mice treated with thioredoxin or saline 3 days after poly(I:C) challenge.
(DOC)

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Author Contributions

Conceived and designed the experiments: NT YH S. Marumo HK SS DK KU S. Muro TH JY MM. Performed the experiments: NT YH S. Marumo HK DK KU. Analyzed the data: NT YH S. Marumo HK SS DK KU S. Muro TH JY MM. Contributed reagents/materials/analysis tools: NT KU TH. Wrote the paper: NT YH JY MM.

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Clinical features and determinants of COPD exacerbation in the Hokkaido COPD cohort study

Masaru Suzuki[1], Hironi Makita[1], Yoichi M. Ito[2], Katsura Nagai[1], Satoshi Konno[1] and Masaharu Nishimura[1], for the Hokkaido COPD Cohort Study Investigators

[1]First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Japan
[2]Department of Biostatistics, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Correspondence to: Masaharu Nishimura, MD, PhD
First Department of Medicine, Hokkaido University School of Medicine
North 15 West 7, Kita-ku, Sapporo 060-8638, Japan
Tel: +81-11-706-5911; Fax: +81-11-706-7899; E-mail: ma-nishi@med.hokudai.ac.jp

Running head: Clinical features of COPD exacerbation

Take home message
Impaired health-related quality of life and weight loss are independent risk factors for COPD exacerbations.

ABSTRACT

Exacerbations are among the major factors that may affect the natural history of chronic obstructive pulmonary disease (COPD). The aim was to investigate the clinical characteristics and determinants of COPD exacerbations in our 5-year observational cohort study that had a very low exacerbation frequency.

A total of 279 patients with COPD participated in the Hokkaido COPD cohort study, and 268 subjects who had clinical data for multiple visits were analyzed. Exacerbation was defined in multiple ways: patient's subjective complaint, symptom definition, requiring prescription change, requiring antibiotic treatment, and requiring hospital admission.

Exacerbation frequency (events/person/year) was 0.78 ± 1.16 (subjective complaint), 0.24 ± 0.47 (symptom definition), 0.20 ± 0.43 (prescription definition), 0.13 ± 0.28 (antibiotic definition), and 0.06 ± 0.19 (admission definition). Exacerbation events did not significantly affect the annual decline in FEV_1. A high St. George's Respiratory Questionnaire total score, especially its Activity score, and a low body mass index were strongly associated with exacerbation-free survival, exacerbation frequency, and development of recurrent exacerbations.

Despite the low exacerbation frequency in our cohort study, impaired health-related quality of life and weight loss were found to be independent risk factors for COPD exacerbations.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation that is usually progressive and is a leading cause of morbidity and mortality worldwide [1]. Exacerbations of COPD are an acute event characterized by a worsening of respiratory symptoms, and they are very important in the clinical course of COPD because they are associated with poor quality of life (QOL), increased mortality, and high socioeconomic costs [1-3]. Recently, Hurst et al. reported that patients who have frequent exacerbations belong to a clinically stable phenotype that is susceptible to further exacerbations [4]. Therefore, it would be critical to determine the clinical characteristics and predictors of exacerbations for better management of COPD patients.

The Hokkaido COPD cohort study is a carefully designed, multi-center, observational cohort, which primarily aims to examine the annual decline in FEV_1 over a period of 5 years based on clinical phenotypes in patients with smoking-related COPD [5, 6]. We have already reported that the rate of annual change in FEV_1 was highly variable among patients with COPD and was not associated with exacerbation frequency [6]. A unique finding of our cohort study was that the exacerbation frequency was much lower than the previous large-scale clinical trials [6]. However, the characteristics and risk of exacerbations in a population with such low exacerbation frequency have not yet been clarified. In this study, the clinical characteristics and determinant of COPD exacerbations were examined in our 5-year observational cohort.

METHODS

Participants

The recruitment of the COPD patients has been described elsewhere [5, 6]. Briefly, 330 subjects with respiratory physician-diagnosed COPD were recruited at Hokkaido University Hospital, Sapporo, Japan, and nine affiliated hospitals from May 2003 to May 2005. All were aged 40 years or older and were either current or former smokers with a smoking history of at least 10 pack-years. Subjects with clinically diagnosed asthma were excluded. Thirty subjects were excluded due to consent withdrawal, or were ineligible for inclusion before visit 1, and a total of 300 subjects were followed. During the first follow-up year, the diagnosis of COPD was reconfirmed in 279 subjects based on the spirometric criteria of the Global Initiative for Chronic Obstructive Lung Disease

(GOLD) guidelines (post-bronchodilator FEV_1/FVC <0.70) [1], and the subjects were eligible for subsequent follow-up. In this study, 268 subjects (GOLD 1, 26%; GOLD 2, 45%; GOLD 3, 24%; GOLD 4, 5%) who had clinical data for multiple visits were analyzed. Of the 268 subjects, 184 (69%) completed a 5-year follow-up period. The reasons for dropout of the initial 279 subjects have been described elsewhere [6]. The median follow-up period was 4.97 years. Characteristics of subjects classified by severity of airflow limitation are shown in Table 1. The Ethics Committee of Hokkaido University School of Medicine approved the study protocol, and written, informed consent was obtained from all participants.

Study protocol

The details of the study protocol of the Hokkaido COPD cohort study have been described elsewhere [5, 6]. Most subjects, except for those with GOLD 1, visited outpatient clinics at each hospital monthly or bimonthly for regular clinical checkups (online supplement Table 1). On the first visit, demographic information, including sex, age, height, weight, smoking history, medical history, and medications, and information on pulmonary symptoms were collected. Every 6 months, any changes in smoking status, medical history, and pharmacotherapy were monitored. Health-related quality of life (QOL) assessed by St. George's Respiratory Questionnaire (SGRQ) [7] was examined every year, and blood was also sampled every year for measurements of circulating blood cell counts, serum immunoglobulin E (IgE), and C-reactive protein (CRP). Spirometry both before and after inhalation of a bronchodilator was performed on every visit. Chest CT scans were performed in the supine position with breath held at full inspiration. Severity of emphysema was visually assessed by three independent pulmonologists according to the modified Goddard scoring system [5, 6, 8].

Assessment of exacerbation

In order to collect exacerbation information, reply-paid postcards were sent to all participants every month, and replies were received from almost all participants (reply rate >99%). The questionnaire items in the postcard were described in the online supplement. If exacerbation was suspected, information was always re-confirmed by telephone interview and/or by the medical charts of subjects when they visited a clinic. In addition, the subjects' medical records were periodically checked, and attending

physicians were asked about the subjects' conditions when necessary.

Exacerbation of COPD was defined in several of the following ways: 1) patient's subjective complaint by reply-paid postcard (any clinical symptoms that did not meet symptom definition criteria); 2) worsening or new onset of either two major symptoms (increased dyspnea, change in sputum purulence, increased sputum volume) or any one major symptom plus any minor symptoms (fever, increased cough, wheezing) compared with baseline (symptom definition); 3) symptom criteria plus requiring prescription change (prescription definition); 4) symptom criteria plus antibiotic treatment (antibiotic definition); and 5) symptom criteria plus hospital admission (admission definition). Radiologically proven pneumonia was not excluded from exacerbation events because many of the patients with severe exacerbation are examined by CT scan in Japan and bronchopneumonia is often detected even if chest X-ray is almost normal.

Statistical analysis

The details of statistical analysis are in the online supplement. Differences among the groups were analyzed using the Student's t-test, the Mann-Whitney U test, or the chi-squared test, where appropriate. Bivariate correlations were analyzed using Spearman's rank correlation coefficient. Factors associated with exacerbation-free survival were analyzed using a Cox proportional hazards model and the Kaplan-Meier method with the log-rank test. Factors associated with exacerbation frequency were analyzed using a Poisson regression model. Factors associated with recurrent exacerbation were analyzed using the Prentice, Williams and Peterson (PWP) total time model that is based on the Cox proportional hazards model for recurrent event data [9]. Here, the total time refers to the time interval from time origin 0 to the occurrence of each event. Significant variables in univariate models were included simultaneously in a multivariate model. Statistical significance was defined as $p<0.05$.

RESULTS

The cumulative number of exacerbation events and the number of subjects who experienced exacerbations during the follow-up period differed depending on the definition criteria (Figure 1). Indeed, the number of exacerbation events became much lower compared to the number of patients' subjective complaints when symptoms

were carefully confirmed. There were 16 events of 243 events (6.6%) whose exacerbation was not picked up by subjective complaint in the postcard but by confirmation of symptoms and prescription changes by interview and/or medical records. The COPD exacerbation frequency (events/person/year) during the follow-up period was 0.78 ± 1.16 (subjective complaint), 0.24 ± 0.47 (symptom definition), 0.20 ± 0.43 (prescription definition), 0.13 ± 0.28 (antibiotic definition), and 0.06 ± 0.19 (admission definition), and the exacerbation frequency was higher in subjects with severe airflow limitation (GOLD 3-4) than in those with mild airflow limitation (GOLD 1-2) (Table 2). There were very few subjects who experienced exacerbations twice or more per year (only 3 subjects by symptom or prescription definitions and none of the subjects by antibiotics or admission definitions). Subjects who experienced at least one exacerbation during the follow-up period had lower lung function, more dyspnea, and higher SGRQ total score (i.e. impaired health-related QOL) compared to subjects who had no exacerbation (online supplement Table 2). Among subjects who experienced at least one exacerbation, 50% (symptom definition), 48% (prescription definition), 42% (antibiotics definition), and 25% (admission definition) of subjects developed multiple exacerbation events during the follow-up period (recurrent exacerbation). The number of exacerbation events was higher in the spring months (March to June) and in the autumn months (October to November) (Figure 2).

Subjects who experienced exacerbations within the first year of follow-up had more frequent exacerbations after the first year of follow-up (Figure 3), which confirmed the recurrent nature of COPD exacerbations. On the other hand, the annual decline in FEV_1 was not affected by exacerbation regardless of its definition and the degree of airflow limitation (online supplement Figure 1), whereas subjects who experienced more than one exacerbation defined by admission criteria per year tended to show a more rapid FEV_1 decline compared to subjects with less exacerbations ($p=0.07$) (online supplement Figure 2). There was no significant correlation between the annual decline in FEV_1 and exacerbation frequency at any definition (data not shown).

A multivariate Cox proportional hazards model showed that low BMI and high SGRQ total score were significant and independent predictors for the early development of the first exacerbation event defined as both prescription change and hospital admission (Table 3 and online supplement Tables 3 and 4), and Kaplan-Meier curves for the classification groups by BMI and SGRQ total score were clearly separated (figure 4).

The multivariate Poisson regression model showed that low BMI, high SGRQ total score, low FEV_1, and low Hb were significantly associated with exacerbation frequency defined as prescription change, and low BMI and high SGRQ total score were significantly associated with exacerbations defined as hospital admission (Table 3 and online supplement Tables 5 and 6). Furthermore, the multivariate PWP total time model showed that high SGRQ total score and low Hb were significant predictors for the development of recurrent exacerbations defined as prescription change, and that older age, low BMI, and high SGRQ total score were significant predictors for the development of recurrent exacerbations defined as hospital admission (Table 3 and online supplement Tables 7 and 8).

Since a high SGRQ total score was significantly associated with all of exacerbation-free survival, exacerbation frequency, and the development of recurrent exacerbations, the SGRQ domain scores of Symptoms, Activity, and Impact were also assessed. The SGRQ Activity score was found to be the only domain that was significantly associated with all of the above analyses (online supplement Table 9).

DISCUSSION

In this paper, the intention was to clarify the clinical characteristics and determinants of COPD exacerbations using the Hokkaido COPD cohort study population. The strongest point of this cohort study is that it was very carefully designed and performed, thus making it possible to collect accurate information regarding each patient's complaints, symptoms, and clinical data during COPD exacerbations. Although COPD exacerbation is defined in the GOLD guidelines [1] as an acute event characterized by a worsening of the patient's respiratory symptoms and leads to a change in medication, a general definition for COPD exacerbation has not been accepted; moreover, several levels regarding the severity of exacerbations are required. Therefore, COPD exacerbation was defined in multiple ways in the present study. It was found that the number of exacerbation events and the number of subjects who experienced exacerbations were very different depending on the definition criteria, especially between patients' subjective complaints and confirmed symptoms. Importantly, the same patients seemed to repeatedly complain about their poor physical conditions even if they did not have enough respiratory symptoms, since the number of exacerbation events was less than the number of subjects who experienced

exacerbation events when the symptom definition was applied (Figure 1). Therefore, it is very important for physicians to confirm patients' respiratory symptoms carefully for the diagnosis of COPD exacerbation. On the other hand, it is possible that we missed some symptomatic events even though the symptom information was re-confirmed by telephone interview and/or by the medical charts. Some subjects might have been shy away from declaring their symptoms accurately to medical staffs although they reported the symptoms on the postcards, which may be a characteristic feature of Japanese. Furthermore, some subjects might not be willing to complain in the postcards so that we might have missed actual exacerbations. We think that enhancement and encouragement of reporting using more sensitive tools such as a daily diary or an electronic personal digital assistant would be ideal for more accurate symptom assessment.

A unique finding of this study is the much lower exacerbation frequency during the study period compared to recent large-scale clinical studies such as TORCH [10], UPLIFT [11], and ECLIPSE [4, 12]. Importantly, the present population included patients with mild airflow limitation (GOLD 1), unlike the above clinical studies that did not recruit GOLD 1 patients, and it was confirmed that exacerbations became more frequent as the severity of airflow limitation increased (Table 2), which was consistent with previous studies [4, 13, 14], suggesting that recruitment of patients with milder airflow limitation may contribute to the lower exacerbation frequency. However, the exacerbation frequency in the present study was still lower than that of previous studies even when compared with patients with the same severity of airflow limitation. Specifically, the mean frequency of exacerbations (events/person/year) defined as prescription change in each GOLD category was 0.14 (GOLD 2), 0.30 (GOLD 3), and 0.77 (GOLD 4) in the present study, whereas the mean was 0.7-0.9 (GOLD 2), 1.1-1.3 (GOLD 3), and 1.2-2.0 (GOLD 4) in previous large-scale clinical studies. Similarly, the mean frequency of hospital admission was 0.06 (GOLD 2), 0.10 (GOLD 3), and 0.09 (GOLD 4) in the present study, whereas the mean was 0.11-0.2 (GOLD 2), 0.25-0.3 (GOLD 3), and 0.4-0.54 (GOLD 4) in previous studies [1, 4, 10, 12]. Such a lower frequency of exacerbation was also observed in the subgroup analysis of the Japanese patients participating the UPLIFT study and in another Japanese report [15, 16]. The frequency of chronic bronchitis in our cohort was also lower compared to the other studies [12, 17]. Thus, national characteristics such as the health care system and socioeconomic