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呼吸不全に関する調査研究

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研究成果の刊行物・別刷

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肺胞腔内にサーファクタント脂質・蛋白が貯留して呼吸困難が進行　J84.0

肺胞蛋白症（PAP）

pulmonary alveolar proteinosis

担当：中田　光

Overview

肺サーファクタント*の生成または分解過程の障害により、肺胞腔内、終末気管支内にサーファクタントやその老廃物が異常貯留をきたす疾患の総称。有病率は人口100万対6.2。壮年期の男性に多い。

誘因・原因 ➡P150

- 肺サーファクタントの生成または分解過程の障害。
- 障害原因により遺伝性、自己免疫性（特発性）、二次性（続発性）に分類されるが、原因が不明な場合も多い。

病態生理 ➡P150

- 肺胞腔内、終末気管支内に肺サーファクタントおよびその老廃物が異常貯留し、両側肺にびまん性病変*がみられる。

症状・臨床所見

- 呼吸困難、咳、痰など。自己免疫性では約1／3が無症状。

検査・診断 ➡P151

胸部X線検査　胸部CT検査　気管支鏡検査　血清マーカー検査　肺生検　肺機能検査

- 胸部X線検査ではびまん性の浸潤影、CT検査でスリガラス様陰影などが認められる。
- 気管支肺胞洗浄による洗浄液（BALF）は、ミルク状（米のとぎ汁状）の外観を呈する。サイトスピン標本*では、好酸性無構造物と泡沫状マクロファージ*がみられる。
- 肺生検で、肺胞腔内の顆粒状無構造物の充満、泡沫状マクロファージの重塊がみられる。

治　療 ➡P151

肺胞洗浄療法　GM-CSF吸入療法

- 自己免疫性では生理食塩水で肺を洗浄する肺胞洗浄療法が標準的治療法。
- 一部の自己免疫性患者でGM-CSF吸入療法の有効性が報告されている。
- 続発性で原因疾患が判明している場合は原因疾患の治療が重要。

予　後

- 治療により予後は良好。自然軽快もある。

用語解説

肺サーファクタント
肺胞の表面張力を減少させ、吸気時に肺胞を広げる作用をもつ界面活性物質。肺胞は球形をしており球面に発生する表面張力が肺胞をつぶす方向にはたらくため、肺胞を広げるにはサーファクタントの役割が重要となる。

びまん性病変
病変が特定域に限定せず、規則性がなく広範囲に広がっている状態。

サイトスピン標本
体液や培養細胞などを遠心分離した標本。

マクロファージ
生体内に侵入した細菌などの異物を捕らえて飲み込み（貪食）、細胞内で異物を分解してその異物に抵抗するための免疫情報をTリンパ球に伝える。大食細胞、貪食細胞ともいう。結合組織内に広範囲に分布し、肝臓や脾臓、リンパ組織や肺などの臓器内にそれぞれの組織に特異的なマクロファージがある。

肺サーファクタント：pulmonary surfactant／肺胞：alveolus／自己免疫性：autoimmune／特発性：idiopathic／続発性：secondary／びまん性病変：diffuse lesion／浸潤影：infiltrative shadow／スリガラス様陰影：ground glass opacity [appearance] ／気管支肺胞洗浄液：bronchoalveolar lavage fluid (BALF) ／マクロファージ：macrophage／生理食塩水：saline

肺胞蛋白症（PAP）

誘因・原因

- 吸気時に肺胞を広げる役割をもつ生理活性物質である肺サーファクタントの生成または分解過程に障害が発生することで起こると考えられている。
- 障害原因により**遺伝性肺胞蛋白症**、**自己免疫性（特発性）肺胞蛋白症**、**二次性（続発性）肺胞蛋白症**に分類される。原因が不明な場合（**未分類肺胞蛋白症**）もある。

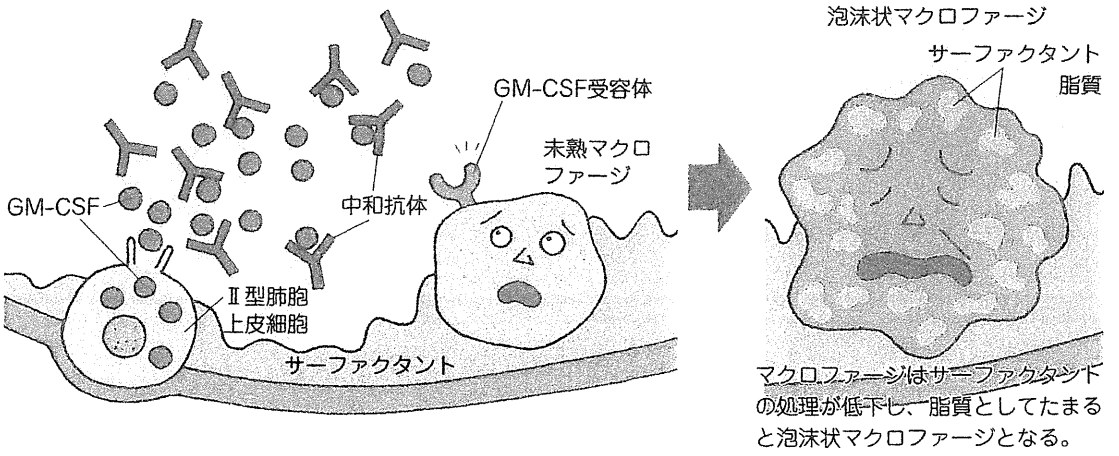
原因による分類

自己免疫性（特発性）肺胞蛋白症	肺胞マクロファージを活性化させる刺激因子（顆粒球マクロファージコロニー刺激因子〈GM-CSF〉）に対する抗体ができ、肺胞マクロファージがサーファクタントを処理できなくなるために起こる。肺胞蛋白症の9割を占める。
二次性（続発性）肺胞蛋白症	原因疾患により肺胞マクロファージのはたらきが低下することで生じる。原因疾患は、骨髄異形成症候群（MDS）、リンパ腫などの血液疾患、結核、非結核性抗酸菌症などの感染症のほか、骨髄性白血病、後天性免疫不全症候群（エイズ）、リジン尿性蛋白不耐症、ベーチェット病など。
遺伝性肺胞蛋白症	サーファクタントの生成または分解過程にかかわる遺伝子の異常により起こる。

病態生理

- 通常、サーファクタントは、Ⅱ型肺胞上皮細胞で合成され、肺胞マクロファージに取り込まれて分解される。肺胞マクロファージの活性化には、Ⅱ型肺胞上皮細胞から分泌される増殖因子である顆粒球（かりゅうきゅう）マクロファージコロニー刺激因子（GM-CSF）が必要である。
- 肺胞蛋白症の9割を占める自己免疫性（特発性）肺胞蛋白症では、顆粒球マクロファージコロニー刺激因子（GM-CSF）に対する中和抗体ができることから、肺胞マクロファージの分化成熟が障害され、サーファクタントを処理できなくなる。その結果、サーファクタントが肺胞内に貯留する現象が起こる（下図）。中和抗体ができる原因は不明。
- 二次性（続発性）では、顆粒球マクロファージコロニー刺激因子（GM-CSF）との関連は明らかではない。
- 遺伝性は、顆粒球マクロファージコロニー刺激因子（GM-CSF）の受容体欠損、サーファクタント蛋白BおよびC、ATP結合カセット輸送体A3の欠損などの遺伝子異常が知られている。

自己免疫性（特発性）肺胞蛋白症の発症機序



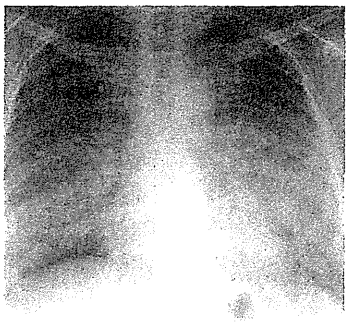
肺サーファクタント：pulmonary surfactant／マクロファージ：macrophage／顆粒球マクロファージコロニー刺激因子：granulocyte macrophage colony-stimulating factor（GM-CSF）／骨髄異形成症候群：myelodysplastic syndrome（MDS）／リンパ腫：lymphoma／結核：tuberculosis（TB）／非結核性抗酸菌症：nontuberculous mycobacterial infection／後天性免疫不全症候群：acquired immunodeficiency syndrome（AIDS）／リジン尿性蛋白不耐症：lysinuric protein intolerance／ベーチェット病：Behçet disease

検査・診断

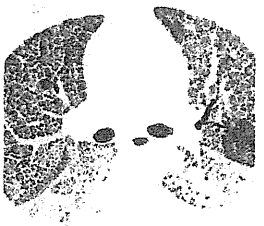
特徴的な検査所見

胸部X線検査	下肺優位のびまん性の浸潤影を認める	胸部CT検査	スリガラス様陰影、メロンの皮様の小葉間隔壁肥厚、小葉内網状影を認める
気管支鏡検査	気管支肺胞洗浄液（BALF）検査にて、ミルク状、米のとぎ汁状の乳白色に混濁した洗浄液を呈する	肺生検	肺胞壁の肥厚、肺胞腔内のサーファクタント充満、泡沫状マクロファージの散見が認められる
血清マーカー検査	自己免疫性では、血清中の抗GM-CSF自己抗体が陽性。血清抗GM-CSF自己抗体以外は続発性でも遺伝性でも高くなる	肺機能検査	Pao_2、%DLco（肺一酸化炭素拡散能力）が低下

- 胸部X線検査では、肺門を中心として両側肺にびまん性浸潤影が認められる。
- 自己免疫性では、CT検査で両肺野に地図状に分布したスリガラス様陰影がみられる。二次性では、均一に分布するスリガラス様陰影が多い。
- 小葉内網状影にともなうメロンの皮様所見（crazy paving appearance）が特徴的。



X線造影像
広範囲に認められるびまん性浸潤影。



HRCT像
小葉間隔壁肥厚、小葉内網状影によりメロンの皮様所見がみられる。

自己免疫性肺胞蛋白症の診断基準

1	肺高分解能CT（肺HRCT）で地図状のスリガラス様陰影を呈する。
2	肺生検または気管支肺胞洗浄液（BALF）が肺胞蛋白症に矛盾しない。
3	血清抗GM-CSF自己抗体が陽性。

治療

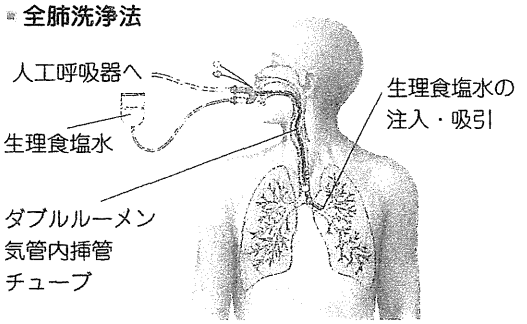
治療の目的

肺胞洗浄療法	生理食塩水を注入して肺胞を洗浄し、貯留したサーファクタントを除去する	GM-CSF吸入療法	自己免疫性（特発性）の場合に、GM-CSF（遺伝子組み換え型）を吸入して肺胞マクロファージの成熟を促す

肺胞洗浄療法

- 全身麻酔下でダブルルーメンの気管内チューブを用いて片側の肺ずつ洗浄する**全肺洗浄法**が標準治療である。
- 陰影がみられた部位まで気管支鏡を挿入して、肺胞を生理食塩水で繰り返し洗浄していくのが**反復区域洗浄**である。

全肺洗浄法



びまん性：diffuse／浸潤影：infiltrative shadow／小葉：lobule／網状影：reticular shadow／メロン皮様所見：crazy paving appearance／気管支肺胞洗浄液：bronchoalveolar lavage fluid（BALF）／生理食塩水：saline

各論18

リンパ脈管筋腫症 (lymphangioleiomyomatosis；LAM)

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1 概念

lymphangioleiomyomatosis（LAM）は1919年Lautenbacherらが TSC 患者で最初に報告し[1]，わが国においては，1970年，山中，斎木が「び慢性過誤腫性肺脈管筋腫症」として報告したのが最初である[2]．1995年，北市らはアジア地区の多数例の臨床像をまとめて報告した[3]．リンパ脈管筋腫症（LAM）は進行性全身性の難治性稀少疾患で妊娠可能年齢の女性に好発する．非遺伝性である孤発性LAM（sporadic LAM，S-LAM）と，常染色体優性遺伝の結節性硬化症（TSC）に伴うLAM（TSC-LAM）に分類される[4]．わが国では2008年，特定疾患治療研究事業として医療費助成制度の対象となった．

2 LAMの病態

LAMは平滑筋様のLAM細胞が，肺嚢胞壁，胸膜，細気管支，血管周囲，体軸リンパ節などで増殖し，組織を障害することが病態の基本である．1993年，16番染色体上の *TSC* 関連遺伝子 *TSC2* 遺伝子，1997年9番染色体上の *TSC1* の遺伝子が同定された．*TSC1* 遺伝子はハマルチン，*TSC2* 遺伝子はチュベリンをコードし複合体を形成し，セリン／スレオニンキナーゼで

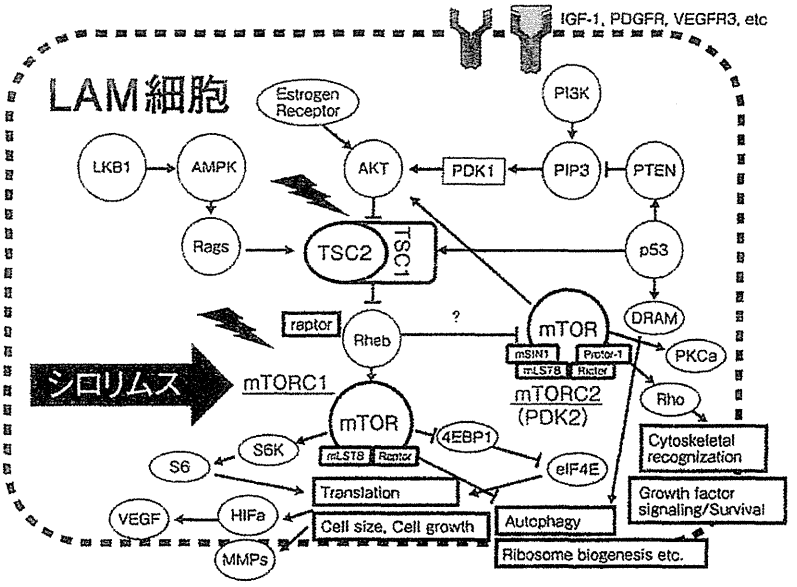

図1　LAM細胞増殖のメカニズム
TSC1，*TSC2* 遺伝子の異常からmTORの抑制が障害されている．シロリムス（ラパマイシン）はmTORを阻害する．

ある，哺乳類ラパマイシン標的タンパク質阻害剤（mTOR）の抑制することで，細胞増殖，血管形成，オートファジーなどを調節している．LAM細胞では*TSC1*，*TSC2*遺伝子の異常のためチュベリン，ハマルチンによるmTORの抑制が欠損し増殖する[4]．LAMは低悪性度の腫瘍性疾患として転移すると考えられている[5]．LAM細胞の増殖にはVEGF，bFGF，IGF，アンギオテンシンなどが関与すると考えられている．またLAM細胞はメタロプロテアーゼを産生し，組織を障害し，修復にも関与するといわれている（図1）[4,6,7]．

3 疫学

日本のLAM有病率は1.2～2.5人/百万人と考えられている[8]．米国では2.5/百万人，イギリスでは0.9/百万人，フランスでは1.3/百万人といわれている[4]．TSC-LAMはS-LAMの10倍程度と推測され[4]，わが国のLAM患者数は3,000～4,500人程度と推定する報告もある．本疾患は，通常生殖可能年齢の女性に発症するが，閉経後の女性でも診断されることがある．TSC-LAMは稀に男性にもみられる[8,9]．

4 症状，徴候

無症状であったのに，気胸で急性発症する場合，徐々に呼吸困難を認め慢性に発症する場合，肺外病変で発症する場合などがある．労作性呼吸困難，胸痛(気胸)，咳，痰，血痰，乳び胸水を認める．初期は無症状の場合も多い．胸郭外病変として，乳び腹水，後腹膜腔～骨盤腔のリンパ脈管筋腫や腎の血管筋脂肪腫（AML）に伴う腹部膨満感，腹痛・腹部違和感，下肢のリンパ浮腫，血尿を認める[9]．

5 検査所見

(1)胸部X線写真では，肺容量減少のない網状粒状影，すりガラス(様)陰影，肺過膨張，肺野の透過性亢進，血管影の減少，胸水貯留を認める（図2左）[9]．

(2)胸部CTでの判定にはHRCTが必要である．HRCTでは，両側性びまん性，散在性，比較的均等に，数mm～1cmの境界明瞭な薄壁嚢胞を認める．気胸，胸水，縦隔リンパ節腫大，胸管の拡張，小粒状影を認めることもある（図2右）[9,10]．

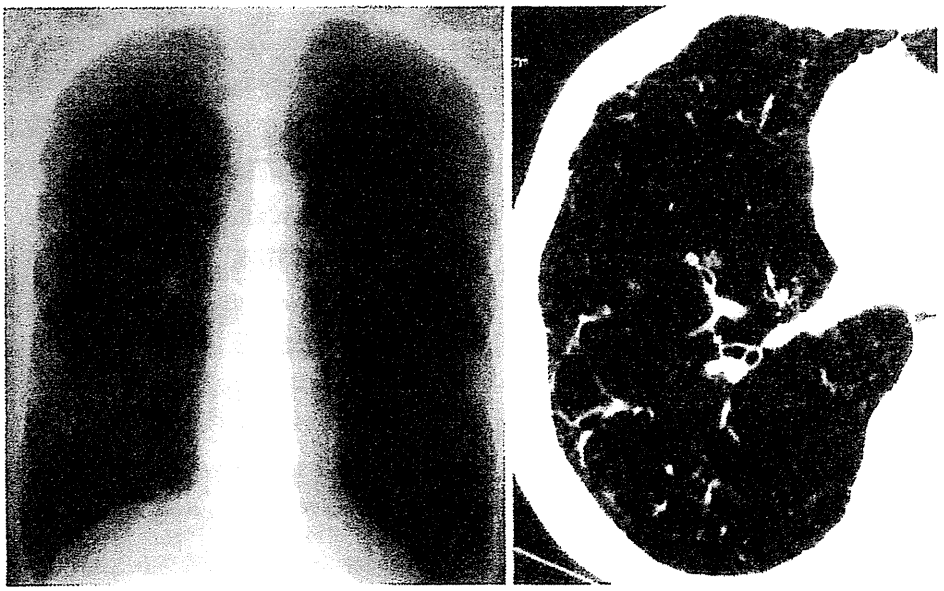

図2　LAM患者の胸部X線像(左)と胸部HRCT像
両側びまん性に網状影，過膨脹所見を認め(左)，嚢胞が多発している(右)．

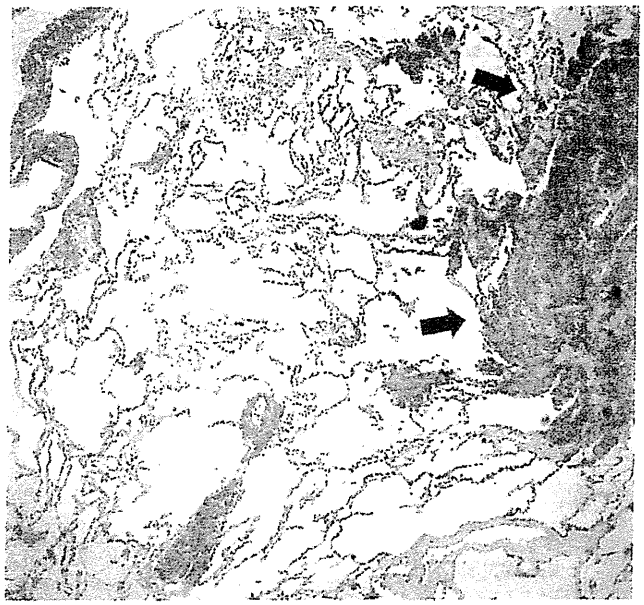

図3 LAM患者の外科的肺生検所見
（α-SMA免疫染色）
α-SMA陽性の平滑筋様細胞塊（矢印）を認める．

(3)呼吸機能検査では，$FEV_{1.0}$，$FEV_{1.0}$/FVC，DLcoの低下，RV，TLCの増加を認める．

(4)腹部骨盤部画像検査では腎，肝などのAML，後腹膜～骨盤腔のリンパ節腫大，腹水貯留を認める．

(5)血液・生化学的検査では，特異的所見に乏しい．研究用試薬であり測定は一般的ではないが，血清中のVEGF-Dが増加することが知られている[11]．

(6)病理組織学的所見では，肺，体軸リンパ節において，結節性にLAM細胞が増殖する（図3）．LAM細胞は，免疫染色で抗α-SMA抗体（図3），抗HMB45抗体，抗estrogen receptor（ER）抗体，抗progesterone receptor（PR）抗体陽性である．リンパ管の新生を伴うことがある．乳び胸水，腹水中にLAM細胞塊を認めることがあり診断に有用である[9]．

診断

2008年，厚生労働省「呼吸不全に関する調査研究班」から「LAM診断基準」[9]，2010年ヨーロッパ呼吸器学会から「LAMの診断と管理のガイドライン」が発行された[12]．呼吸不全に関する調査研究班による「LAM診断基準」は難病情報センターホームページ（http://www.nanbyou.or.jp/entry/339）からダウンロード可能である．喫煙歴のない若年女性で，肺気腫所見，気胸を認めた場合LAMの可能性を考慮する．LAMは，特異的肺外病変（腹部の骨盤部の腎AML，後腹膜骨盤腔のリンパ節腫大の画像診断，TSC）を含む特徴的臨床像，HRCTの胸部画像所見などが揃っている場合は，臨床診断も可能であるが，時に鑑別に苦慮する場合もある．わが国の診断基準では，病理による確定診断が推奨されている[9]．

ヘマトキシリンエオジン染色所見とHMB-45陽性所見で病理診断確実，HMB-45陰性の場合はα-SMA陽性とER陽性あるいはPR陽性で病理診断ほぼ確実と分類している[9]．図4に診断基準のアルゴリズムを示す．TSCの診断[13]は，難病情報センターホームページ（http://www.nanbyou.or.jp/entry/243）を参照のこと．

鑑別すべき疾患として，ブラ，ブレブ，慢性閉塞性肺疾患，ランゲルハンス細胞組織球症，シェーグレン症候群に伴う肺病変，リンパ球性間質性肺炎，嚢胞性肺病変を呈するアミロイドーシス，空洞形成性転移性肺腫瘍などの嚢胞性肺疾患，Birt-Hogg-Dube症候群があげられる[9]．

⑦ 治療，管理

LAMの治療は，基本的にはLAM細胞の増殖抑制とそれに伴う組織の破壊の対策が基本であるが，有効な根本的治療法はまだ証明されていない．適応あれば肺移植の対象疾患である．個体差が大きく，個々の患者の経過などを踏まえて利益と損失を考慮し治療方針をたてる．他分野（内科，移植外科，泌尿器科，婦人科，心療内科，理学療法，コメディカルなど）と連携し包括的な治療と管理が求められる（図5）[13-15]．

2008年，厚生労働省「呼吸不全に関する調査研究班」から「LAMの治療と管理の手引き」が発行され[14]，前述のヨーロッパ呼吸器学会「LAMの診断と管理のガイドライン」[12]が発行されている．LAMの進行は個々の患者で差が大きく，肺病変，肺外病変の進行に注意しながら個別的に治療，管理を行うことが必要である．以下は「LAMの治療と管理の手引き」を要約する[14]．

1) 気管支拡張剤として，チオトロピウム，サルメテロール，ツロブテロール，テオフィリンなどが用いられる．
2) ホルモン療法として抗エストロゲン療法が実施されているが，エビデンスは乏しく効果は個別的である．LAMの進行を確実に防止できる有効な治療法がなく，実施できる治療としてホルモン療法しかないため，患者が治療を望む場合，ホルモン療法を実施する．ゴナドトロピン放出ホルモンアゴニストとプロゲステロン療法が行われるがわが国で保険適応は無い．
3) 呼吸困難例，呼吸不全例では，呼吸リハビリテーション，適応あれば長期酸素療法を行う．
4) 経過を通じて70%程度の患者が気胸を生じる．繰り返す場合は，早い段階で外科的臓側胸膜補強術（被覆術），内科的胸膜癒着術，外科的胸膜癒着術などを考慮する．強力な胸膜癒着術は，肺移植術の際に出血，手術時間の延長の問題を生じる可能性があり，近年，外科的臓側胸膜補強術（被覆術）が好まれる傾向がある．
5) 乳び胸水を認める場合，低脂肪食，あるいは中鎖脂肪酸の補充と無脂肪食を行う．量が多く自覚症状が強い乳び胸水例は，胸膜癒着術も行われる．
6) リンパ浮腫を認める場合，弾性ストッキング，リンパマッサージを行う．
7) AMLについては腫瘍径に応じた治療管理基準がある．4 cm未満，自覚症状なければ年1回の画像検査，腫瘍径4～5 cm以上，自覚症状ない場合，6ヵ月ごとの画像チェックを行う．腫瘍径4～5 cm以上，自覚症状がある場合，腫瘍の塞栓術か外科的摘出術を考慮．泌尿器科，腎臓内科，消化器外科と連携する．
8) LAMは最終的には肺移植の対象疾患である．長期酸素療法が必要となる場合，肺移植を考慮する．しかしながら移植後，移植肺に再発することがあるといわれている．
9) LAMは若年女性に認められ，社会的，家庭内での損失や影響は大きい．病変は多臓器にわたり，精神社会的なケアを含め，多分野（内科，移植外科，泌尿器科，婦人科，心療内科，理学療法，コメディカルなど）が連携し，包括的な治療と管理が求められる[14, 15]．
10) 妊娠中に気胸，乳び胸腹水などのリスクがあるが，妊娠出産は必ずしも禁忌ではない．妊娠の可否は，LAMの病勢に及ぼす影響と，呼吸機能障害の程度を考慮し慎重に考える．妊娠，出産に関して，TSC-LAM患者では，遺伝相談を考慮する．また，呼吸器科医と産科医の両者によって観察されるべきである．
11) 経口避妊薬ピル，ホルモン補充療法など，エストロゲンを含む薬剤はLAMが悪化する事があり避けること．
12) 航空機での旅行は気胸悪化のリスクが

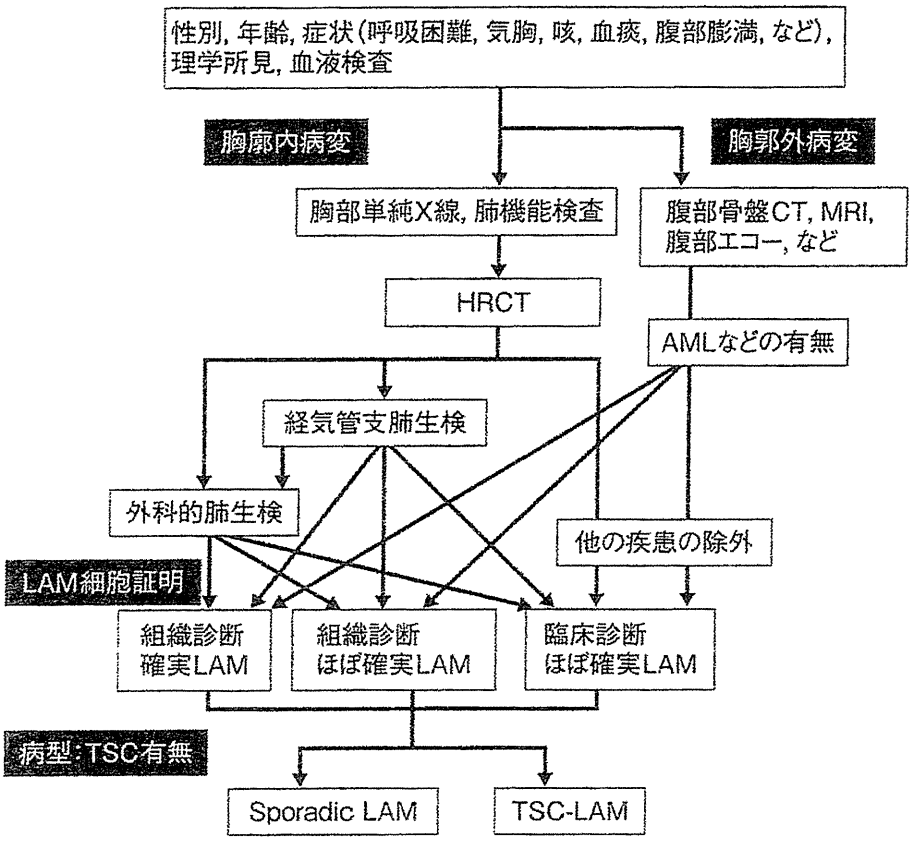

図4 LAM診断のアルゴリズム

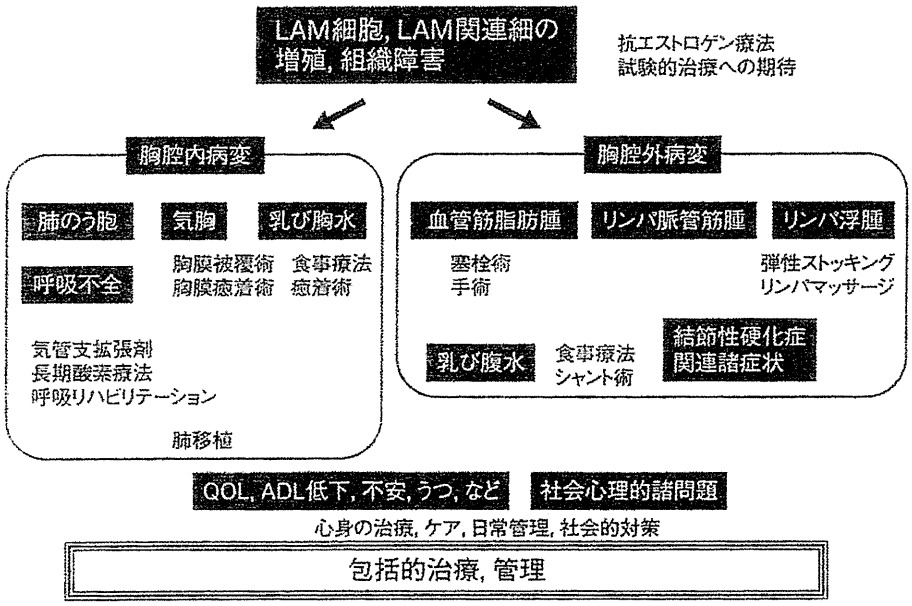

図5 LAMの病態に基づく治療管理

り，呼吸不全の患者では，機内の気圧低下により，搭乗中酸素吸入が必要となる可能性がある．

13）骨粗鬆症を認める場合はその治療を行う．

14）インフルエンザワクチン，肺炎球菌ワクチンは有用と考えられる．

⑧ 新たな試験的治療

Bissler らは，シロリムス（ラパマイシン）を用いて AML を標的として，「シンシナテイ血管筋脂肪腫シロリムス試験（CAST 試験）」を実施した．AML を有する 21 名の患者にシロリムスが投与され，AML は 40～60%に縮小した．また 12 例の患者で肺機能は改善し LAM に対する効果が期待されることとなった[16,17]．

CAST 試験の成果を受けて，シロリムスが LAM 患者の肺機能（$FEV_{1.0}$ の変化）に及ぼす影響を明らかにするため，リンパ脈管筋腫症に対するシロリムスの有効性に関する国際多施設共同試（MILES 試験）を実施した[18]．米国，カナダ，日本が参加し実施された．その結果，1ヵ月の $FEV_{1.0}$ の変化量は，シロリムス群でプラセボ群に対してシロリムス群で有意差が認められた（$P < 0.001$）．投与期間 1 年間の $FEV_{1.0}$ の変化量もシロリムス群でプラセボ群に対し有意に低かった．FVC の変化量もシロリムス群でプラセボ群に対し有意に高かった（$P = 0.001$）．血清 VEGF-D 濃度，健康関連の QOL のスコアなども投与期間中シロリムス群で改善した．シロリムスは LAM 患者の肺機能を安定化し，血清 VEGF-D 濃度を低下させ，そして症状が軽快して健康関連 QOL を改善した[18]．

⑨ 経過，予後

呼吸不全は慢性に進行するが，突発的に気胸，腹部症状の悪化が認められる．わが国の LAM 患者の 5 年生存率は 91%，10 年生存率は 76%である[8]．

⑩ 社会資源と患者支援

特定疾患として医療費補助の対象疾患である．基準を満たす呼吸機能障害の場合，身体障害制度を利用することができる．これら社会資源は基準を満たせば利用可能である．米国の患者会を中心に患者の輪が世界に広がっている．わが国でも患者会が設立され，患者への情報提供などを行っている．患者と医療関係者が一同に参加する勉強会（LAM 勉強会）を 2003 年から大阪と東京で年 1 回開催し，厚生労働省研究班の活動の一環として定着している．

⑪ 症例

症　例▶ 43 歳，女性．職業：特記事項なし．喫煙歴：なし

既往歴▶ 33 歳　右自然気胸，他院にてブラ多発指摘．胸膜湯着術施行．

家族歴▶既婚．息子，娘あり．正常分娩．

粉じん吸入歴▶特記事項なし．

現病歴▶ 38 歳頃から労作時呼吸困難を認めていたが放置していた．

43 歳頃から徐々に呼吸困難増強し当院外来受診した．HRCT にて LAM を疑われた．その後，左気胸のため緊急入院した．入院後，左胸腔持続ドレナージ施行．その後安定期に経気管支肺生検を施行したが明らかな LAM 細胞を認めなかった．退院時の動脈血液ガス pH 7.45，$PaCO_2$ 32.4Torr，PaO_2 86.1Torr（room air），肺機能 %VC 77.2%，%FVC 73.2%，$FEV_{1.0}$% 37.5%，%RV 150%，%DLco 38.0%．外科的肺生検は行わず臨床診断 LAM と考えた．ヒスロン（メドロキシプロゲステロン）を投与したが効果不十分のためその後中止．47 歳から長期酸素療法開始．50 歳左気胸のためドレナージ，胸膜癒着施行．プレレリン点鼻開始．53 歳肺移植登録．その後入退院を繰り返し呼吸不全徐々に悪化した．58 歳永眠．肺移植登録時の胸部 X 線写真，HRCT 像を図 2 に示す．

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PLOS ONE

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

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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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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

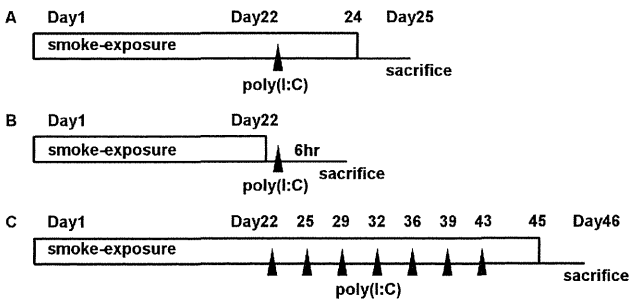

Figure 1. Time course of cigarette smoke exposure and poly(I:C) challenge. (A) Mice were exposed to cigarette smoke (CS) or air to day 24. Poly(I:C) or saline was challenged on day 22, and mice were sacrificed on day 25. (B) In CS-exposed mice, poly(I:C) was challenged on day 22. CS exposure was continued 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

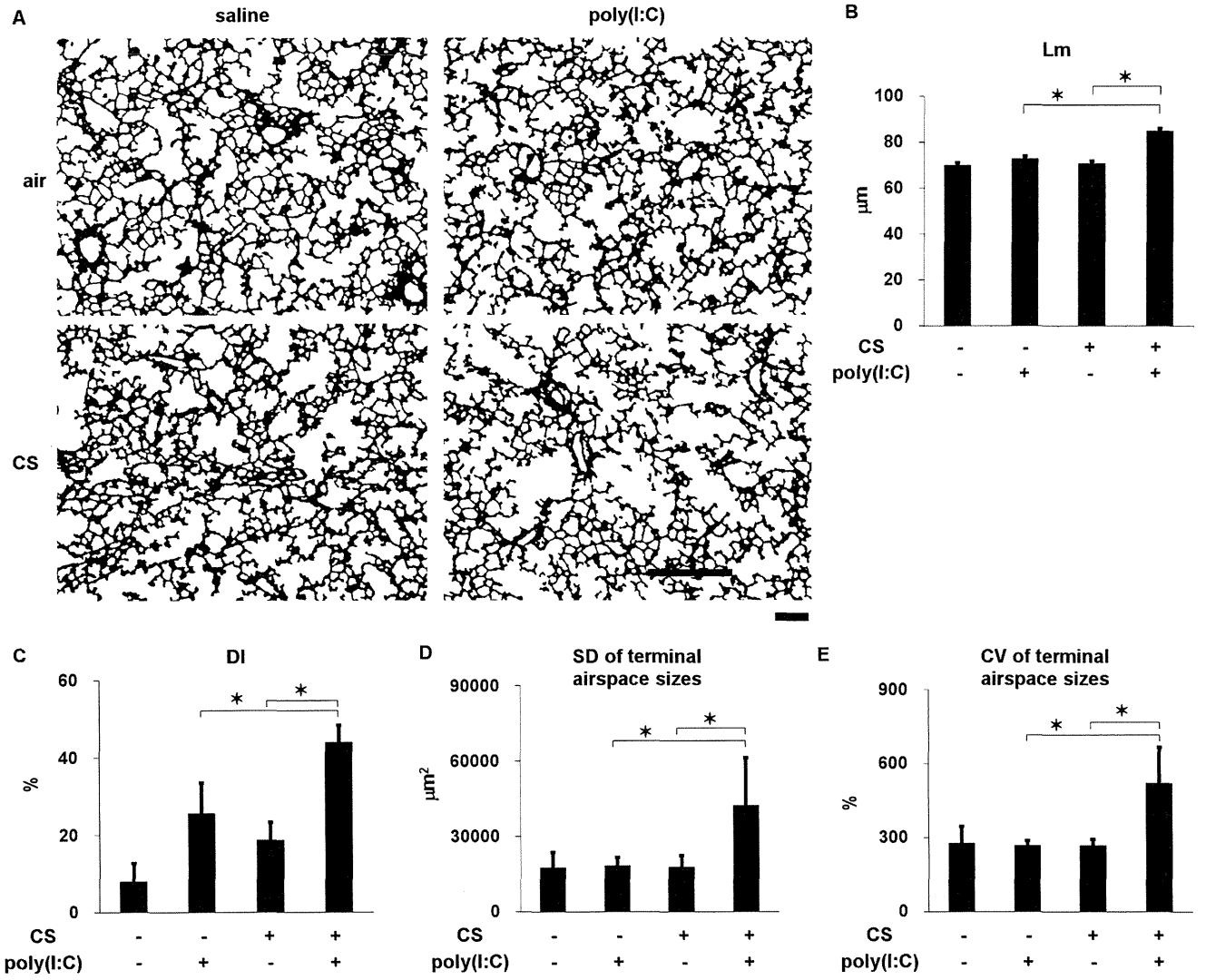

Figure 2. Lung morphometry in C57Bl/6 mice cigarette exposed to cigarette smoke or air and challenged with poly(I:C) or saline seven times. (A) Representative binary images of lung photomicrographs (×4). (B) Mean linear intercept (Lm). (C) Destructive index (DI). (D) Standard deviation (SD) and (E) coefficient 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).

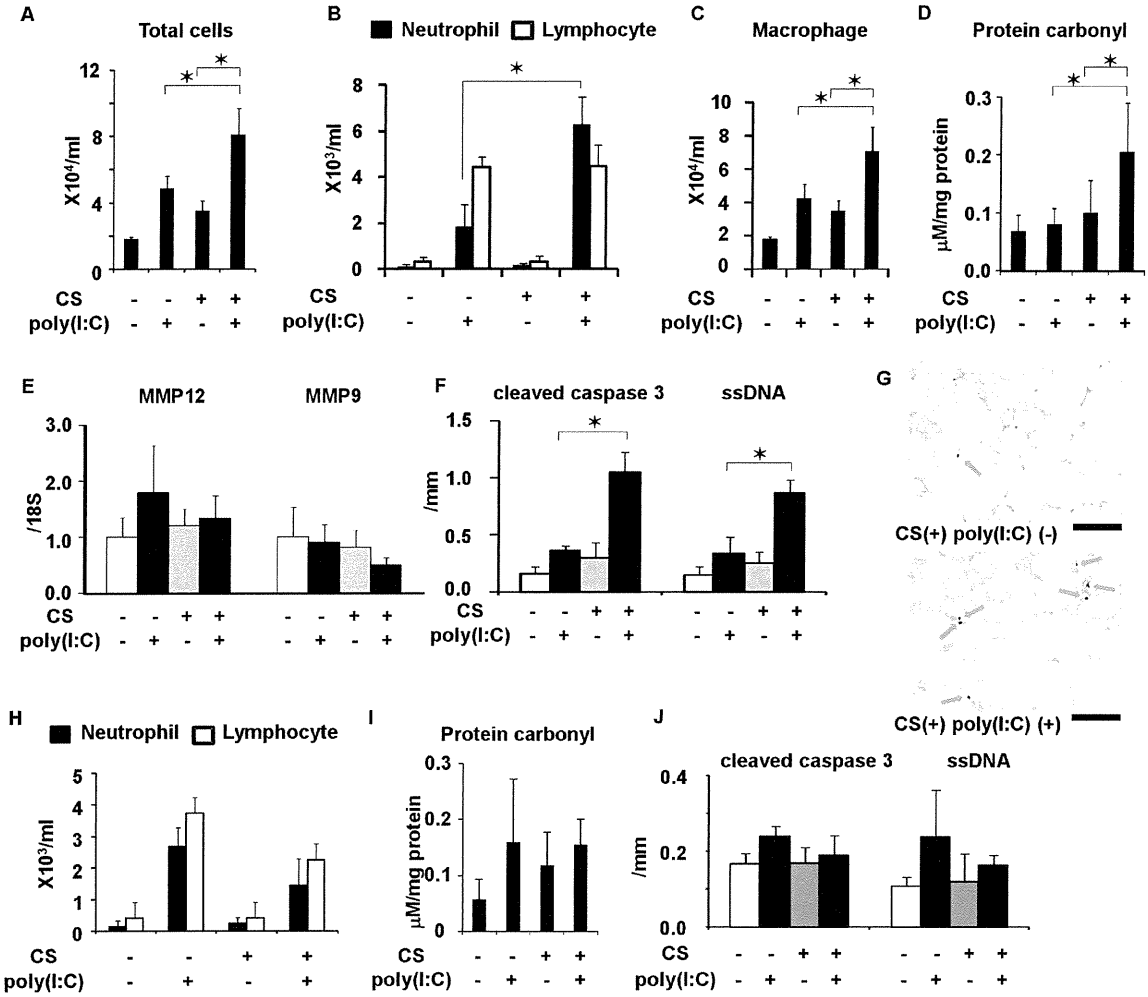

Figure 3. Comparison of impact of poly(I:C) between C57Bl/6 and NZW mice exposed to cigarette smoke. (A) Total cell counts, (B) neutrophil and lymphocyte counts, and (C) macrophage count, and (D) protein carbonyl in BALF. (E) mRNA expression of MMP-12 and MMP-9 in lung homogenates and (F) cleaved caspase 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.

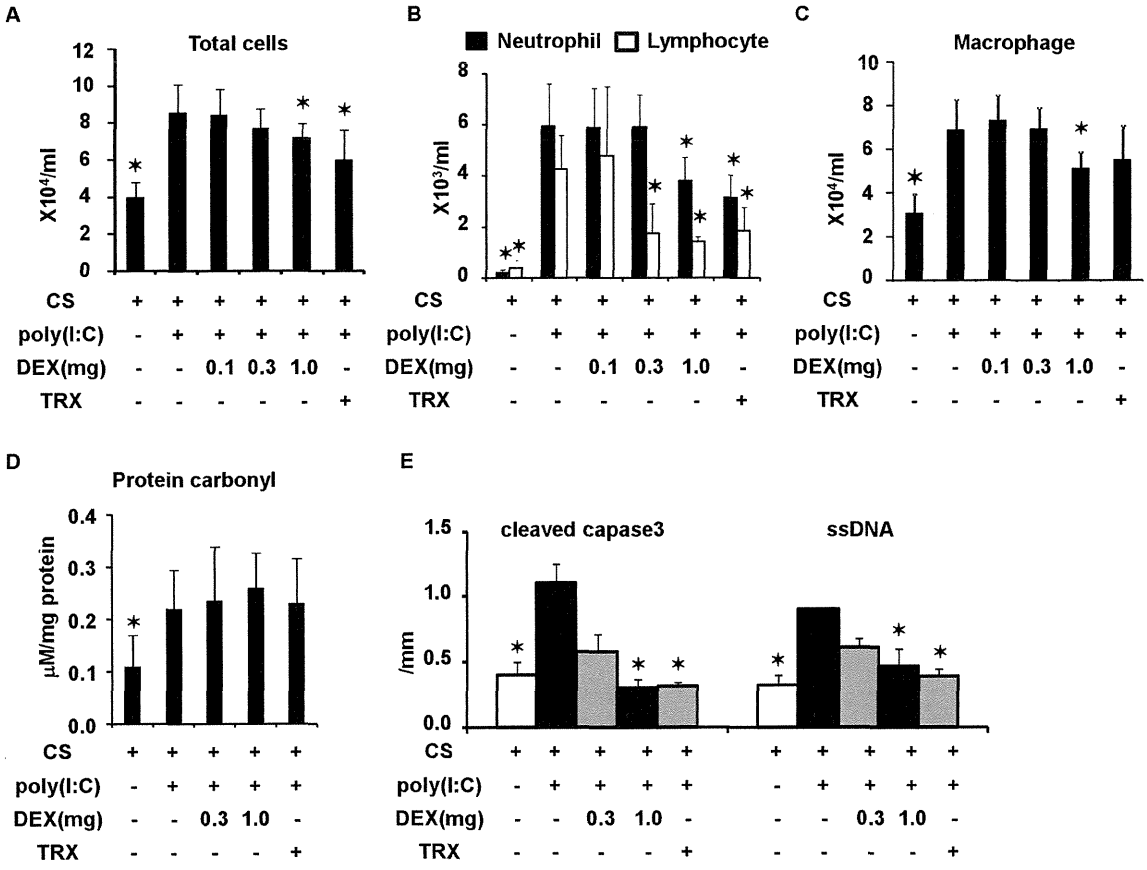

Figure 4. Effects of dexamethasone (DEX) at 0.1, 0.3, and 1 mg/kg and TRX in C57Bl/6 mice exposed to cigarette smoke and challenged with poly(I:C). (A) total cell counts, (B) neutrophil and lymphocyte counts, (C) macrophage count, and (D) protein carbonyl in BALF, and (E) cleaved caspase 3- and ssDNA-positive 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).

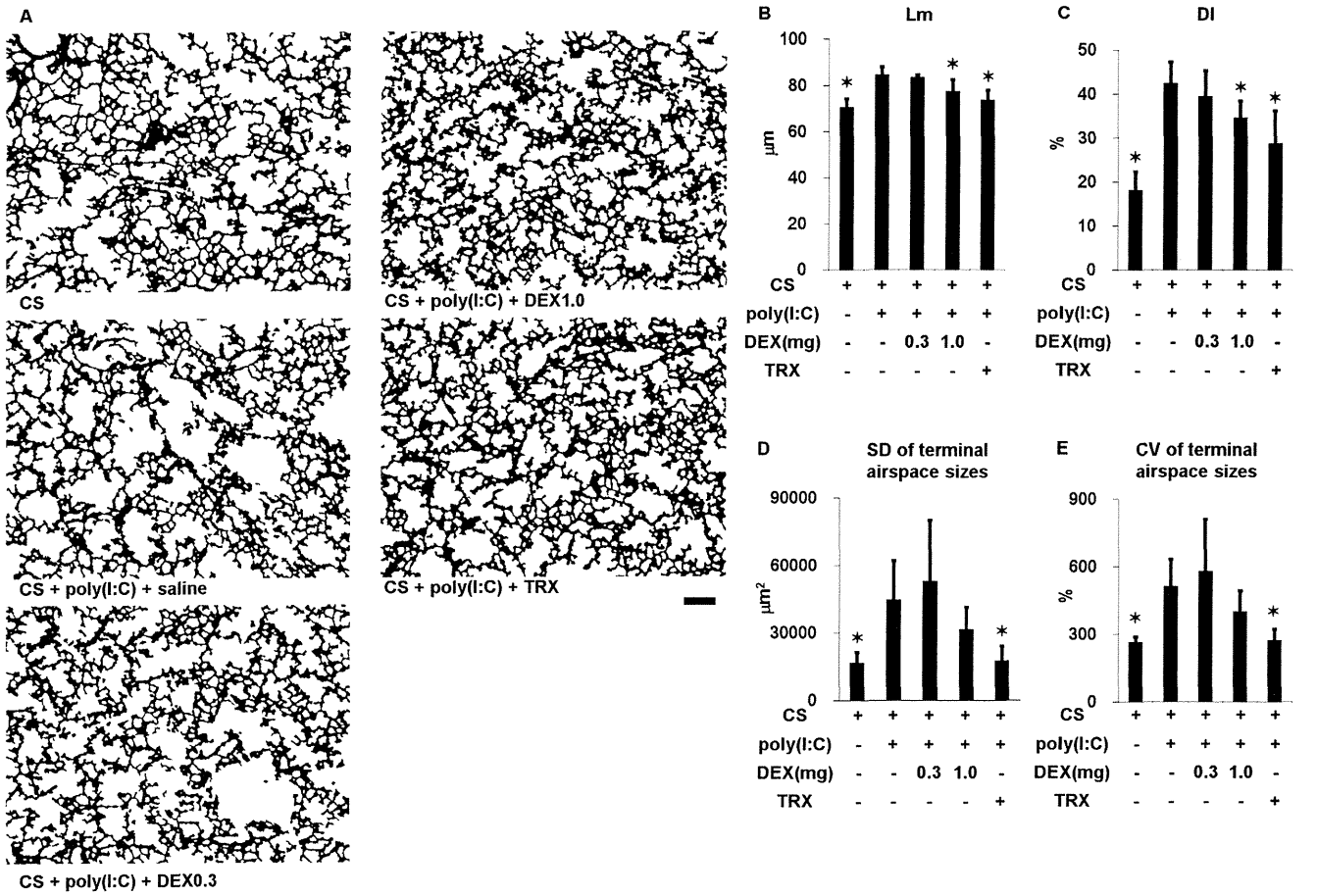

Figure 5. Lung morphometry in mice exposed to cigarette smoke and in those exposed to cigarette smoke, challenged with poly(I:C) and treated with dexamethasone (DEX) at 0.3 and 1 mg/kg, TRX or saline. (A) Representative binary images of lung photomicrographs (×4). (B) Mean linear intercept (Lm). (C) 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.

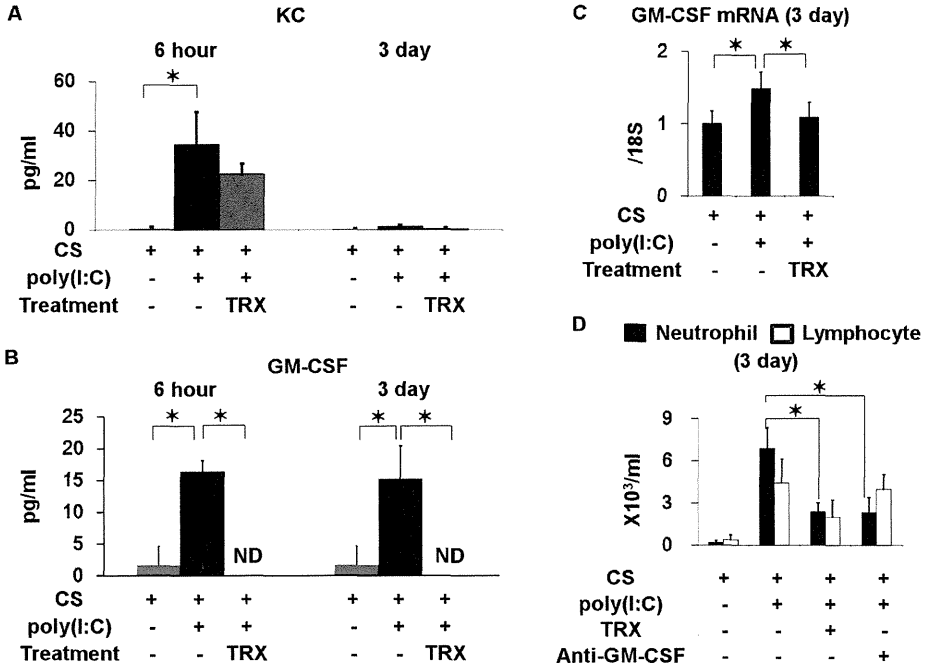

Figure 6. Effects of TRX on inflammatory cytokines in BALF from exposed to cigarette smoke and challenged with poly(I:C). (A) KC and (B) GM-CSF in BALF obtained 6 h and 3 days after poly(I:C) challenge. (C) Messenger RNA of GM-CSF in lung homogenates 3 days after poly(I:C) challenge in mice exposed to 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

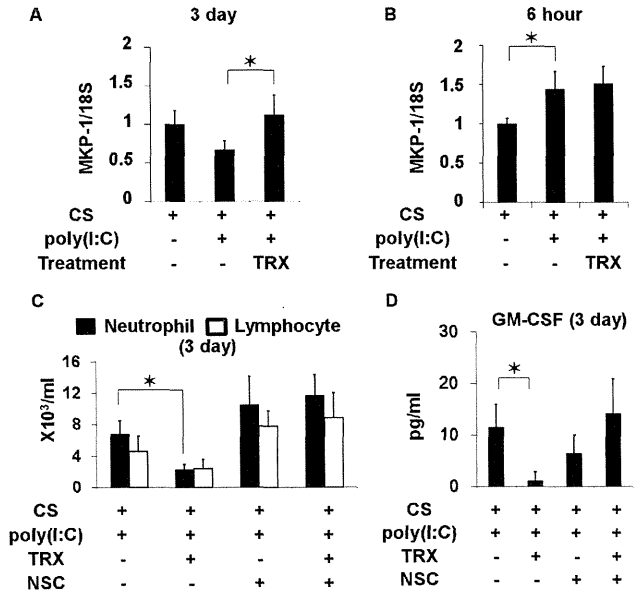

Figure 7. Pulmonary mRNA expression of MKP-1 in mice exposed to cigarette smoke then challenged with poly(I:C), and profiles of inflammatory cells and GM-CSF in BALF from mice treated with MKP-1 inhibitor. (A) MKP-1 mRNA in mice treated with TRX and saline at 3 days after poly(I:C) challenge. (B) MKP-1 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

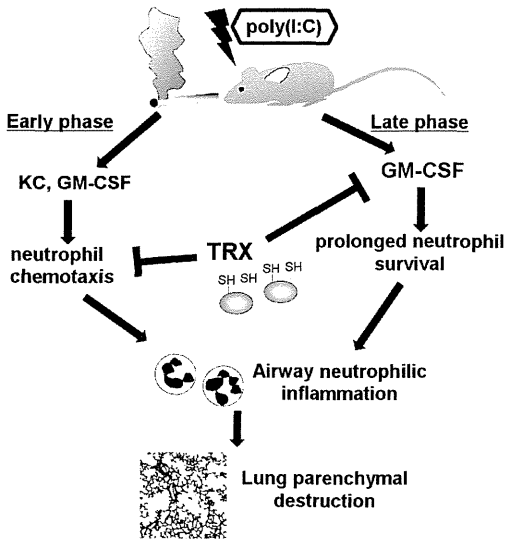

Figure 8. Estimated mechanism of dual regulation of poly(I:C)-induced neutrophilic inflammation by recombinant TRX in mouse lungs exposed to cigarette smoke. Poly(I:C)-induced neutrophilic inflammation consists of two phases. Poly(I:C) induces neutrophilic chemokines such as KC and GM-CSF that cause 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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