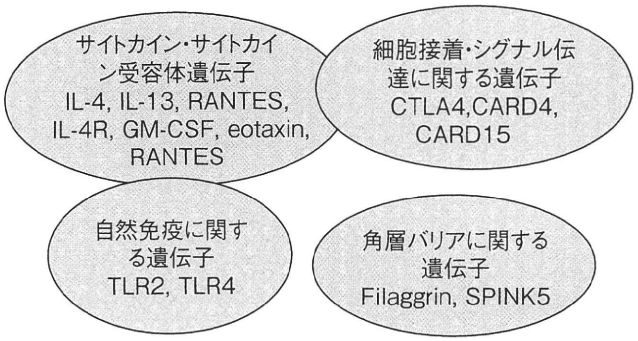

図1 アトピー性皮膚炎の発症に関与する主な候補遺伝子

アに関する遺伝子多型などが存在することが明らかにされている（図1）。筆者らは特にサイトカイン遺伝子多型について検討し，IL-12，IL-13，eotaxin などの遺伝子多型頻度で有意な差を認めている[2)〜4)]。また，自然免疫に関与する mannose binding lectin は，抗菌活性に関与するレクチンであるが，本邦では健常人との間に有意差はなかったが，海外の検討では有意差が報告されており，この例のように人種間でこれらの遺伝子多型頻度は異なる可能性がある[5)6)]。

アトピー性皮膚炎の発症は多因子遺伝子によるものであるが，これに対し皮膚炎の生じる疾患でひとつの遺伝子異常のみによって皮膚症状が生じる疾患も近年明らかにされている。高 IgE 症候群は，出生時より再発を繰り返す湿疹，重症ウイルス感染症，高 IgE 血症を生じる症候群であり，その原因遺伝子として Tyk2 遺伝子変異が報告された[7)]。これは Th1 細胞の分化を誘導する IL-12 の産生に関与する tyrosine kinase に遺伝子変異が生じるために，IL-12 のシグナルが正常に伝達されず，この結果 Th1 への分化が阻害され Th2 サイトカインバランスに傾くことによって，IgE の過剰産生を生じると説明される（図2）。また皮膚炎を生じる IPEX 症候群（immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome）の原因遺伝子としては，抑制性 T 細胞の FoxP3 遺伝子異常が明らかにされた[8)]。この疾患では Treg 細胞の IL-10 産生や granzyme 発現が抑制され自己反応性 T 細胞の機能が制御されないため，自己免疫疾患や皮膚炎を生じると考えられる。このような症候群とは異なり，アトピー性皮膚炎は多因子遺伝疾患でありいくつかの遺伝子異常がその発症に関与しているが，最近ではサイトカイン異常とともに角質のバリアに関する遺伝子異常が注目を浴びている。

表皮角化細胞はプロフィラグリンを産生し，プロフィラグリンより形成されるフィラグリンは，角質で天然保湿因子として作用し，皮膚のバリア機能の形成に重要である。尋常性魚鱗癬は乳幼児に発症し，四肢，体幹に角栓を伴い角質増生を生じる疾患である。尋常性魚鱗癬ではフィラグリン産生低下がみられるが，それに伴ってフィラグリンの遺伝子多型として，R510X 変異群，2282del4 変異群が報告されている。一方，アトピー性皮膚炎でも 18〜46％の患者でフィラグリン遺伝子の変異が存在することが報告されている。これまで日本人のアトピー性皮膚炎に特有な遺伝子多型が 8 型存在すること，またフィラグリン遺伝子変異群では，重症群では TEWL に代表される角質バリア機能の障害が高度であり，この群におけるバリア機能低下にフィラグリン遺伝子異常が関与することが報告された[9)]。バリア機能の低下によってアレルゲンの易侵入性が生じ，角層のセリンプロテアーゼの亢進，サイトカインネットワークの活性化が生じるのではないかと推測される。さらに Palmer はフィラグリン遺伝子変異群が，有意に喘息発症群に多く，喘息の発症とも相関することを報告した[10)11)]。この結果は，アトピー性皮膚炎の重症度がフィラグリン遺伝子多型と相関していること，また同時に喘息の発症が皮膚の乾燥と関与していることを示唆するデータであり，上皮組織における喘息などアレルギー疾患を説明できる可能性を示している。

❹ サイトカインの皮膚炎症における役割

アトピー性皮膚炎の発症に関わるサイトカインと抗原刺激などの環境因子との関係を，動物モデルを用いた実験から検証することができる。皮膚サイトカインを過剰産生し，皮膚炎を自然発症するマウスに，IL-18 遺伝子発現マウス，TSLP 遺伝子発現マウスなどがあり，これらは生後 2 ないし 6 週頃よりアトピー性皮膚炎を自然発症する[12)13)]。これらのマウスはいずれも SPF 環境下

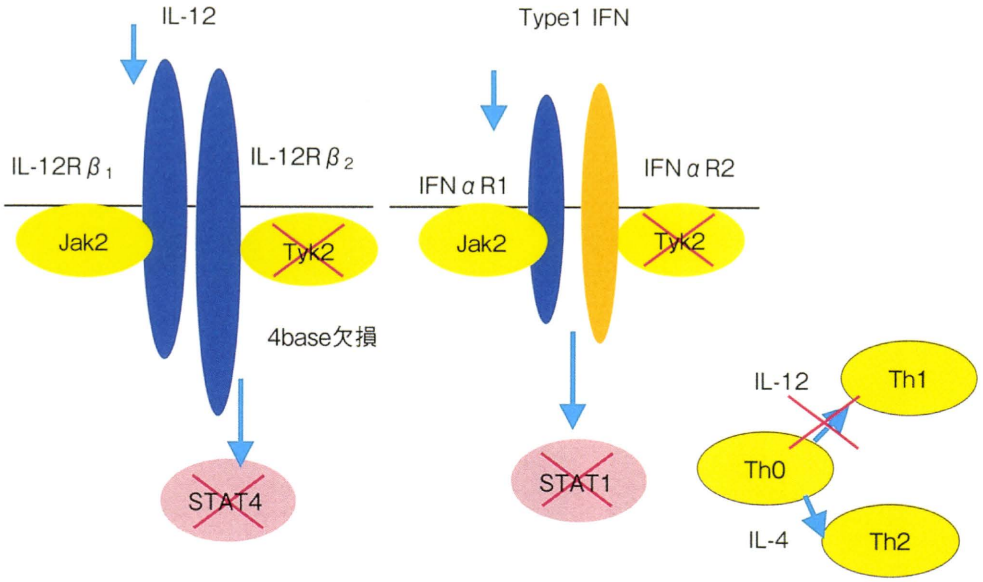

図2　高 IgE 症候群の原因遺伝子 Tyk2 遺伝子変異（文献 7）より）

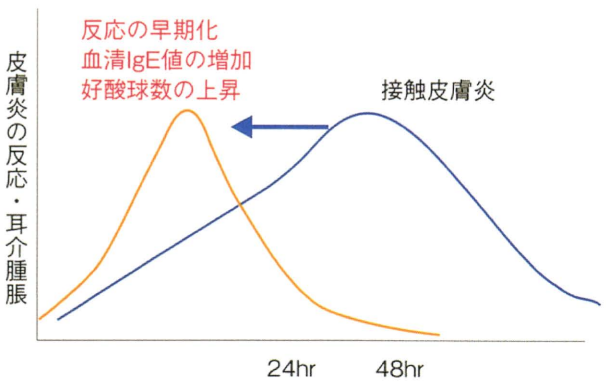

図3　アレルギーは環境で変わる
（反復する刺激で Th2 反応に変化する）（文献 14）より）

（環境抗原のない状態）においても顕著な皮膚炎を生じることから，サイトカインの産生異常が持続することよって，皮膚炎が自然発症すると考えられる。一方，これまで正常のマウスで抗原刺激を繰り返して惹起する接触皮膚炎モデルにおいて，抗原反復によって遅延型反応は次第に即時型反応に近づき，反応のピークが早期に現れること，Th2 細胞が皮膚に浸潤し血清 IgE 値が高値になることが報告されている[14]（図 3）。筆者らは，TARC を遺伝的に過剰産生するマウスを作成し，同様な反復抗原塗布による接触皮膚炎発症実験を行った。その結果，正常のマウスでみられる遅延型反応と比較し，顕著な肥満細胞浸潤を伴う皮膚炎の増悪と，血清 IgE 値の著明な増加を認めた（図 4）[15]。この現象は，TARC などの Th2 ケモカインを持続的に産生する背景において，ハプテンという環境抗原の負荷を繰り返すことによって，より顕著なアレルギー状態を誘導し，この皮膚炎の持続がさらにさまざまな Th2 反応を増強すること（肥満細胞浸潤や血清 IgE 値の上昇を示している）と考えられる。すなわちアトピー性皮膚炎においても，アレルギーに関与する遺伝的背景のもとに，皮膚炎が持続するという状態は，血清 IgE 値の上昇やアレルゲンに対する易感作性を生じ，次の免疫異常を誘導するのではないかと推測できる。

このことは，アトピー性皮膚炎患児の臨床経過を経年的に調査した研究からも明らかにされた。Fukiwake らは，石垣島において乳幼児のアトピー性皮膚炎患者について年齢別に 4 年間の経過を観察し，皮膚炎の推移と諸検査値の推移を検討した[16]。4 年間で皮膚炎を生じた群，自然治癒した群，アトピー性皮膚炎が持続した群，検査期間を通じて皮膚炎をまったく生じなかった群の 4 群に分類し，血清 IgE 値，TARC 値を比較した（図 5）。興味あることに，0 歳時に皮膚炎を生じ 4 年後にアトピー性皮膚炎が維持した群でのみ，血清 IgE が高値，血清 TARC 値が高値を示したが，一方他の群ではこれらの異常はなかった。このことは出生後よりアトピー性皮膚炎が持続することによって，血清 IgE 高値，血清 TARC 値の上昇が誘

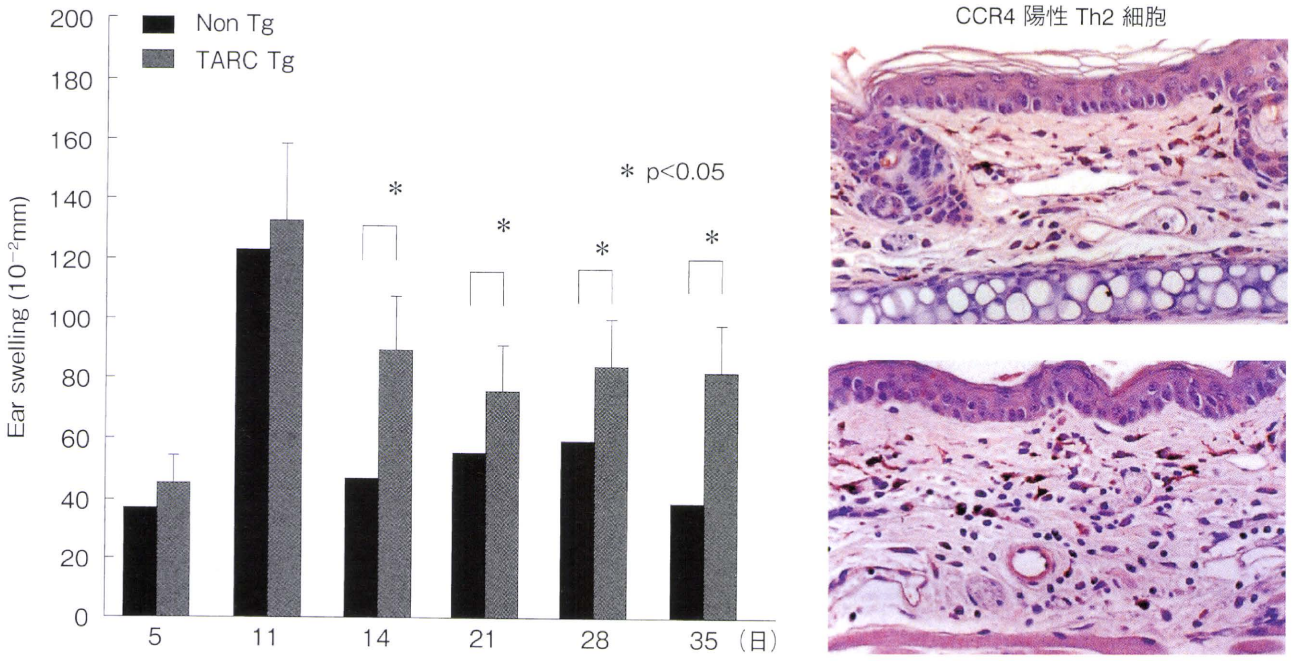

図4　慢性皮膚炎モデル（反復刺激）における TARC 遺伝子導入マウスの反応（文献 15)より）

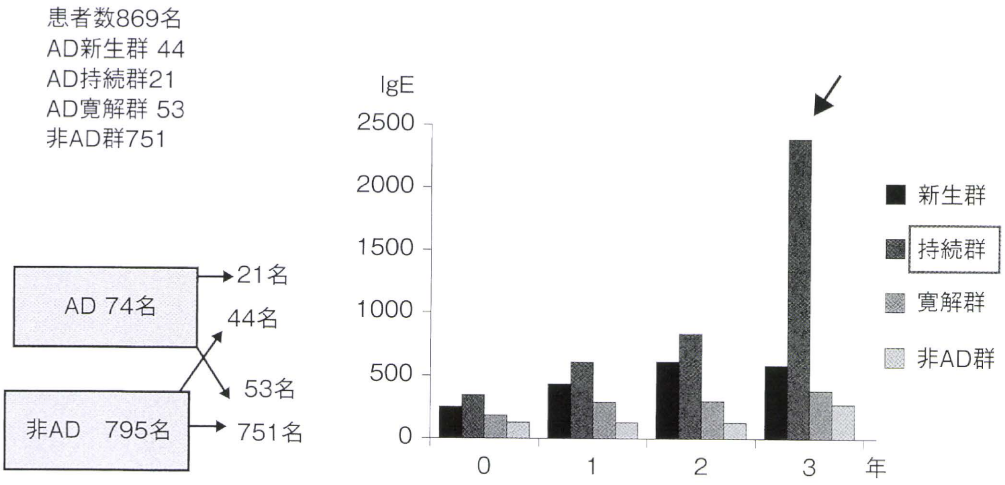

図5　アトピー性皮膚炎の持続群で血清 IgE 値が高値
［乳児 AD（1〜4 歳）：2001〜2004 年までの 3 年間の観察］（文献 16)より）

導され皮膚炎が遷延化する状態になったと考えられる。このように環境因子を反復して塗布する動物モデル，TARC を持続的に発現する動物モデルでの実験や，アトピー性皮膚炎患児での経時的推移と検査値を合わせて考えると，アトピー性皮膚炎の悪化の要因として，環境因子，発汗，ストレスなど生理的な悪化因子と同時に，アトピー性皮膚炎の長期間持続という状態がさらにアレルギー反応を増悪させるのではないかととらえることができる。このようにアトピー性皮膚炎の病態には遺伝因子の関与（必ず発現されるとは限らない）と環境因子による皮膚炎の増悪（個体によって異なる）が関与しているが，さらに皮膚炎の持続・局所的な皮膚の変化（免疫異常）が，次のアレルギー状態を誘導すると考えられる（図 5)。

❺ アトピー性皮膚炎の環境因子

アトピー性皮膚炎の発症・増悪には，上述した

ように遺伝的な関与が存在するが，さらにさまざまな環境因子が関与している。アトピー性皮膚炎は幼小児期に自然寛解例が多い一方，逆に思春期に発症や増悪する例があるなど個人差のある臨床経過を示す。また発疹の発症部位として幼小児期で肘・膝などに生じ，成人で顔面に苔癬化紅斑を生じるなど皮疹の部位が年齢とともに異なって変化する。このような臨床経過の推移には環境因子が大きく関与していると考えられる。

アトピー性皮膚炎の発症と環境因子を検討した臨床研究で，いくつかの環境因子の関与が明らかになっている。1歳6カ月男子（n=546）におけるアトピー性皮膚炎発症と環境因子を検討した研究から，①母のアトピー性皮膚炎歴罹患あり（オッズ比2.8），②4カ月でのアトピー性皮膚炎あり（オッズ比5.4），③1歳6カ月で保育所通園歴あり（オッズ比3.1）で，アトピー性皮膚炎の発症に有意な相関がみられる。また1歳6カ月の女子（n=477）では，①4カ月でのアトピー性皮膚炎あり（OR：7.1），②4カ月までの母乳（OR：2.3）との相関があり，また3歳児でのアトピー性皮膚炎の発症因子として，①家族でペットを飼育している家庭では発症頻度が低いことが示されている。これらの結果は，家庭においてさまざまな環境因子の関与を示しているが，生後4カ月以降で皮膚炎が持続しているという臨床経過については，男女ともに発症に関与しており，皮膚炎の持続がその発症の時期に関係していることを示す[17]。

⑥ アトピー性皮膚炎の治療

アトピー性皮膚炎の治療目標は，「症状がなく，あるいはあっても軽微であり，日常生活に支障がなく，薬物療法もあまり必要としない。あるいは軽微ないし軽度の症状は持続するも，急性に悪化することはない状態」である[18]。

上述したように，アトピー性皮膚炎の病態をサイトカインの制御からみた場合，皮膚炎が持続する状態は，アレルギー状態を悪化する方向にシフトしていくため，これらを長期にわたり悪化させずに維持していくことの重要性が理解できる。すなわち，悪化因子としての抗原への曝露が持続することによって，即時型反応が誘導されやすくなり，サイトカインレベルの上昇が次の炎症を誘導していくと考えられる。したがってアトピー性皮膚炎の治療は，これらの維持する皮膚炎をいかに制御していくかということが中心となり，ステロイド外用薬やタクロリムス軟膏による治療や，寛解時期において免疫調節薬の間歇的投与による予防的治療，スキンケアと掻破の予防としての抗ヒスタミン薬によるバリア機構を改善することが，治療の中心となる。悪化因子は個々において異なっており，乳児以降では，ダニなどの環境アレルゲン，ストレス，接触因子などが悪化因子となるためこれらの解明と除去が有効であり，少量の抗原による頻回の刺激を除去することは，皮膚炎の持続という点からは，局所的な皮膚の変化を予防する上で重要である。同時に，薬剤使用に関する患者教育や，治療の目標などの患者教育も重要である。さらに，治療の目標として「皮膚炎を悪化させない」ことを再確認し，症状の悪化時にのみ皮膚炎を鎮静化する（reactive therapy）と同時に，寛解維持期において皮膚炎を予防していく治療（proactive therapy）が主体となることが重要であろう（図6）。

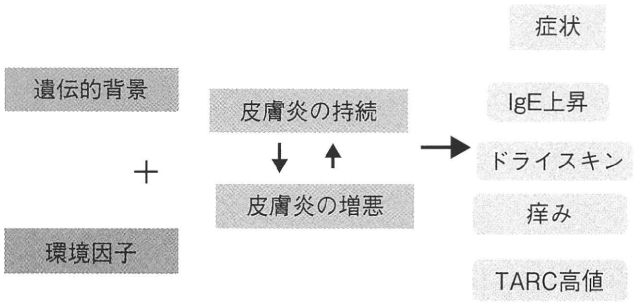

図6　アトピー性皮膚炎の病態

⑦ まとめ

遺伝子多型解析からみたアトピー性皮膚炎の病態，サイトカイン調節からみたアトピー性皮膚炎の病態について述べた。皮膚炎の持続する状態は，さまざまな皮膚構築（浸潤細胞など）の変化・リモデリングを誘導し，アレルギーを次の状態に悪化させる。このような変化を抑制・予防することが，寛解期におけるアトピー性皮膚炎の長期維持

に重要であり，急性増悪期における炎症の鎮静化と同様に重要である。患者・家族への外用薬や治療理解を深めることにより，長期寛解が可能となるであろう。また，将来的な治療ターゲットとして，アトピー性皮膚炎関連蛋白（転写因子・サイトカイン）制御，遺伝子発現と表現型の解析，候補遺伝子を有する患者に対する予防なども新たな研究対象として発展することが期待される。

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TARCの読み方・検査値

TARC(thymus and activation regulated chemokine)

中村晃一郎

Key words：TARC/CCL17，アトピー性皮膚炎，SCORAD

1. ケモカインについて

白血球が炎症部位に浸潤するためには，白血球が血管内皮細胞に接着するステップ，血管内皮細胞を通過するステップ，更にケモカインによって白血球が炎症局所に遊走集積する3つのステップが必要である(**図1**)．第1のステップでは白血球のインテグリン発現，第2のステップでは血管内皮細胞のICAM-1，ELAM-1発現が必要であり，第3のステップでは炎症部位から産生される白血球走化因子(ケモカイン)が必要である．ケモカインは白血球走化・活性化因子の総称であり，炎症反応の形成に不可欠である[1]．

ケモカインが様々な領域の炎症性疾患で重要であることは，動脈硬化症，多発性硬化症，糸球体腎炎などの報告からも明らかにされている．またアトピー性皮膚炎や乾癬などの炎症性皮膚疾患においてもケモカインの重要性が明らかにされている．例えばTh1細胞がその病態の主体を占める乾癬では，ケラチノサイトよりIL-8/CXCR8やIP-10/CXCR10が産生され，Th1細胞の浸潤や，好中球による角層下微小膿瘍の形成に関与している．

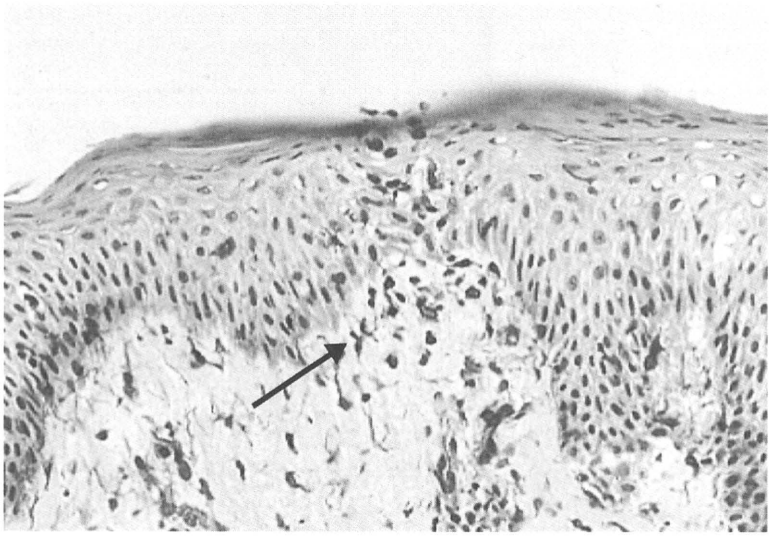

図1　アトピー性皮膚炎病変部におけるCCR4陽性細胞の浸潤
矢印：CCR4陽性細胞を示す．

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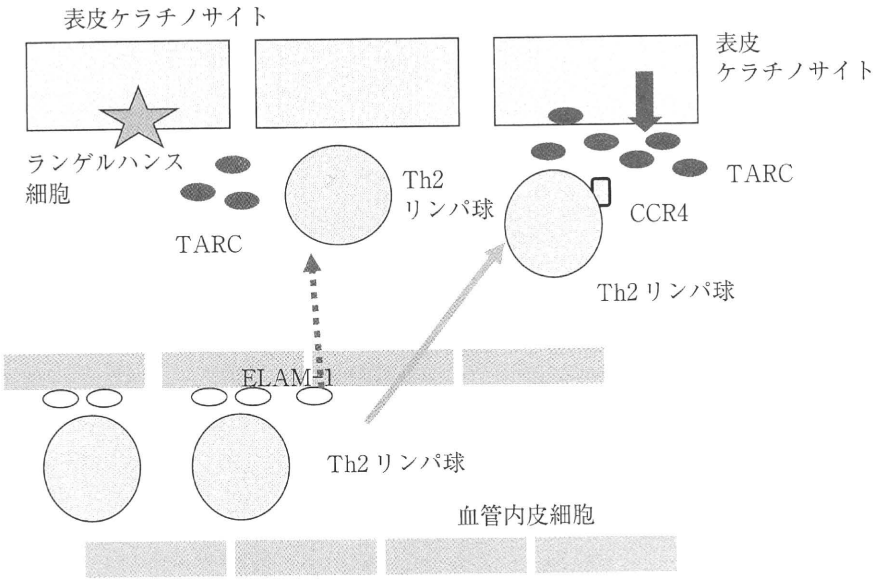

図2　炎症性皮膚組織におけるTARC産生と浸潤細胞
表皮ケラチノサイト，樹状細胞よりTARCが産生され，CCR4陽性T細胞が浸潤する．

2. TARC/CCL17について

ケモカインはその構造式によってCCおよびCXCの2種に分類される．TARC(thymus and activation regulated chemokine)/CCL17(以下，TARC)は，CCケモカインに属し，主にCCR4受容体を発現するT細胞に対して走化活性化作用を有する．著者らは，アトピー性皮膚炎のモデルマウスであるNc/Ngaマウスの病変部表皮ケラチノサイトでTARC発現が亢進し，同時に真皮にCCR4陽性T細胞が浸潤することを報告した[2]（**図2**）．更にアトピー性皮膚炎患者で血清TARC値は健常人に比べて高値を示していた[3,4]．

a. 健常人における血清TARC値

ヒト血清TARC値は，2009年に塩野義製薬により製品化されたTARC ELISAキット(アラポートTARC)で測定が可能である．アラポートキットによるTARC測定では，基準値を‘平均値＋1.96σ’とした場合，2歳以上(健常人)で平均値304 pg/mL，標準偏差値：224 pg/mL(基準値743 pg/mL)であった[5]．興味あることに新生児(健常児)の血清TARC値の基準値は高値であり，1歳未満の基準値は1,367 pg/mL，1歳から2歳未満で基準値998 pg/mLであった．このように健常人の血清TARC値は年齢に応じて変化し，新生児では高値を示し，健常人では成長とともにTARC値が減少すると考えられる．

表1　TARCの保険適用概要
（2008年7月1日より）

(1) 測定項目：ヒトTARC定量
(2) 測定目的：血清中のヒトTARC量の測定
　(アトピー性皮膚炎の重症度評価の補助)
(3) 保険点数：200点(月1回に限り算定)
(4) 免疫学的検査判断料：144点(月1回につき)

b. アトピー性皮膚炎における血清TARC値

アトピー性皮膚炎は，幼小児期に発症し，慢性に経過するアレルギー性皮膚疾患である．2009年アトピー性皮膚炎診療ガイドラインでは，鑑別疾患を考慮しながら診断を行い，重症度評価に基づいて治療方針が決定される．また重症度や病勢の評価のための検査値として，‘末梢血好酸球数，血清LDH値，TARC値が有用であり，特にTARCが病勢を鋭敏に反映する’と述べられている[6]．2008年7月より血清TARC値は‘アトピー性皮膚炎の重症度評価の補助’として保険適用の検査項目として使用できるようになった(**表1**)．

著者らの検討でも，成人アトピー性皮膚炎の

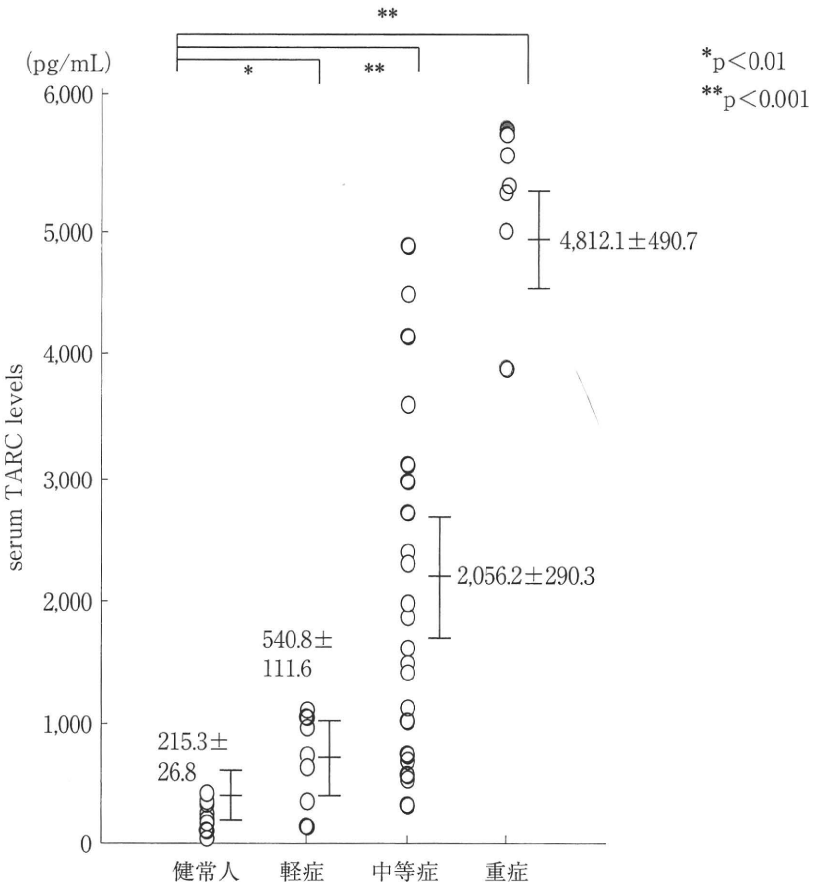

図3 アトピー性皮膚炎の重症度別血清TARC値(文献[3]より引用)
軽症，中等症，重症の順に血清TARC値は有意に上昇する．

血清TARC値は重症度と相関し，血清IgE値，末梢血好酸球数と正の相関を示した[3]．血清TARC値は，軽症群で平均540.8±111.6 pg/mL，中等症群で2,056.2±290.3 pg/mL，重症群で4,812.1±490.7 pg/mLであった(**図3**)．なお，血漿TARC値は血清TARC値と同様に重症度と正の相関を示すが，血清TARC値は，血小板より産生されるTARCを含むため，炎症細胞によって産生されるTARCの全体的な炎症を反映すると考えられる[7]．

アラポートTARCキットでアトピー性皮膚炎患者のTARC値を測定した大規模な検索では，軽症平均213±121 pg/mL，中等症503±422 pg/mL，重症1,363±1,388 pg/mLであり，SCORAD (SCORing Atopic Dermatitis)と正の相関を示した(**図4**)．血清TARC値，IgE値，LDH値の鋭敏性の比較検討では，TARC値，LDH値，好酸球数のカットオフ値をそれぞれTARC: 450 pg/mL，LDH: 200 U/L，好酸球数：6％に設定した場合，重症群のTARC値はカットオフ値の10倍となるのに対して，LDH値では重症群は1.7倍，末梢血好酸球数は2.0倍であり，このデータからTARCがより鋭敏なマーカーであることが示された．なお血清TARC値の軽症，中等症のカットオフ値は，700 pg/mLと考えられた．

海外の報告でも，血清TARC値がアトピー性皮膚炎の重症度と相関することが示されており，血清TARC値はSCORAD，皮疹の範囲，皮疹の強度と正の相関を示しているといえる[8]．

c. 治療における血清TARC値の推移

アトピー性皮膚炎の標準治療は，寛解導入期にステロイド外用薬を中心とした薬物療法であり，寛解維持期にスキンケア，原因悪化因子の除去が主体である．急性増悪期においてステロイド軟膏やタクロリムス軟膏などの免疫調節薬によって皮疹の改善を認める．これらの薬物

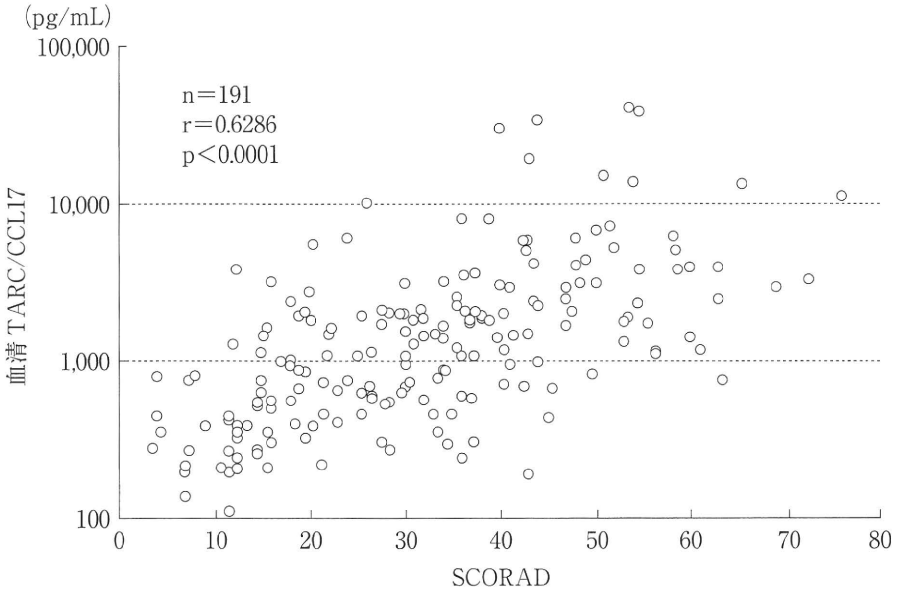

図4　アトピー性皮膚炎患者の血清TARC値と重症度SCORADとの相関
アトピー性皮膚炎患者の血清TARC値は重症度と有意に相関する．
（文献[7]より引用）

療法によって皮疹の改善した症例では，血清TARC値は速やかに減少することから，血清TARC値は，治療による皮疹の評価に優れていることが示される．また治療経過における血清TARC値の推移は，血清IgE値，末梢血好酸球数の変動に比較して大きな変動を示し，鋭敏であった．一方治療が奏効せず，皮疹が改善しなかった症例では，血清TARC値が高値を維持した（**図5**）[7]．このことから，血清TARC値は，治療経過による病勢を鋭敏に反映していることが理解され，治療の有効性を評価するうえでも有効なマーカーであると考えられる．

d. アトピー性皮膚炎における血清TARC値の推移とその産生細胞

新生児のアトピー性皮膚炎患者についてその臨床経過を経年的に追跡した興味深いデータがある．Fukiwakeらは，健常児，アトピー性皮膚炎の新生児を観察し，その皮疹の推移，血清TARC値の推移を検討した[9]．健常児，アトピー性皮膚炎患児いずれも新生児期に血清TARC値は高値を示したが，アトピー性皮膚炎患児で4年間の経過に自然軽快した患者群の血清TARC値は，経過とともに減少した（平均644.2 pg/mLより448.7 pg/mLに減少）．一方4年間の観察でアトピー性皮膚炎が持続している患児群の血清TARC値は，4年後にも高値を維持した（平均691.7 pg/mLより682.0 pg/mLに変動）．このことは血清TARCが高値を持続することによってアトピー性皮膚炎が遷延化する可能性を強く示唆している．

TARCの産生細胞としては表皮ケラチノサイトが関与しており，アトピー性皮膚炎の急性病変，慢性病変で表皮ケラチノサイトのTARC mRNA発現亢進が認められた[10]．このことは表皮ケラチノサイトがCCR4陽性細胞の遊走に関与しており，表皮から真皮への濃度勾配によってT細胞が浸潤する可能性を示している．なお著者らの検討ではアトピー性皮膚炎患者より採取した単球由来樹状細胞がTARCを産生し，*in vitro*で樹状細胞のTARC産生量は皮疹の重症度と相関していた[11]．皮膚病変において，表皮ケラチノサイト以外にランゲルハンス細胞や真皮樹状細胞（単球由来樹状細胞に相当する細胞）が病変部へのTh2陽性細胞の遊走にかかわっている可能性を示唆している[12]．また著者らは*in vitro*で抗アレルギー薬添加により末梢血単核球由来のTARC産生が抑制されることを明らかにしており，抗アレルギー薬が何らかのメカニ

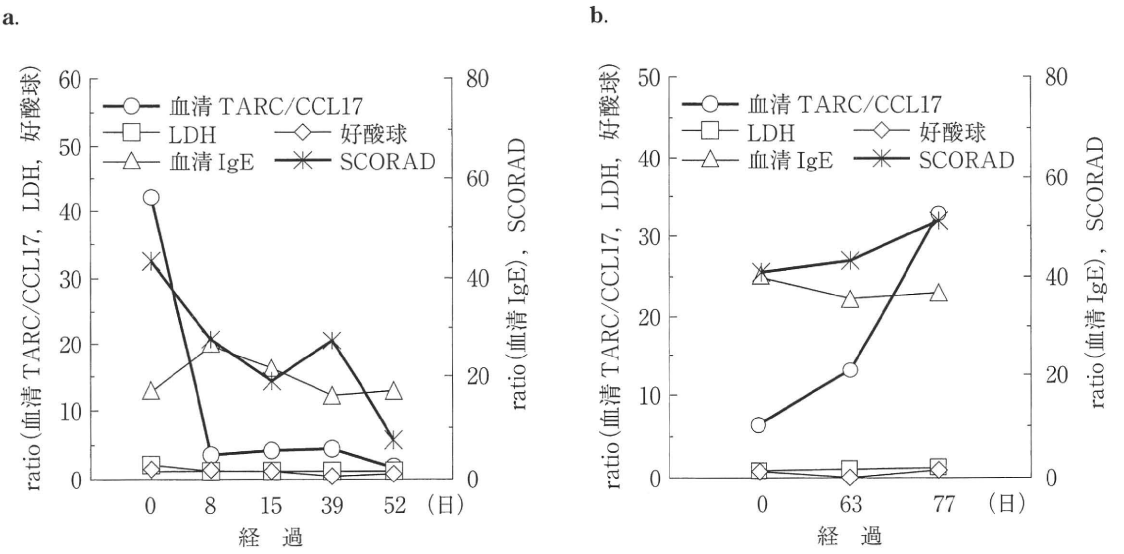

図5 治療前後での血清TARC値，LDH値，末梢血好酸球数の比較(文献[7]より引用)
a: 治療奏効例で，重症度スコア改善，血清TARC値の減少を認める．
b: 治療抵抗例で，重症度スコアの悪化，血清TARC値の増加を認める．

ズムによって末梢血の単核球のTARC産生を抑制しかゆみの制御に関与する可能性が考えられる[13]．

e. アトピー性皮膚炎における血清TARC値の意義

このように血清TARC値はアトピー性皮膚炎の病勢を極めてすばやく反映するマーカーである．血清TARC値のアトピー性皮膚炎における意義として，以下のことが考えられる．血清TARC測定は，①初診時のアトピー性皮膚炎の重症度評価として優れている．②臨床経過におけるアトピー性皮膚炎の病勢を評価することができる．③アトピー性皮膚炎と鑑別疾患との区別に用いることが可能である．④臨床的治療薬の有効性を判定することが可能である．⑤臨床診療において患者に検査値を提示することによって，外用薬や内服薬などの治療のアドヒアランスを向上することが期待できる．

以上のようにTARC値はアトピー性皮膚炎の病勢のマーカーであり，血清TARC値を治療中に活用することによって，治療の効果の指標となると考えられる．またTARC値を治療目標の指標としても活用することができ，診療における治療のアドヒアランスを上げるうえでも有意義であると考えられる．

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TSLP Promoter Polymorphisms are Associated with Susceptibility to Bronchial Asthma

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Running title: An association study of *TSLP* in bronchial asthma

This article has an online data supplement that is accessible from this issue's table of contents online at www.atsjournals.org.

AT A GLANCE COMMENTARY

TSLP initiates Th-2-mediated immune responses and plays crucial roles in allergic inflammation. The influence of genetic changes in this crucial cytokine on the etiology of asthma is unclear.

What This Study Adds to the Field

Our findings suggest that functionally *TSLP* polymorphisms contribute to the disease susceptibility of asthma. The susceptible functional allele might contribute to the Th2-polarized immunity in asthma during viral respiratory infections. We also found synergistic suppression of poly(I:C)-induced TSLP production by dexamethasone and salmeterol.

ABSTRACT

Rationale: Thymic stromal lymphopoietin (TSLP) triggers dendritic cell-mediated T helper (Th) 2 inflammatory responses. A single nucleotide polymorphism (SNP), rs3806933, in the promoter region of the *TSLP* gene creates a binding site for the transcription factor activating protein (AP)-1. The variant enhances AP-1 binding to the regulatory element and increases promoter-reporter activity of *TSLP* in response to poly(I:C) stimulation in normal human bronchial epithelium (NHBE).

Objectives: We investigated whether polymorphisms including the SNP rs3806933 could affect the susceptibility to and clinical phenotypes of bronchial asthma.

Methods: We selected three Tag SNPs and conducted association studies of the *TSLP* gene using two independent populations (639 childhood atopic asthma patients and 838 controls, and 641 adult asthma patients and 376 controls, respectively). We further examined effects of corticosteroids and a long-acting β_2-agonist (LABA) (salmeterol) on expression levels of the *TSLP* gene in response to poly(I:C) in NHBE.

Measurements and Main Results: We found promoter polymorphisms, rs3806933 and rs2289276, significantly associated with disease susceptibility in both childhood atopic and adult asthma. The functional SNP rs3806933 was associated with asthma (meta-analysis, P = 0.000056; odds ratio, 1.29; 95% confidence interval, 1.14-1.47). A genotype of rs2289278 was correlated with pulmonary function. We also found that the induction of *TSLP* mRNA and protein expression induced by poly(I:C) in NHBE was synergistically impaired by a corticosteroid and salmeterol.

Conclusions: *TSLP* variants are significantly associated with bronchial asthma and pulmonary function. Thus, TSLP might be a therapeutic target molecule for combination therapy.

Abstract: 244 words

The body of the manuscript: 3100 words

Key Words: asthma; TSLP; bronchial epithelial cells; combination therapy; genetic polymorphisms

Abbreviations: confidence interval, CI; linkage disequilibrium, LD; odds ratio, OR; single nucleotide polymorphism, SNP; T helper type, Th; untranslated region, UTR.

INTRODUCTION

Thymic stromal lymphoprotein (TSLP) is an epithelial cell-derived cytokine that triggers dendritic cell-mediated T helper (Th) 2 inflammatory responses and plays an important role in the initiation and maintenance of the allergic immune response (1-6). A recent study has shown that TSLP is

released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells (7). In humans, TSLP is highly expressed by airway epithelial cells during allergic inflammation (2), and the *TSLP* expression in asthmatic airways is correlated with both the expression of Th2-attracting chemokines and disease severity (3).

Large numbers of association studies on asthma and asthma-related phenotypes using genetic polymorphisms have been conducted in different populations (8). Recent studies have shown roles of human genetic polymorphisms of the *TSLP* gene. A variant in *TSLP* was associated with reductions in IgE in response to cockroaches and total IgE in a sex-stratified analysis (9). A functional SNP, rs3806933, has been identified in the regulatory element of *TSLP* (10). The variant creates a binding site for AP-1, and affects the transcriptional efficiency of the long form of *TSLP* induced by stimulation with poly(I:C) in bronchial epithelial cells (10).

The majority of exacerbations of asthma coincide with respiratory viral infections, most commonly by rhinoviruses (RVs) (11). DsRNA, a TLR3 ligand, is a potent stimulus for innate antiviral immune responses, and poly(I:C) is thought to mimic the effects of dsRNA (12). Inflammatory mediators IL-1β and TNF-α regulate human *TSLP* gene expression, and human *TSLP* mRNA levels increase after exposure to Toll-like receptor (TLR) 2, TLR3, TLR8, and TLR9 ligands in airway bronchial epithelial cells (13, 14). A suppressive effect of glucocorticoid on the expression of TSLP induced in airway epithelial cells by stimulation with the TLR3 ligand and Th2 cytokines has been reported (14). In addition, several clinical studies have shown that the combination of an inhaled corticosteroid and long-acting β-adrenergic agonist (LABA) is more efficacious in asthma than either alone and reduces exacerbations (15-18). We investigated the effects of dexamethasone (DEX) and salmeterol (SAL) on the induction of TSLP by poly(I:C).

In this study, we found that the promoter polymorphisms rs3806933 and rs2289276 were significantly associated with susceptibility to both childhood atopic and adult asthma using a case-control association study. We also found a significant correlation between lung function and the genotype of rs2289278. Functional analyses of the related variant rs2289276 were conducted. We further found that corticosteroids and LABA (salmeterol) synergistically suppressed the expression levels of *TSLP* mRNA and TSLP protein production induced by dsRNA in bronchial epithelial cells.

Some of the results of these studies have been previously reported in the form of an abstract (19).

METHODS

Additional details on methods are provided in the online supplement.

Study Subjects and Genotyping

All subjects with asthma were diagnosed according to the criteria of the National Institutes of Health (National Heart, Lung, and Blood Institute) as described (20-22). Data for forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), and FEV_1:FVC were available for subjects with adult asthma. The clinical parameters of the participants are summarized in Table 1. Genomic DNA was prepared in accordance with standard protocols. Genotyping was performed by the TaqMan allele-specific amplification

(TaqMan-ASA) method (Applied Biosystems, Foster City, CA). All individuals were recruited with written informed consent to participate in the study in accord with the rules of the process committee at the Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN).

Statistical Analysis

A total of 23 polymorphisms in the *TSLP* gene have been identified in the Japanese population (10). Pairwise linkage disequilibrium (LD) was calculated as D'/LOD and r^2 and three Tag SNPs, rs3806933, rs2289276 and rs2289278, were selected among seven SNPs with a frequency of greater than 10% by using the Haploview 4.1 program (http://www.broad.mit.edu/mpg/haploview/) (10) (Figure 1A). The functional promoter SNP rs3806933 had an allele-specific effect on expression through altering affinity for AP-1, and the SNP was in strong LD with rs2289276 (r^2 = 0.82) (Figure 1B). To test the association between *TSLP* variants and bronchial asthma, we conducted contingency chi-square test. We applied Bonferroni corrections, the multiplication of *P* values by three, the number of Tag SNPs. In the association study, corrected *P* values of less than 0.05 were judged to be significant. The Mantel-Haenszel method was used to combine allele frequency data sets. ORs with 95%CIs were also calculated. Haplotype frequencies for three loci were estimated, and haplotype association tests were performed using Haploview 4.1. We further investigated associations between asthma-related phenotypes. We examined associations between asthma-related phenotypes (eosinophil count, serum total IgE, lung functions and disease severity) and variants within patients with asthma as described (22, 23). Comparisons in mRNA expression analysis and protein expression analysis were performed with Student's t-test. Statistically significant differences in the luciferase assay were assessed with the Bonferroni-Dunn test with two-factor factorial analysis of variances (ANOVA). Statistical significance was defined at the standard 5% level.

Computational Analysis of Transcription Factor-Binding Sites and Biotinylated Oligonucleotide Precipitation Assay

TRANSFAC® Professional 10.3 (http://www.biobase.de/pages/) was used to predict putative transcription factor-binding sites under the minimizing condition of the sum of false positives and false negatives.

Biotinylated oligonucleotide precipitation assay was conducted as described (10). The oligonucleotides for the precipitation assay are listed below: -82C oligo, 5'-TGGCCCCTAAGGCAGGCCTTACAG-3'; -82T oligo, 5'-TGGCCTCTAAGGCAGGCCTTACAG -3'. AP-2α was detected by immunoblotting with an anti-AP-2α antibody (C-18; Santa Cruz Biotechnology, Inc.).

Quantitative Real-Time RT-PCR, Enzyme-Linked Immunosorbent Assay (ELISA) and Luciferase Assay

The expression of *TSLP* was determined by real-time quantitative RT-PCR using SYBR Premix Ex Taq (Takara, Shiga, Japan). TSLP in culture supernatants was measured using ELISA kits (R&D Systems Inc., Minneapolis, MN), and the reporter luciferase assays were conducted as described (10).

RESULTS

Identification of *TSLP* Polymorphisms and Haplotypes Associated with Asthma Susceptibility

All genotype and allele frequencies are shown in Table 2, and those for the control and asthma groups were in Hardy-Weinberg equilibrium. We found that the functional SNP rs3806933 was associated with childhood atopic asthma (P = 0.0063; odds ratio, 1.25; 95% confidence interval, 1.07-1.47) and adult asthma (P = 0.0023; odds ratio, 1.37; 95% confidence interval, 1.12-1.67). Another SNP, rs2289276, was also significantly associated with childhood atopic asthma (P = 0.00066; odds ratio, 1.33; 95% confidence interval, 1.13-1.57) under allelic model (Table 2). The directions of associations of the two SNPs were similar in both of the populations. We combined the results using Mantel-Haenszel meta-analysis, and observed the most significant association at rs3806933 (meta-analysis, P = 0.000056; odds ratio, 1.29; 95% confidence interval, 1.14-1.47) (Table 2). We also found a significant association under dominant model at rs3806933 (meta-analysis, P = 0.00013; odds ratio, 1.37; 95% confidence interval, 1.16-1.62) and at rs2289276 (meta-analysis, P = 0.00019; odds ratio, 1.36; 95% confidence interval, 1.16-1.61) (Table 2). We further conducted an association study using the adult asthma cases that were diagnosed in childhood (see Table E1 in the online data supplement). We confirmed a significant association between rs3806933 and adult asthma diagnosed in childhood with a similar direction of association. A recent study has shown a female-specific association between a variant of the *TSLP* gene and total serum IgE and IgE to cockroach (9). We further conducted a sex-stratified analysis, however we could not determine the female-specific effect on the associations. Although we surveyed associations between the three SNPs and asthmatic patients who had high eosinophil counts, high serum IgE levels and disease severity, we could not find any association. However, the rs2289278 C allele was significantly correlated with decreased FEV_1:FVC in adult asthma (P = 0.00021 by the Jonckheere-Terpstra test) (Figure 2). We next constructed the haplotypes of the three SNPs and estimated the frequency of each haplotype in controls and those with bronchial asthma (Table E2). We identified three common haplotypes in the population. Haplotype T-T-C of *TSLP* was significantly associated with childhood atopic asthma and adult asthma. We obtained P values of 0.00070 and 0.032, respectively, by using the Haploview 4.1 program.

To test the generalizability of our findings in other ethnic populations, we compared our results with two recent genome-wide association studies (GWAS) of asthma (24, 25). Markers rs3806932, rs2289276, and rs11466741 were included in the Illumina panels used in the studies. Marker rs3806933 was in complete LD with rs3806932 (D' = 1.00 and r^2 = 1.00), and rs2289276 was in complete LD with rs11466741 (D' = 1.00 and r^2 = 1.00) in a Japanese population (10). However, we could not replicate the association between these *TSLP* variants and asthma in the African- and European-ancestry samples (Table E3).

Transcription factor binding to the rs2289276 SNP

We predicted a potential allelic difference in cis-acting regulatory function in transcription via a bioinformatics approach, and rs2289276

(-82C/T) was found to possibly alter the affinity of a transcription factor, AP-2α, between two alleles (Figure 3A). The sequence containing the -82C SNP on the protective allele corresponded to the putative binding site to AP-2α, a possible transcription suppression factor (26). We next examined the binding of AP-2α protein to the sequences containing the -82C/T SNP, and binding was clearly detectable in both oligonucleotides without stimulation (Figure 3B). The binding ability of -82T was diminished compared with that of -82C, regardless of poly(I:C) stimulation, suggesting that the higher AP-2α binding to -82C (on the protective allele) might have reduced its transcriptional activity through repressive effects on the transcription.

The Induction of *TSLP* mRNA and Protein Expression Induced by Poly(I:C) in NHBE is Synergistically Impaired by a Corticosteroid and Salmeterol

We investigated the effects of dexamethasone (DEX) and salmeterol (SAL) on the induction of *TSLP* by poly(I:C). We first confirmed, by ELISA, the capability of DEX to suppress poly(I:C)-induced TSLP protein production in normal human bronchial epithelial (NHBE) cells. The addition of DEX 0.5 h before or simultaneously with poly(I:C) stimulation dramatically reduced TSLP production in a dose-dependent manner (Figure E1). Even if DEX was added to the NHBE medium at 1 h after poly(I:C) stimulation, TSLP production was also significantly impaired (more than 50% suppression) (Figure E1). Next we investigated effects of SAL on expression of the *TSLP* gene and TSLP protein production. NHBE cells were stimulated with or without poly(I:C) for 4 h. DEX (1 μM) and/or SAL (1 μM) was added to medium 0.5 h before the stimulation with poly(I:C). Relative expression of *TSLP* in NHBE was normalized with *GAPDH* expression. The *TSLP* mRNA expression was decreased by DEX and/or SAL. The greatest suppressive effect was observed by concurrent exposure of the cells to DEX and SAL (Figure 4A). Expression data are representative of three independent experiments, and similar results were obtained using NHBE cells from three individuals.

We also measured the protein levels of TSLP under the same conditions. NHBE were stimulated with or without poly(I:C), and the concentrations of TSLP in supernatants 24 h after stimulation were measured by ELISA. DEX and SAL were added to the medium 0.5 h before the stimulation with poly(I:C) at the indicated doses. Synergistic suppression of TSLP protein production was also observed by concurrent exposure to DEX and SAL, and DEX and/or SAL caused a dose-dependent decrease in TSLP protein production (Figure 4B). Data represent mean ± SD. Data are representative of two independent experiments performed using NHBE cells from two individuals, and similar results were obtained. These results implied that DEX and SAL might synergistically suppress TSLP production in human airway epithelial cells during viral respiratory infections.

Effects of Dexamethasone and Salmeterol on Luciferase Transcription

Rs3806932 (-1914T/G) was in complete LD with rs3806933 (-847C/T) (D' = 1.00 and r^2 = 1.00) (10). The promoter activity of reporter constructs containing the -1914G-847T-82T (minor) haplotype showed significantly greater activity than the other haplotype,

-1914A-847C-82C (major) in response to poly(I:C) as described (10). We here examined the effects of DEX and SAL on the promoter activities of these reporter constructs (Figure 5A). DEX and SAL suppressed reporter activities and their synergistic suppression of luciferase activities was also observed by concurrent exposure to DEX and SAL in both of the haplotypes (Figure 5B). However, there was no difference in the luciferase activities between the major and minor haplotypes under concurrent exposure to DEX and SAL ($P = 0.096$ by the Bonferroni-Dunn test with two-factor factorial ANOVA).

DISCUSSION

We have reported that a functional promoter SNP (rs3806933) of *TSLP* that creates a binding site for the transcription factor AP-1 enhances AP-1 binding to the regulatory element (10). The functional variant also increases promoter-reporter activity of long-form *TSLP* in response to poly(I:C) stimulation in NHBE(10). In this study, we found that the *TSLP* functional polymorphism was associated with both childhood atopic and adult asthma in a Japanese population. There is a synergistic relationship between viral infection and allergen sensitization and exposure in provoking exacerbations and the major trigger for exacerbations in both children and adults is viral infection (27-29). Clinical studies suggest that asthmatics are more susceptible to rhinovirus infections than normal individuals and have longer duration of lower respiratory tract symptoms when infected with rhinoviruses (30-32). TSLP appears to be involved in the development of bronchial asthma through functional genetic polymorphisms that might contribute to Th2-polarized immunity through higher TSLP production by bronchial epithelial cells in response to viral respiratory infections.

A recent study has shown that *TSLP* expression is increased in asthmatic airways and correlates with lung function (3). The numbers of both epithelial and submucosal cells expressing *TSLP* mRNA correlated inversely with FEV_1 (3). We found a significant correlation between the rs2289278 genotype and lung function (FEV_1:FVC). The rs2289278 variant is located in intron 2 of long-form *TSLP* and the 5' untranslated region of short-form *TSLP*. However, the genetic influences of the polymorphism on the function of the *TSLP* gene in asthma are unclear, and further investigation of the functional role of the variant needs to be conducted.

A recent study has shown that a SNP in *TSLP* is associated with cockroach-allergy IgE in Costa Rican girls and with total IgE in girls in two populations (9). The study has shown significant evidence of linkage to IgE produced in response to cockroach allergy on chromosome 5q23 in female subjects (9). *TSLP* is located near the linkage peak and has female-specific effects on lung disease in mice (33). It has also been reported that rs2289276 is associated with reductions in IgE in the response to the cockroach and total IgE (9). In our Japanese population, we could not find an association between total IgE and the rs2289276 SNP among asthmatic subjects in this study and female-specific effect on the associations. Although inconsistent associations may reflect sample size, phenotype heterogeneity, or gene-environment interactions in the population, further replication studies of these findings are needed.

We could not replicate our findings in the populations that were examined in two recent GWAS (24, 25).

There were differences of minor allele frequencies of the variants among the control populations. The MAFs of the three variants, rs3806932, rs2289276, and rs11466741, in the Japanese population were 0.29, 0.26 and 0.26, respectively (10). To test for replication at the level of the gene rather than the SNP, further fine mapping around the *TSLP* gene and association studies seem to be needed.

We could not replicate our findings in European and African-ancestry populations for which recent GWAS's were performed (24, 25). There were differences of minor allele frequencies of the variants among the control populations. The MAFs of the three variants, rs3806932, rs2289276, and rs11466741, in the Japanese population were 0.29, 0.26 and 0.26, respectively (10), compared to 0.43, 0.29, and 0.29 in the CAMP population, and 0.63, 0.18, and 0.31 in the African American population, and 0.66, 0.17, and 0.28 in the African Caribbean population. Failure to observe a SNP-for SNP replication in ethnically diverse populations is not uncommon, and can result from variation in allele frequencies, population admixture, heterogeneity of the phenotype, and environmental factors. Our failure to replicate at the SNP level is therefore not surprising, and it is likely that variants other than those tested in this study are the causal variants. Elsewhere it has been argued that a gene-based approach, rather than a SNP-for SNP, may provide evidence for genetic analysis at the functional level (25). Our findings suggest that replication at the level of the gene rather than the SNP, and further fine mapping around the TSLP gene are warranted.

We previously have shown that a functional rs3806933 SNP in the promoter region of long-form *TSLP* (-847T) creates a binding site for AP-1 and enhances AP-1 binding to the regulatory element (10). In this study, we found the binding of AP-2α protein, a possible transcription suppression factor, to the sequences containing the rs2289276 (-82C/T) SNP and higher binding to -82C (on the protective allele). Both of the SNPs, rs3806933 (-847C/T) and rs2289276 (-82C/T), were significantly associated with asthma susceptibility. We also identified a common disease-susceptible haplotype, T(-847)-T(-82)-C(1560), in both childhood atopic and adult asthma. The two different transcription factors on the two promoter SNPs might lead to preferential transcription from the susceptible haplotype T-T-C through their cooperative effects in bronchial epithelial cells. In this study, we did not examine the functional effects of polymorphisms rs3806932, rs2289277, rs10073816, and rs11466741, which were in strong LD with the related variants, rs3806933 and rs2289276. The functions of these linked polymorphisms remain to be elucidated.

Combination therapy with LABA and inhaled corticosteroids reduces exacerbation frequency in asthma, and it is also efficacious as intervention therapy in asthma exacerbations (16-18). In this study, SAL was able to suppress TSLP production in NHBE cells and, furthermore, we demonstrated synergistic suppression of poly(I:C)-induced TSLP production by DEX and SAL in NHBE cells. Respiratory viral infections are associated with the majority of exacerbations of bronchial asthma, and a recent study has shown that combined corticosteroid/β_2 agonists synergistically suppress rhinovirus-induced neutrophil (CXCL8) and lymphocyte (CCL5, CXCL10)

chemokine production in airway epithelial cells (34). Glucocorticosteroids inhibit both NF-κB and AP-1 through glucocorticoid-induced leucine zipper protein (35, 36), and an NF-κB binding site has been identified 3.7kb upstream from the start of *TSLP* transcription (13). Although further investigation of the molecular mechanisms mediating suppressive effects of DEX and/or SAL on TSLP and chemokine production is needed, combination therapy might exert an anti-Th2 polarized inflammatory effect during respiratory viral infections through suppressive effects on TSLP and production of other chemokines in airway epithelial cells.

In summary, we identified *TSLP* as a susceptibility gene for childhood atopic and adult asthma by means of a case-control study with SNPs, and demonstrated that glucocorticoid/LABA treatment synergistically suppressed poly(I:C)-induced TSLP in NHBE. Our data strongly support the important role of TSLP in bronchial asthma and the clinical benefits of combination therapy in asthma management.

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