されていたが，その後のフォローアップにより，症状スコア，薬物スコア，症状薬物スコア，新規アレルゲン感作に関してはSCIT終了12年後においても有意に抑制され，治療終了後の効果継続作用が長期的に確認された[19),20)]。また小児季節性アレルギー性鼻炎に対するSCITは喘息発症を予防することが報告されていたが(PAT-study)，その後の調査により，この効果はSCIT終了7年間に渡り持続することが明らかとなった[21),22)]。

Ⅴ．SITの作用機序

SCITは種々の免疫担当細胞に働き，アレルギー性鼻炎の病態を抑制する[23)]。スギ花粉症へのSCITに関しても，これまでに飛散期における血清スギ特異的IgE抗体価の上昇抑制作用，好塩基球からの抗原特異的なヒスタミン遊離抑制作用，花粉飛散期における血清IL-4値や可溶性VCAM-1値，可溶性CD23値の上昇抑制作用などがみられる[14),24)]。さらにSCITの重要かつ中心的な作用機序として，アレルゲン特異的なT細胞機能を調節し免疫寛容を誘導することが挙げられる[23)]。

Th2細胞への作用としては，特にIL-5産生の制御が重要である。スギ花粉症では，SCITの奏功群は不良群に比較して末梢血単核細胞からのIL-5発現が有意に低下する。さらにIL-5産生抑制には共抑制シグナル分子BTLA (B and T lymphocyte attenuator) の発現上昇が関与する[14)]。またイネ科花粉症ではSCITを行うことにより，花粉飛散期の鼻粘膜内IL-5発現が非SCIT群と比較し有意に低下する。

さらに最近では，SCITによって誘導された制御性T細胞と，本細胞が産生するIL-10やTGF-β (transforming growth factor-β) などの制御性サイトカインによる免疫偏向/抑制が，効果発現に中心的に関与することが示されている[23)]。例えば，イネ科花粉の免疫療法を1年間行った患者のリンパ球はアレルゲン刺激に対してIL-10を有意に多く産生し，この産生には免疫療法群で増加した$CD25^+$ $CD4^+$細胞，すなわち制御性T細胞の関与が示唆されている[25)]。ダニアレルギーやシラカンバ花粉症においても免疫療法70日後に制御性T細胞が誘導され，本細胞がアレルゲン特異的増殖応答を抑制し，さらにこの抑制は抗IL-10抗体または抗TGF-β抗体の添加で解除される[26)]。

Ⅵ．SCIT効果の予測因子

免疫療法の有効例では治療後早期（数日～1カ月）よりIL-10産生が亢進することから，IL-10産生を指標として免疫療法の効果が予測できる可能性がある[27)]。また最近の報告では，治療前の検査値で血清総IgE量が低値で，かつ特異的IgE抗体価が高値なアレルギー性鼻炎患者ではSCITの効果が高く，特に特異的IgE抗体価/血清総IgE量の比が16.2以上では高い敏感度および特異度でSCITの有効性が予測可能との報告がされた[28)]。本邦での追試，解析が望まれる。

おわりに

以上，アレルギー性鼻炎に対するSCITのアップデートについて概説した。季節性鼻炎（花粉症），特に成人例に対するSCITの有効性と安全性についてはメタ解析にて確立された。しかしながら，メタ解析を評価する際にはSelection biasやPublication biasなどに注意する必要がある。最近ではエンドポイント設定も含めた，免疫療法の効果を判定し報告するためのガイドラインも示さ

BTLA (B and T lymphocyte attenuator)　TGF-β (transforming growth factor-β)

れている[29]。一方，小児アレルギー性鼻炎を対象としたSCITの効果に関するメタ解析では，エビデンスレベルの高い論文が少ないことなどから一定の結論は得られていない[30]。我が国においても，小児鼻炎例に対するSCITの効果と安全性，あるいは長期予後や自然経過への修飾作用など，未解決な事項は多く，今後の検討が必要である。

文　献

1) Ring J, Gutermuth J : 100 years of hyposensitization : history of allergen-specific immunotherapy (ASIT). Allergy 2011 (doi : 10.1111/j.1398-9995.2010.02541.x)
2) WHO見解書　アレルゲン免疫療法：アレルギー疾患の治療ワクチン．アレルギー 47 : 749, 1998
3) 鼻アレルギー診療ガイドライン作成委員会：第5章 治療．鼻アレルギー診療ガイドライン－通年性鼻炎と花粉症－ 第6版，ライフ・サイエンス，東京，2008，p34-62
4) Zuberbier T, Bachert C, Bousquet PJ et al : GA2LEN/EAACI pocket guide for allergen-specific immunotherapy for allergic rhinitis and asthma. Allergy 65 : 1525, 2010
5) Casale TB, Busse WW, Kline JN et al : Omalizumab pretreatment decreases acute reactions after rush immunotherapy for ragweed-induced seasonal allergic rhinitis. J Allergy Clin Immunol 117 : 134, 2006
6) Muller U, Hari Y, Berchtold E : Premedication with antihistamines may enhance efficacy of specific-allergen immunotherapy. J Allergy Clin Immunol 107 : 81, 2001
7) Durham SR, Walker SM, Varga EM et al : Long-term clinical efficacy of grass-pollen immunotherapy. N Eng J Med 341 : 468, 1999
8) Des Roches A, Pardis L, Menardo JL et al : Immunotherapy with a standaerdized Dermatophagoides pteronyssinus extract. V. Duration of the efficacy of immunotherpy after its cassation. Allergy 51 : 430, 1996
9) Tabar AI, Gracia BE, Rodriguez A et al : A prospective safety-monitoring study of immunotherapy with biologically standardized extracts. Allergy 48 : 450, 1993
10) Dreborg S, Agrell B, Kjellman NI et al : A double-blind, multicenter immunotherapy trial in children, using a purified and standerdized Cladosporium herbarum preparation. I. Clinical results. Allergy 41 : 131, 1986
11) Varney VA, Edwards J, Tabbah K et al : Clinical efficacy of specific immunotherapy to cat dander : a double blind placebo controlled trial. Clin Exp Allergy 27 : 860, 1997
12) Varney VA, Tabbah K, Marvolen G et al : Usefulness of specific immunotherapy in patients with severe perennial rhinitis induced by house dust mite : a double-blind, randomized, placebo-controlled trial. Clin Exp Allergy 33 : 1076, 2003
13) Calderon MA, Alves B, Jacobson M et al : Allergen injection immunotherapy for seasonal allergic rhinitis. Cochrane Database Sys Rev 24 : CD001936, 2007
14) 岡野光博：スギ花粉飛散後の花粉症．治療 93 : 395-400, 2011
15) Okano M, Otsuki N, Azuma M et al : Allergen-specific immunotherapy alters the expression of BTLA, a co-inhibitory molecule, in allergic rhinitis. Clin Exp Allergy 38 : 1891, 2008
16) Sone T, Dairiki K, Morikubo K et al : Recognition of T cell epitopes unique to Cha o 2, the major allergen in Japanese cypress pollen, in allergic patients cross-reactive to Japanese cedar and Japanese cypress pollen. Allergol Int 58 : 237, 2009
17) Yilmaz M, Bingol G, Altintas D et al : Effect of SIT on quality of life. Allergy 55 : 302, 2000
18) Nagao M, Hiraguchi Y, Hosoki K et al : Allergen-induced basophil CD203c expression as a biomarker for rush immunotherapy in patients with Japanese cedar pollinosis. Int Arch Allergy Immunol 146 (suppl 1) : 47, 2008
19) Eng PA, Reinhold M, Gnehm HPE : Lon-term efficacy of preseasonal grass pollen immunotherapy in children. Allergy 57 : 306, 2002
20) Eng PA, Boer-Reinhold M, Heijnen IAFM, Gnehm HPE : Twelve-year follow-up after discontinuation of preseasonal grass pollen immunotherapy in childhood. Allergy 61 : 198, 2006
21) Moller C, Dreborg S, Ferdousi HA et al : Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivis (the PAT-Study). J Allergy Clin Immunol 109 : 251, 2002
22) Jacobsen L, Niggemann B, Dreborg S et al : Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma : 10-year follow-up on the PAT study. Allergy 62 : 943, 2007

23) Akdis CA, Akdis M : Mechanisms of allergen-specific immunotherapy. J Allergy Clin Immunol 127 : 18, 2011
24) 湯田厚司, 西村真治, 坂倉康夫ほか：スギ花粉症免疫療法でのHRTシオノギキットによる好塩基球ヒスタミン遊離率の変化. アレルギー 51 : 634, 2002
25) Francis JN, Till SJ, Durham SR : Induction of IL-10+ CD4+ CD25+ T cells by grass pollen immunotherapy. J Allergy Clin Immunol 111 : 1255, 2003
26) Jutel M, Akdis M, Budak F et al : IL-10 and TGF-β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 33 : 1205, 2003
27) Savolainen J, Laaksonen K, Rantio-Lehtimaki A et al : Increased expression of allergen-induced in vitro interleukin-10 and interleukin-18 mRNA in peripheral blood mononuclear cells of allergic rhinitis patients after specific immunotherapy. Clin Exp Allergy 34 : 413, 2004
28) DiLorenzo G, Mansueto P, Pacor ML et al : Evaluation of serum s-IgE/total IgE ratio in predicting clinical response to allergen-specific immunotherapy. J Allergy Clin Immunol 123 : 1103, 2009
29) Bousquet PF, Brozek J, Bachert C et al : The CONSORT statement checklist in allergen-specific immunotherapy-a GA²LEN paper. Allergy 69 : 1737, 2009
30) Roder E, Berger MY, de Groot H et al : Immunotherapy in children and adolescents with allergic rhinoconjunctivitis : a systematic review. Pediatr Allergy Immunol 19 : 197, 2008

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原　著　　第29回日本耳鼻咽喉科免疫アレルギー学会特別企画　若手研究者奨励賞：最優秀賞

スギ花粉症に関する鼻上皮細胞の網羅的遺伝子解析

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現在スギ花粉症は日本で最も多いアレルギー疾患となっている。スギ花粉症に関連する遺伝子を同定するために，スギ花粉症患者と吸入抗原6種類に対する特異的IgE陰性のコントロールを対象に2009年度スギ飛散期に鼻上皮細胞を擦過し，mRNAのプロファイルをIllumina 500GXシステム（イルミナ）を用いて網羅的遺伝子発現解析を行った。発現解析はスギ花粉症患者群とコントロール群において，発現差が4倍以上かつ$p<0.05$であるものを有意としたところ18遺伝子を同定できた。Intelectin 1はスギ花粉症患者で17.4倍の発現を認めた。定量的polymerase chain reactionでもスギ花粉症患者で有意な上昇を認めた。2010年度鼻上皮細胞でのIntelectin 1の発現を再度定量的PCRで確認した。その結果前年度と同様に，スギ花粉症患者はコントロール群に比べ有意な発現亢進を認めた。しかしスギ特異的IgE陽性症状未発症者では，コントロール群と同様低い発現であった。次に通年性アレルギー性鼻炎患者の下甲介粘膜を用いて免疫染色を行ったところ，intelectin 1は鼻上皮細胞で産生されていることが分かった。また培養鼻粘膜擦過片からの鼻上皮細胞初代培養株において，Intelectin 1はIL-4及びIL-13の刺激により発現された。以上のことからIntelectin 1はアレルギー性鼻炎の症状（発症）に関する分子であることが示唆された。

キーワード：マイクロアレイ，定量的PCR，鼻上皮細胞，季節性アレルギー性鼻炎，免疫組織染色

略語：cRNA, complementary ribo nucleic acid; 定量的PCR, polymerase chain reaction; IL-4, Interleukin4; IL-13, Interleukin13; IL-17RB, Interleukin 17 receptor B

はじめに

スギ花粉症は，2月～4月にかけて日本各地で猛威をふるう季節性アレルギー性鼻炎である。我々が行った最新の疫学調査では，スギ特異的IgE陽性者（CAPスコア2以上）は55.5%（855名／1,540名）であり，全体の39.6%（610名）がスギ花粉飛散時期にアレルギー症状を有していた[1]。

マイクロアレイはプローブに特定のmRNAと一致するDNA鎖を採用することで，一度に数千から数万種類の遺伝子発現情報を同定することができる優れた解析法である。この手法により，各疾患に関連する細胞や疾患動物モデルにおける，病態関連特異的な遺伝子発現を網羅的に解析することできる。これまで我々はスギ花粉飛散時期に採取した末梢血にてマイクロアレイを行った[2]。その結果，スギ花粉症患者では，末梢血から分離したCD4陽性T細胞のIL-17RB（Interleukin-17 receptor B）発現が，非アレルギーであるコントロール群と比較して有意に上昇していたことを見出した。この結果はWangらの報告とも一致していた[3]。一方で気道局所における遺伝子発現解析では，喘息患者由来の気管支組織を用いた研究で，79遺伝子の発現変化を認めたと報告された[4]。末梢血よりも炎症反応の主体となる組織での遺伝子発現変化を把握することは，病態の解明に大きく貢献できる可能性が高い。そこで我々は2009年度のスギ花粉飛散時期に，スギ花粉症発症者，スギ特異的IgE陽性未発症者，非アレルギー者から鼻粘膜擦過法にて鼻粘膜上皮細胞を採取し，マイクロアレイ法によりmRNA発現解析を行った。さらに2010年度のサンプルを使用して2009年度サンプルで得られた結果の追認を行った。

対　象

福井大学アレルギーデータベースからスギ花粉症患者群（血清スギ特異的IgEがCAPスコアで2以上かつ毎年スギ花粉飛散時期にスギ花粉症の症状を認め，スギ花粉症と診断がされている），感作陽性未発症者群（血清スギ特異的IgEがCAPスコアで2以上であるが，今まで全く花粉症の症状がない），非アレルギー群（7項目の吸入アレルゲンに対する血清特異的IgEが全て陰性であり鼻炎の症状を認

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表 1　2009 年度及び 2010 年度の対象者の内訳

	2009 年		2010 年		
	スギ花粉症患者	コントロール	スギ花粉症患者	感作未発症者	コントロール
合計（人）	32	25	17	14	16
男性（人）	16	13	8	4	6
女性（人）	16	14	9	10	10
年齢（歳）	36.8（25–50）	32.1（18–47）	30.4（20–47）	27.2（19–44）	26.2（19–41）
RIST (total IgE)	95.6	45.6	75.5	66.7	25.5
スギ特異的 IgE	14.6	0.34 以下	16.2	3.6	0.34 以下
皮内反応テスト（陽性/陰性）			17/0	7/6	0/16
鼻汁好酸球（陽性/陰性）			10/7	4/10	0/16

めていない）であるコントロール群を抽出した。CAP スコアの測定はスギ，ヤケヒョウヒダニ，コナヒョウヒダニ，カモガヤ，ブタクサ，アスペルギルス，カンジダの 7 項目が測定されている。さらにスギ花粉症の症状の有無，気管支喘息やアトピー性皮膚炎，食物アレルギーなどの既往も調べられている。スギ花粉症患者群及び感作陽性未発症者群とも，血清スギ特異的 IgE のみ CAP スコアで 2 以上であり，測定した他の 6 項目の血清特異的 IgE は陰性である人を選んだ。さらに 3 群とも気管支喘息，アトピー性皮膚炎，食物アレルギーの既往は認めていない人を選んだ。

対象者とは本研究の内容を書面と口頭にて説明し，文書で同意を得た。2009 年対象者はスギ花粉症患者群 32 名，非アレルギー群 25 名，2010 年対象者はスギ花粉症患者群 17 名，感作陽性未発症者群 14 名，非アレルギー群 16 名であった。（対象者の背景因子を表 1 に示す）

本研究は福井大学医学部倫理委員会における承認を得て行った。

方　法

鼻粘膜上皮採取

鼻粘膜上皮細胞は 2009 年度および 2010 年度とも，スギ花粉飛散時期（3 月中旬）に施行した。採取は下甲介粘膜を，細胞診用ブラシ（MEDICAL PACKAGING, Camarillo, CA, USA）で数回擦過し，鼻粘膜上皮細胞を採取した。採取した鼻粘膜上皮細胞をすぐに TRIzol（Invitrogen, Leek, the Netherlands）に溶解し，–80°C で保存した。

RNA 抽出および cRNA 合成

採取した鼻粘膜上皮細胞より，miRNeasy Mini キット（QIAGEN, Valencia, CA, USA）を用いて total RNA を抽出した。この total RNA（100–500n）から，Illumina TotalPrep RNA Amplification Kit（Applied Biosystems, Foster City, CA, USA）により，ビオチンラベル化した cRNA を合成した。

マイクロアレイ解析

鼻粘膜上皮細胞より合成した cRNA を用いたマイクロアレイは，HumanRef-8 ver3 BeadChip（Illumina, San Diego, CA, USA）によって行い，アレイの蛍光強度を BeadsStation 500X 遺伝子発現解析システム（Illumina）により検出した。このチップは，約 24,500 種類のアノテーション済み RefSeq（Build 36.2, Release 22）転写産物をターゲットとするプローブが掲載されており，網羅的に mRNA の発現量を調べることが可能となっている。

定量的 polymerase chain reaction（PCR）

cDNA 合成

鼻粘膜上皮細胞より抽出した total RNA から，Oligo(dT)$_{20}$ Primer（TOYOBO, Osaka, Japan），5X RT Buffer, dNTP Mixture（10 mM, TOYOBO），ReverTra Ace（100 U/μL, TOYOBO），RNase OUT（40 U/μL. Invitrogen）を用いて，逆転写反応を 42°C，1 時間行った後，65°C，10 分間で酵素失活反応を行い cRNA 合成した。

定量的 PCR 反応

合成した cDNA を Nuclease-free Water により 40 倍に希釈して，定量的 PCR に使用した。遺伝子の内在性コントロールには Glyceraldehyde-3-phosphate dehydrogenase（GAPDH）を用いた。qPCR™ Mastermix Plus QuickGoldStar（Eurogentec, Seraing, Belgium），20× GAPDH probe（Applied Biosystems），20× TaqMan expression probe（Applied Biosystems），cDNA からなる反応液を ABI PRISM 7900HT Sequence Detection System（Applied Biosystems）にかけ定量的 PCR を行った。PCR 反応におけるプロトコールは，95°C 10 分後 PCR による増幅サンプルが指数関数的に起こる領域で，一定の増幅産物になるサイクル数（Threshold Cycle; Ct）を検出した。各遺伝子発現量は GAPDH の発現量に対する比を ΔΔCt 法で算出した。

皮内反応テスト

スギ抗原に対する皮内反応テストは，2010年度スギ花粉飛散時期の対象者に施行した。前腕部に皮内反応検査用スギ抗原液（Torii Phrmaceutical CO. LD, Tokyo, Japan）と対照液をそれぞれ0.02 mlずつ皮内に注入し，その15分～30分後に判定を行い，スギ抗原注入部付近で皮膚の発赤径が20 mm以上又は，膨疹径が9 mm以上を陽性とした。

免疫組織染色

福井大学医学部附属病院耳鼻咽喉科・頭頸部外科において，手術時に採取された通年性アレルギー性鼻炎患者の下甲介粘膜で免疫組織化学的染色を行った。手術時に採取された下甲介粘膜は直ちにホルマリンで固定され，その後パラフィンにて固定されている。体を4 μmの厚さに切離し，Intelectin 1に対するポリクローナル抗体（R&D Systems, Minneapolis, US）と，ヒツジIgGに対する2次抗体（R&D）を使用した。

細胞培養

鼻粘膜上皮細胞初代培養細胞株は，通年性アレルギー性鼻炎患者から樹立した。RNA用の鼻粘膜上皮細胞回収と同様に数回擦過し，すぐにペニシリン（100 unit/ml）とのストレプトマイシン（100 μg/ml）を含む培養液（Reagent Pack, Takara, Otu, Japan）に回収した。回収した鼻粘膜上皮細胞を37°C，5% CO_2のインキュベーターにて培養した。培養した鼻粘膜上皮細胞にLPS（100 ng/ml）（invitrogen, Carlsbad, US），IFN-γ（20 ng/ml）（AbD Serotec, Oxford, UK），TNF-α（10 ng/ml）（Cell Signaling Technology, Beverly, US），ヒスタミン（1×10^{-4} M/ml）（Nakarai tesque, Kyoto, Japan），IL-4（10 ng/ml）（R&D），IL-13（10 ng/ml）（R&D），IL-33（10 ng/ml）（R&D）を添加し，刺激後15時間後に細胞を回収し，RNA抽出した。

統計解析

検定はノンパラメトリック法にて行った。網羅的遺伝子発現解析は，BeadStudioソフトウェア（version 3.1, Illumina）を用い，検出した蛍光強度をシグナル値として出力した。シグナル値とともに出力されるDetection p-valueは検出結果の信頼性の指標であり，$p<0.01$であればシグナル値の信頼性があるとされる。よってこれらの値を基に結果の信頼性について検討した。また，Detection p-valueを基にFlagを付加し，$p<0.01$をPresent，$p>0.05$をAbsentとした。PresentとAbsentの中間である$0.01 <$ Detection p-value < 0.05はMarginalとした。BeadStudioソフトウェアにより出力された各サンプルのシグナル値より，バックグラウンドを引いた値の解析は，GeneSpring GX Software Version 10.0（Agilent Technologies, Santa Clara, CA, USA）によって行った。シグナルの最小値は5とし，それ以上の値をシグナル値として採用した。チップ間のノーマライズは，シグナル値からバックグラウンドを引いた値の75%タイルに合わせて補正するMedian Shift補正を行った。またBaseline補正はBaseline to median of all samplesにより行った。スギ花粉症患者群とコントロール群の遺伝子発現量が4倍以上の増加あるいは減少があった遺伝子を抽出した。発現量の比較についてMann-Whitney検定を行い，多重比較補正はBemjamini Hochberg false discovery rate（FDR）を行い，q-value < 0.05を統計学的な有意水準とした。

定量的PCRの結果の統計解析では，Wilcoxon検定（有意水準5%）を用いた。定量的PCRと網羅的遺伝子発現解析の結果の相関は，mRNA発現量の相対値と網羅的遺伝子発現解析によりノーマライズした各遺伝子の発現量について，Spearman順位和相関係数を用いて検証した。

結　果

遺伝子発現解析

2009年度対象者に行ったマイクロアレイの結果ではスギ花粉症患者群とコントロール群との間で4倍以上有意な発現変化を認めた遺伝子は18遺伝子であった。そのうち13遺伝子はスギ花粉症患者群において，コントロール群と比較して有意に発現が上昇していた。

その中で遺伝子発現レベルが10倍以上の差を認めた4遺伝子の中に一つがIntelectin 1であった。Intelectin 1はコントロール群と比較して17.6倍発現が上昇していた。定量的PCRの結果ではスギ花粉症患者群がコントロール群と比較して20.4倍有意に上昇していた（図1a, $p<0.0001$）。

2010年度対象者の鼻粘膜上皮搾過細胞におけるIntelectin 1の発現量を定量的PCRにて行った。その結果，コントロール群と比較して感作陽性未発症者は1.8倍上昇していたが有意な変化は認めなかった。一方スギ花粉症群ではコントロール群と比較して192.4倍（$p<0.0001$）上昇していた。また，スギ花粉症患者群は感作陽性未発症者と比べても有意にIntelectin 1の発現は高い結果であった（図1b, $p<0.001$）。

2010年度対象者においてスギ抗原に対する皮内反応で陽性と示した24名と陰性を呈した22名でIntelectin 1の発現を比較すると，陽性者は陰性者より146.1倍（図2a, $p<0.01$）高かった。感作陽性未発症者で皮内反応陽性者7名と陰性者6名とでは有意な発現変化は認めなかった（図2b）。

また，鼻汁好酸球陽性者14名と陰性者33名との比較では，陽性者が陰性者と比べ61.4倍Intelectin 1の発現が有意に高かった（図3a, $p<0.0001$）。また感作陽性未発症者の鼻汁好酸球陰性者10名と比較して，陽性者4名のIntelectin 1の発現が5.7倍有意に高かった（図3b, $p<0.01$）。

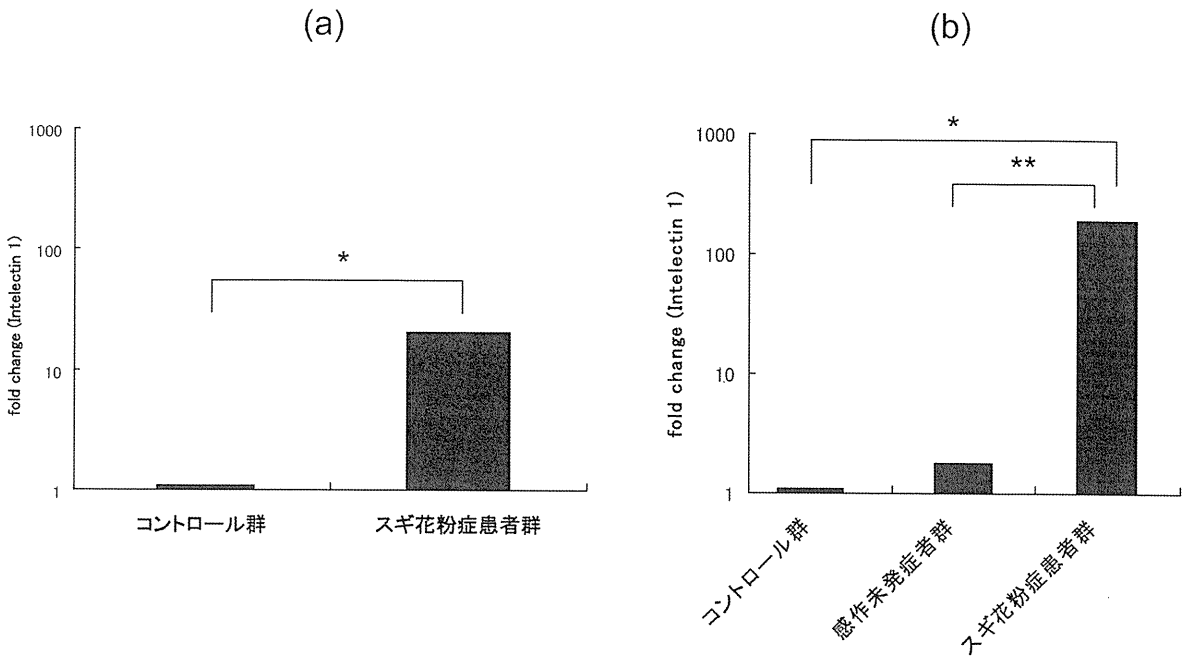

図1　(a) 2009年度 Intelectin 1 の発現量（定量的 PCR）。コントロール群を基準としたスギ花粉症群の Intelectin 1 の発現量。* $p<0.0001$。(b) 2010年度 Intelectin 1 の発現量(定量的 PCR)。コントロール群を基準としたスギ花粉症群及び感作陽性未発症者群の Intelectin 1 の発現量。* $p<0.0001$，** $p<0.001$。

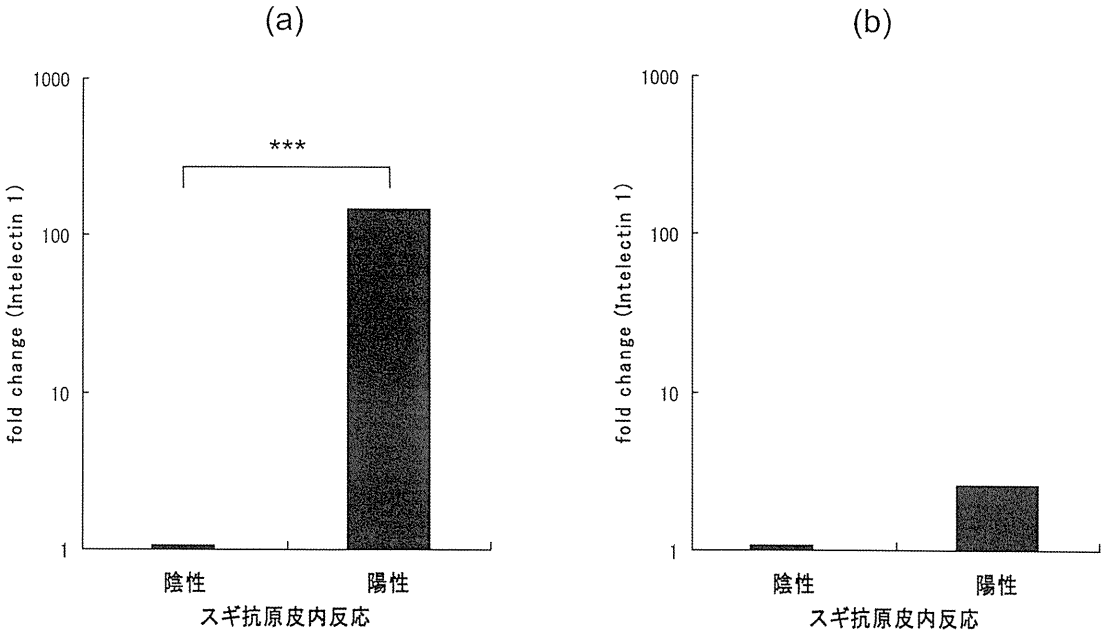

図2　2010年度 Intelectin 1 の発現量（定量的 PCR)。(a) 2010年度すべての対象者においてスギ抗原皮内反応陰性者を基準とした皮内反応陽性者の Intelectin 1 の発現量。*** $p<0.01$。(b) 感作陽性未発症者群におけるスギ抗原皮内反応陰性者を基準とした皮内反応陽性者の Intelectin 1 の発現量。

免疫染色

通年性アレルギー性鼻炎患者由来の鼻下甲介粘膜における免疫染色では，Intelectin 1 が鼻下甲介粘膜の上皮に局在していることが分かった。一方，粘膜下組織は染色はされなかった（図4)。

細胞実験

鼻粘膜上皮細胞の採取直後は Intelectin 1 の発現は確認できたが，初代培養した鼻粘膜上皮細胞では Intelectin 1 の発現が認められなかった。初代培養は鼻粘膜採取後平均10日間培養を行った後実験に用いた。そこで LPS，IFN-γ，

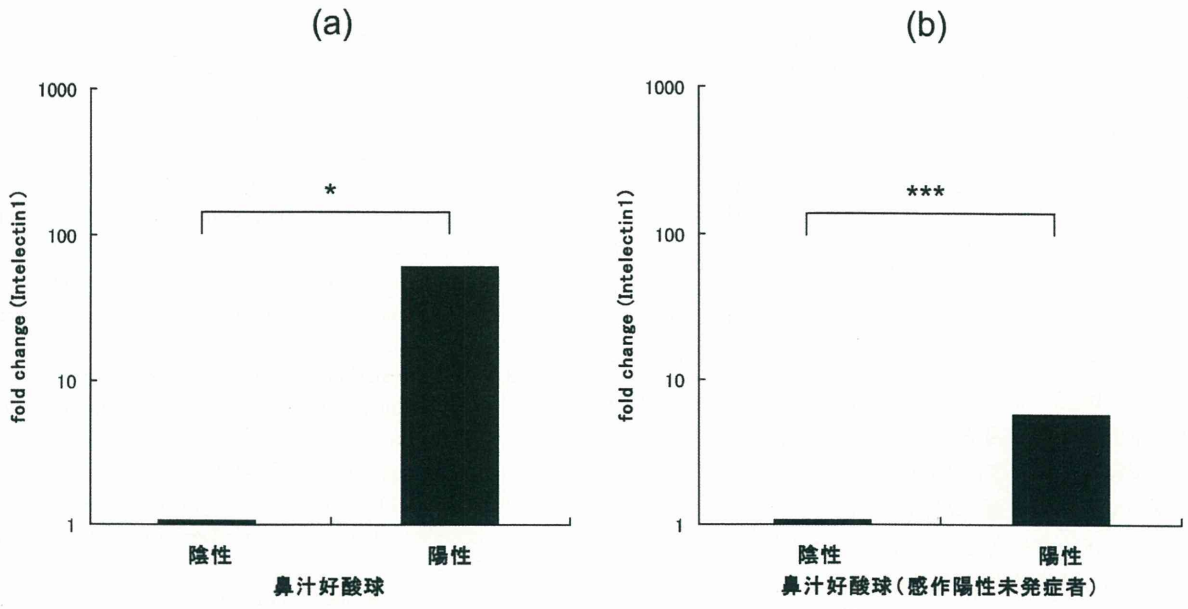

図3 2010年度 Intelectin 1 の発現量（定量的 PCR）。(a) 鼻汁好酸球陰性者を基準とした鼻汁好酸球陽性者の Intelectin 1 の発現量。* $p<0.0001$。(b) 感作陽性未発症者群における鼻汁好酸球陰性者を基準とした鼻汁好酸球陽性者の Intelectin 1 の発現量。*** $p<0.01$。

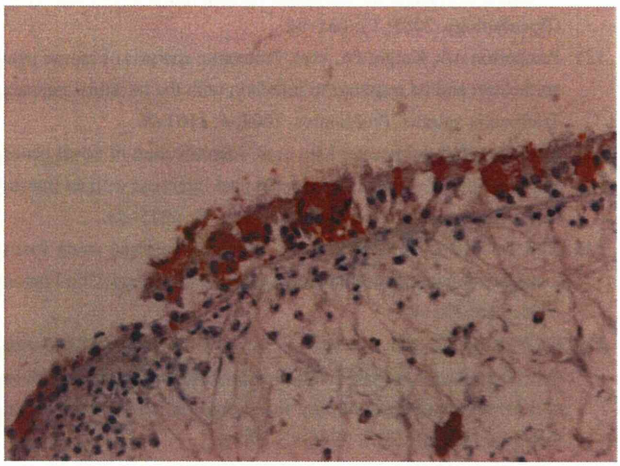

図4 Intelectin 1 免疫染色（通年性アレルギー性鼻炎患者由来の下甲介粘膜）。Intelectin 1 は下甲介粘膜の上皮細胞に存在している。

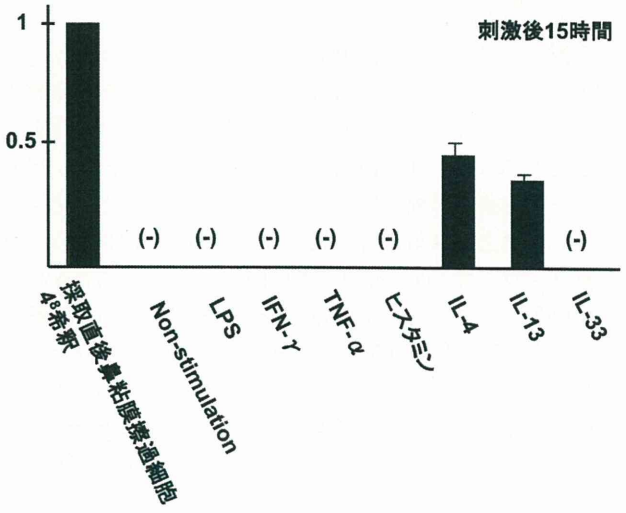

図5 初代培養鼻粘膜上皮細胞における Intelectin 1 の発現。IL-4 と IL-13 の刺激により Intelectin 1 の発現は誘導される。

TNF-α，ヒスタミン，IL-4，IL-13，IL-33 で 15 時間刺激をすると，IL-4 と IL-13 の刺激で Intelectin 1 の発現が認められた（図5）。

考　察

本研究ではスギ花粉症飛散時期に，スギ花粉症患者の鼻上皮細胞において多量の Intelectin 1 が発現していることが判明した。

Intelectin 1 は常染色体 1q.21.3 にコードされ，ヒトとマウスでは Intelectin 1 と Intelectin 2 の 2 つの Intelectin が同定されている。ヒトでは顎下腺，小腸，大腸，胸腺でその発現が確認されており，分泌型の糖タンパクとして存在し，いくつかの機能を有している。Intelectin 1 は Omentin や小腸における Lactofferin receptor とも呼ばれ，鉄の吸収やインスリン刺激による糖代謝や脂肪代謝に関連する[5-7]。また Intelectin は arabinogalactan を認識し，結合する[5,8]。arabinogalactan は細菌や真菌，原虫に存在するが，ヒトでは存在しないため[9-11]，細菌や真菌などに結合し，生体の防御機構に関与していると考えられる。さらに腸管における鞭虫に対する免疫反応に関連するとも考えられている[12-15]。腸管免疫防御機構において，寄生虫排除が IgE を介することは有名なことである。IgE 産生に関しては，アレルギー性鼻炎も寄生虫感染もほとんど同じような機序が考え

られる。鼻上皮細胞において，Intelectin 1 の発現が IL-4 と IL-13 の Th2 サイトカインで亢進すること，マウスの気道上皮細胞でも Intelectin 1 が IL-13 により誘導されること，ヒト喘息患者での気管支上皮で Intelectin 1 の発現が上昇していること[14-16]，そして Intelectin 1 の遺伝子多型と喘息との関連が報告されていること[17]から，Intelectin はアレルギー性鼻炎において重要な分子である可能性が高いと思われる。

感作陽性未発症者の存在は，アレルギー性鼻炎発症予防の観点からもとても注目されている。我々は皮内反応テストと鼻汁好酸球の２つのアプローチにて Intelectin 1 が感作陽性未発症者の同定因子になりえないか検討した。その結果，感作陽性未発症者内の皮内反応テスト陽性：陰性では差を認めず，鼻汁好酸球浸潤の有無の方が関連していた。しかしスギ花粉症患者では，Intelectin 1 の値も有意に高く，ちょうど感作陽性未発症者では約 1/3 程度なので，ある一定量の Intelectin 1 産生が好酸球浸潤，そして発症に関与するかもしれない。鼻汁好酸球はアレルギー性鼻炎の鼻粘膜の腫脹や鼻汁との関連や，アレルギー性鼻炎の診断との有意な相関を示す報告がある一方で[18,19]，そのパラメーターとしては臨床的には不適切であるとの報告がある[20]。鼻汁好酸球テストは，手技的要素や採取の条件により，その感度が一定しないと考えられる。今回スギ花粉症患者群で鼻汁好酸球が陰性であった４名はこれらの要因に含まれるかもしれないが，Intelectin 1 と同時に測定すると，アレルギー性鼻炎の診断の感度をより明確にできるとも考えられる。気道上皮と好酸球，及び Intelectin 1 の関連では，OVA 感作マウスで Intelectin 1 を抑制すると，下気道に走化される好酸球の減少と，下気道の炎症が抑制されたとの報告もある[15]。鼻を含め気道上皮は細菌，真菌，花粉などの様々なアレルゲンにさらされており，生体はそれらに対する防御反応を有している。好酸球はアレルゲンなどの刺激により放出されるサイトカインやケモカインで走化され，上皮における炎症反応の主役として働く。スギ花粉に対する生体の反応として Intelectin 1 の発現が上昇し，それに伴い好酸球浸潤が起こり，局所の炎症が増強するストーリーが考えられる。Intelectin 1 を標的とした新しいアレルギー治療を模索中である。

文　献

1) Sakashita M, Hirota T, et al. Prevalence of allergic rhinitis and sensitization to common aeroallergens in a Japanese population. Int Arch Allergy Immunol. 2009; 151: 255–61.
2) Matsumoto Y, Noguchi E, et al. Upregulation of IL17RB during natural allergen exposure in patients with seasonal allergic rhinitis. Allergol Int. 2011; 60(1): 87–92.
3) Wang H, Mobini R, et al. Allergen challenge of peripheral blood mononuclear cells from patients with seasonal allergic rhinitis. Clin Exp Allergy. 2010; 40(8): 1194–202.
4) Laprise C, Sladek R, et al. Functional classes of bronchial mucosa genes that are differentially expressed in asthma. BMC Genomics. 2004; 5(1): 21.
5) Tsuji S, Uehori J, et al. Human intelectin is a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall. J Biol Chem. 2001; 276(26): 23456–63.
6) Tan BK, Pua S, et al. Decreased plasma omentin-1 levels in Type 1 diabetes mellitus. Diabet Med. 2008; 25(10): 1254–5.
7) Suzuki YA, Shin K, et al. Molecular cloning and functional expression of a human intestinal lactoferrin receptor. Biochemistry. 2001; 25; 40(51): 15771–9.
8) Tsuji S, Yamashita M, et al. Differential structure and activity between human and mouse intelectin-1: Human intelectin-1 is a disulfide-linked trimer, whereas mouse homologue is a monomer. Glycobiology. 2007; 17: 1045–51.
9) Pedersen LL, Turco SJ. Galactofuranose metabolism: A potential target for antimicrobial chemotherapy. Cell Mol Life Sci. 2003; 60: 259–66.
10) Abeygunawardana C, Bush CA, et al. Complete structure of the cell surface polysaccharide of Streptococcus oralis C104: A 600-MHz NMR study. Biochemistry. 1991; 30: 8568–77.
11) Leitao EA, Bittencourt VC, et al. Beta-galactofuranose-containing O-linked oligosaccharides present in the cell wall peptidogalactomannan of Aspergillus fumigatus contain immunodominant epitopes. Glycobiology. 2003; 13: 681–92.
12) Pemberton AD, Knight PA, et al. Proteomic analysis of mouse jejunal epithelium and its response to infection with the intestinal nematode, Trichinella spiralis. Proteomics. 2004; 4: 1101–8.
13) Datta R, deSchoolmeester ML, et al. Identification of novel genes in intestinal tissue which are regulated post infection with an intestinal nematode parasite. Infect Immun. 2005; 73: 4025–33.
14) Kuperman DA, Lewis CC, et al. Dissecting asthma using focused transgenic modeling and functional genomics. J Allergy Clin Immunol. 2005; 116: 305–11.
15) Gu N, Kang G, et al. Intelectin is required for IL-13-induced monocyte chemotactic protein-1 and -3 expression in lung epithelial cells and promotes allergic airway inflammation. Am J Physiol Lung Cell Mol Physiol. 2010; 298(3): L290–6.
16) Zhen G, Park SW, et al. IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. Am J Respir Cell Mol Biol. 2007; 36(2): 244–53.
17) Pemberton AD, Rose-Zerilli MJ, et al. A single-nucleotide polymorphism in intelectin 1 is associated with increased asthma risk. J Allergy Clin Immunol. 2008; 122(5): 1033–4.
18) Malmberg H. Symptoms of chronic and allergic rhinitis and occurrence of nasal secretion granulocytes in university students, school children and infants. Allergy. 1979; 34: 389–94.
19) Zeiger RS, Heller S. development of nasal basophilic cells and nasal eosinophils from age 4 months through 4 years in children of atopic parents. J Allergy Clin Immunol. 1993; 91: 723–34.
20) Crobach MJ, Hermans J, et al. The diagnosis of allergic rhinitis: how to combine the medical history with the results of radioallergosorbent tests and skin prick tests. Scand J Prim Health Care. 1998; 16: 30–6.

Microarray analysis in nasal epithelial cells that are related with Japanese cedar pollinosis

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ABSTRACT

Seasonal allergic rhinitis caused by Japanese cedar pollen (SAR-JC) is one of the most common allergic diseases in Japan. To identify the genes that related to SAR-JC, we collected nasal samples by brushing inferior turbinate before and during pollen season in 2009. RNA profiles of the samples were analyzed by microarray analysis with Illumina 500GX (Illumina) Subjects were SAR-JC patients (SAR group) and controls without any allergic symptoms/environmental allergens specific IgE (Control group).

We defined over four times fold change between SAR-JC group and Control group as a significant. Eighteen genes were found to be significantly up-regulated after the exposure of allergen. The expression Intelectin 1 exhibited 17.4 times in SAR-JC group compared to Control group. Real-time reverse transcription-polymerase chain reaction (RT-PCR) verified the same result. We also got the same result in 2010 samples from SAR-JC group. Immunohistochemical staining of inferior turbinate showed that Intelectin1 was expressed in nasal epithelial mucous cells from patient with perennial allergic rhinitis. The stimulation of IL-4 and IL-13 induced Intelectin 1 expression by human cultured nasal epithelial cells. These results indicated that Intelectin 1 might be the candidate gene related to onset of allergic rhinitis.

Key words: microarray, quantitative real-time PCR, nasal epithelial cell, seasonal allergic rhinitis, immunohistochemical staining

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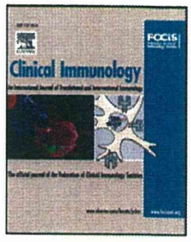

Sublingual administration of *Lactobacillus paracasei* KW3110 inhibits Th2-dependent allergic responses via upregulation of PD-L2 on dendritic cells

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KEYWORDS
Allergy;
Dendritic cells;
Vaccination;
Sublingual administration

Abstract Lactic acid bacteria have potential in immunomodulation therapy, but their clinical efficacy and underlying mechanisms are unclear. We aimed to clarify the anti-allergic immune responses induced by intragastric and sublingual administration of heat-killed *Lactobacillus paracasei* KW3110 and *Lactobacillus acidophilus* L-92. The KW3110 strain (but not the L-92 strain) enhanced ovalbumin (OVA)-induced expression of CCR-7 and PD-L2 in murine dendritic cells (DCs), and strongly inhibited IL-5 and IL-13 production *in vitro* in co-cultures with Th2-skewed $CD4^+$ T cells from DO11.10 transgenic mice. Sublingual administration of low-dose KW3110 (but not L-92) to OVA-sensitized mice selectively suppressed serum IgE production and Th2 cytokine expression in cervical lymph nodes, and significantly improved symptoms after OVA provocation *in vivo*. KW3110 probably accelerates DC migration into the regional lymph nodes and inhibits Th2 cytokine production through enhanced CCR-7 and PD-L2 expression. Thus, sublingual KW3110 administration may be effective in reducing allergic inflammation.

Abbreviations: PBMC, Peripheral blood mononuclear cell; SLIT, Sublingual immunotherapy; DC, Dendritic cell; OVA, Ovalbumin; BM, Bone marrow; LPS, Lipopolysaccharide; CLN, Cervical lymph node; ILN, Inguinal lymph node; ELISA, Enzyme-linked immunosorbent assay; ELISPOT, Enzyme-linked immunospot; TLR, Toll-like receptor; LGG, *Lactobacillus rhamnosus* GG; FACS, Fluorescein-activated cell sorter; FITC, Fluorescein isothiocyanate; GM-CSF, Granulocyte macrophage colony-stimulating factor; TCR, T cell receptor; OD, Optical density

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1. Introduction

An increased prevalence of allergic rhinitis has been observed in many countries over the recent years [1]. A variety of medications have been used to relieve the symptoms of this condition; however, these drugs do not treat the underlying disease and have a high risk of adverse events, particularly when taken over a long period [2]. Antigen-specific immunotherapy can effectively change the natural course of allergic disease, prevent the development of other allergic diseases, and reduce new allergic sensitization [3]. However, conventional subcutaneous administration necessitates frequent visits to the physician and is associated with a risk, albeit low, of anaphylactic shock [4].

The efficacy of lactic acid bacteria in suppressing the development of allergic diseases has been demonstrated [5]. For example, when expectant mothers were treated with *Lactobacillus rhamnosus* GG (LGG), and their infants were also treated with LGG from birth to 6 months of age, there was inhibition of the development of atopic dermatitis at ages 2 and 4 years [6]. In a study of infants with milk allergy treated with LGG, placebo or other *Lactobacillus* strains, LGG significantly enhanced IFN-γ production from peripheral blood mononuclear cells (PBMCs) [6]. However, in another study, there was no evidence of significant clinical benefit or differences in cytokine production from PBMCs in infants treated with LGG compared to a placebo [7].

Xiao et al. [8] observed marked improvements in nasal symptoms and modulation of Th2-skewed immune responses after 13 weeks of administration of the probiotic strain *Bifidobacterium longum* for treatment of Japanese cedar pollinosis. There is no other evidence of benefit after 22 weeks of administration of LGG for treatment of birch pollinosis [7]. The use of different probiotics and the study methodologies, such as dose, period of administration, and study sample size may explain these contradictory results. Intestinal health and factors such as diet and antibiotic therapy can also have a significant influence, which makes it difficult to evaluate the roles of probiotics [8,9].

In this study, we examined 2 strains of heat-killed lactic acid bacteria, *Lactobacillus paracasei* KW3110 and L-92, which are widely used in Japan and are reported to produce unique and different immune responses *in vitro*. KW3110 induces higher levels of IL-12 *in vitro* than the LGG strain, making it a more effective strain [9]. KW3110 may inhibit Th2 cytokine-mediated allergic inflammation through mechanisms independent of toll-like receptors (TLRs) [10,11], since the induction of IL-12 is not abrogated in bone marrow (BM)-derived dendritic cells (DCs) in TLR2-, TLR4- and TLR9-deficient mice [12]. L-92 activates DCs in a TLR2-dependent manner [13] and may exhibit anti-allergic activity through induction of regulatory T cells *in vivo* [14]. We administered the *Lactobacillus* strains directly to the oral mucosa, rather than as probiotics, and examined the influence of the KW3110 and L-92 strains on allergic responses in antigen-sensitized mice *in vivo* and *in vitro*.

We have previously observed higher CCR-7 and PD-L2 expression in mature DCs upon stimulation with KW3110 but not with L-92, which accelerated DC migration to draining lymph nodes and increased inhibitory signals for Th2 cytokine production. Similar immunomodulation was observed in cervical lymph nodes (CLNs), which play an important role in allergic rhinitis as draining lymph nodes, after sublingual administration of KW3110 *in vivo*. This therapeutic approach may therefore be effective for allergic rhinitis.

2. Material and methods

2.1. Mice

Seven-week-old female BALB *c* mice were purchased from SLC Inc. (Hamamatsu, Japan) and were maintained under specific pathogen-free conditions. The use of these mice was approved by the Chiba University Institutional Animal Care and Use Committee and the experiments were conducted in conformity with the guidelines of the committee.

2.2. Reagents and medium

The heat-killed KW3110 [11] and L-92 [14] strains were obtained from the Central Laboratories for Frontier Technology, Kirin Holdings Co. (Kanagawa, Japan), and from the R&D Center, Calpis Co. (Yokohama, Japan) respectively. Ovalbumin (OVA) (grade 5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). We used RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, L-glutamine (2 μM), penicillin (100 $U.mL^{-1}$), streptomycin (100 $\mu g.mL^{-1}$), HEPES (10 mM), 2-mercaptoethanol (55 μM), 1% non-essential amino acids, and 1 mM sodium pyruvate (all from Gibco BRL, Grand Island, NY, USA) in the cell culture experiments. OVA was dissolved in endotoxin-free D-PBS (Wako Pure Chemical Industries, Osaka, Japan), which had an endotoxin level below the detection limit (0.05 ELISA units per milligram protein). The reagents used for stimulation were also tested for endotoxin contamination; these also had levels below the detection limit.

2.3. Maturation of DCs

DCs from murine BM were cultured in a 100-mm bacteriological petri dish with 10 $ng.mL^{-1}$ granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ) [15]. The medium was replaced twice. The immature DCs were stimulated with 1 $mg.mL^{-1}$ of OVA in endotoxin-free PBS with or without 1 $\mu g.mL^{-1}$ KW3110 or L-92, and 10 $\mu g.mL^{-1}$ LPS (Lipopolysaccharide) (O111:B4, Sigma-Aldrich), and cultured for 24 h in 24-well plates at 1×10^6 cells per well. The DCs were analyzed by fluorescein-activated cell sorter (FACS) analysis of surface markers (FACSCalibur; Becton Dickinson, Sunnyvale, CA).

2.4. Phagocytosis by DCs *in vitro*

KW3110 or L-92 (1 mg of each) was suspended in 1 mL of 100 mM carbonate buffer (pH 9.5), reacted with fluorescein 5(6)-isothiocyanate (100 $\mu g.mL^{-1}$) (Sigma-Aldrich) at 37 °C for 60 min, and then washed with sterile PBS [16]. Fluorescein isothiocyanate (FITC)-labeled *Lactobacillus* (1 $\mu g.mL^{-1}$) was cultured with immature DCs (1×10^6 $cells.mL^{-1}$) for 24 h. After washing with PBS, the DCs were fixed with a cold methanol:acetone (1:1) solution for 10 min and then stained with biotin-labeled CD11c (N418; BioLegends, San Diego, CA, USA), followed by Cy5-coupled streptavidin. For

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FACS analysis, incubated DCs were stained with Cy5-labeled CD11c and MHC class II Alexa555, and then fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 (in 50 mM NaCl, 5 mM EDTA, and 0.02% NaN_3; pH 7.5) for 10 min on ice. After blocking with 3% BSA in PBS for 15 min, the cells were washed thoroughly with PBS supplemented with 1% FCS and 0.1% NaN_3. Phagocytosis of DCs was examined by confocal microscopy (Olympus, Tokyo, Japan) and FACS analysis. We used the fluorescence intensity of immature DCs after incubation for 24 h with labeled *Lactobacillus* as an indicator of *Lactobacillus* uptake.

2.5. Cytokine production in the co-culture of DCs with Th2 cells

Th2-skewed $CD4^+$ T cells were prepared from OVA-specific T cell receptor (TCR) (DO11.10) transgenic mice in a BALB *c* background, expressing TCR α/ß specific for OVA peptide, presented in the context of I-Ad [17]. Immature DCs (5×10^5) prepared from BALB *c* mice using the method described above, were pre-incubated with 0.4 μM $OVA_{323-339}$ peptide (Loh15) with 1 μg mL of KW3110 or L-92 for 24 h, and were then co-cultured with Th2-skewed $CD4^+$ T cells (1×10^5 cells) for 48 h. After preincubation, some DCs were treated for 30 min with 10 $\mu g.mL^{-1}$ of a neutralizing rat monoclonal antibody directed against PD-L2 (TY25 [18], rat IgG2a; BioLegends, San Diego, CA, USA) or a control isotype rat IgG2a monoclonal antibody. The cells were washed 3 times to remove excess antibody before co-culture. The culture supernatants were collected for ELISA analysis of cytokine production.

2.6. Migration of DCs after sublingual administration

We administered 0.5 mg of FITC-labeled KW3110 with 100 μg of OVA in 0.01 mL PBS sublingually to BALB *c* mice, using a micropipette tip placed under the tongue, while holding the back of the mouse for 20 s. The oral mucosa and CLNs were obtained, and freshly frozen 6-μm thick sections were fixed in a cold methanol:acetone (1:1) solution for 10 min. These were examined after staining with biotin-labeled CD11c followed by Cy5-coupled streptavidin.

2.7. *In vivo* immunization study protocol

BALB *c* mice were sensitized intraperitoneally with 100 μg of OVA and 4 mg of alum (Pierce, Rockford, IL, USA) once a week for 3 weeks. From 1 week after the last sensitization, 500 μg of OVA in 20 μL PBS was administered intranasally for 7 consecutive days [19]. Sensitized mice (n=18 in each group) received 5 mg or 0.5 mg of KW3110 or L-92 in 0.1 mL PBS, administered through a gavage tube for 7 consecutive days at the same time every day. A second group of sensitized mice (n=16 in each group) received 0.5 mg of KW3110 or L-92 in 0.01 mL PBS by sublingual administration for 7 consecutive days, as described above. After the last nasal administration of OVA, the behavior of the mice was recorded by video camera, and sneezing and nasal rubbing events were counted for 5 min [19]. The mice were then sacrificed and the serum, CLNs, spleens, inguinal lymph nodes (ILNs), and BM were collected. Serum OVA-specific IgE Ab was analyzed by ELISA.

2.8. Treatment with a neutralizing anti-PD-L2 antibody

We investigated the preventive effect of treatment *in vivo* with a neutralizing anti-PD-L2 antibody. The OVA-sensitized mice were injected intravenously with 250 μg of anti-mouse PD-L2 mAb (TY25, rat IgG2a) or rat control IgG2 mAb 12 h after the last sensitization. We then administered 0.5 mg of KW3110 sublingually for 7 consecutive days.

2.9. Analysis of cytokine production from $CD4^+$ T cells in CLNs

Single-cell suspensions were prepared from CLNs and incubated with biotinylated anti-CD4 antibody (GK1.5; BioLegends, San Diego, CA, USA) at 4 °C for 30 min, followed by incubation with anti-biotin beads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ cells were sorted using the MACS systems (Miltenyi Biotec) and suspended in complete RPMI 1640 medium. Cells were then cultured at a density of 1.5×10^5 cells per well in the presence of 1 $mg.mL^{-1}$ of OVA with $CD4^+$ T cell-depleted and irradiated splenic feeder cells (5×10^5 cells per well) in round-bottom 96-well microculture plates for 48 h. Cytokines in the supernatants were then analyzed by ELISA.

2.10. Detection of OVA-specific IgE-secreting cells

The number of OVA-specific IgE-secreting cells among CLN, ILN, spleen, and BM cells was evaluated in 96-well filtration plates (Multiscreen; Millipore Corp., Bedford, MA, USA) coated with 50 $\mu g.mL^{-1}$ OVA or BSA in PBS at 4 °C overnight, and then blocked with 10% FCS in RPMI. CLN cells (3×10^5 cells per well) were incubated on the plates at 37 °C under 5% CO_2. After incubation for 5 h, the plates were washed with PBS containing 0.1% Tween 20 (Sigma Chemical Co.) and then stained with alkaline phosphatase-conjugated anti-IgE antibodies (23 G3; Southern Biotechnology, Birmingham, AL, USA). Alkaline phosphatase activity was visualized using a BCIP/NBT phosphatase substrate (KPL, Gaithersburg, MD, USA). The plates were scanned and spots were counted automatically using an ELISPOT image analysis system (CTL Analyzers LLC, Cleveland, OH, USA).

2.11. ELISA analysis

Cytokine levels in the culture supernatant were measured by ELISA. Flat-bottom 96-well Nunc-Brand Immuno plates (Nalge Nunc International) were coated overnight at 4 °C with affinity-purified anti-IL-4 Ab (11B11), anti-IL-5 Ab (TRFK5) (all from Mabtech AB, Nacka, Sweden) or anti-IL-13 Ab (the ELISA Ready-SET-Go! Kit; eBioscience, San Diego, CA, USA). The wells were then washed and blocked with 1% BSA in PBS, and the culture supernatants were titered onto a treated plate and incubated overnight at 4 °C. Bound cytokines were detected using biotin-conjugated anti-IL-4 Ab (BVD6-24G2), anti-IL-5 Ab (TRFK4) (all Mabtech Ab), or anti-IL-13 Ab (eBioscience) followed by streptavidin–alkaline phosphatase

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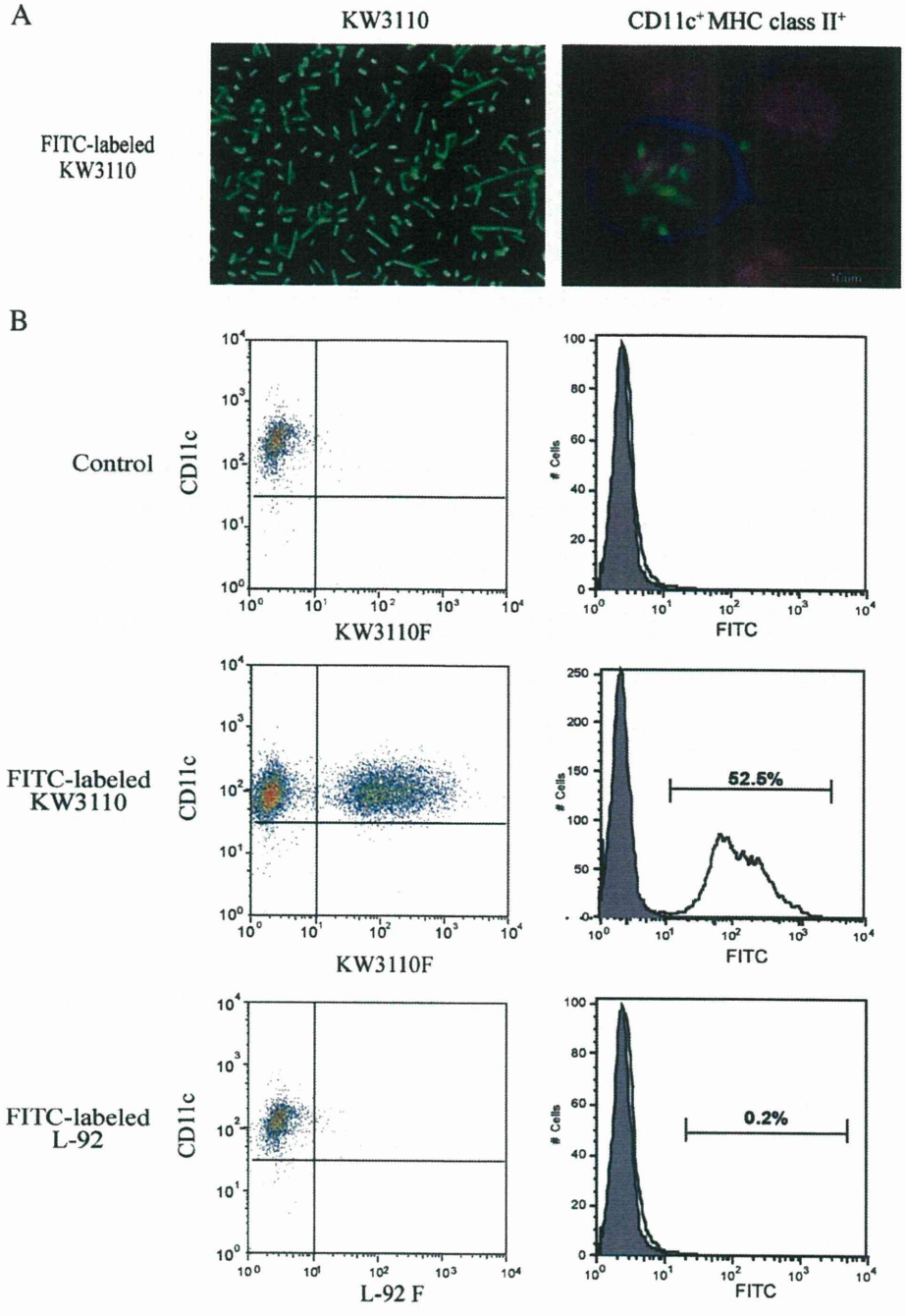

Figure 1 Phagocytosis of immature DCs analyzed by confocal microscopy (A) (green: KW3110, white: DAPI (nuclear), blue: CD11c-Cy5, red: MHC class II) and flow cytometry; (B) magnifications are 200× and 400× for the left and right images respectively. DCs phagocytosed many KW3110 bacteria in their intact form, and 52% of the cultured $CD11c^+$ DCs were found to contain FITC-labeled KW3110 after 24 h, based on FACS analysis. Only a few L-92 bacteria were phagocytosed.

(AP). The plates were developed with *p*-nitrophenyl phosphate tablets (Pierce), with the reaction terminated by the addition of 2N NaOH. Specific absorbance was measured and optical density (OD) was quantified at 410 nM using a Multiskan JX plate reader (Thermo LabSystems, Beverly, MA, USA). OVA-specific IgE was measured with a mouse anti-OVA IgE antibody assay kit (Chondrex, Redmond, WA, USA).

2.12. Flow cytometric analysis

Four-color cytometry was performed as follows. Briefly, cells were harvested from culture plates, preincubated with 10 $mg.mL^{-1}$ of unlabeled anti-CD16/32 (24G2) (BD Biosciences), and then stained on ice with a combination of Abs. We used combinations of FITC-conjugated mouse CD11c (N418)

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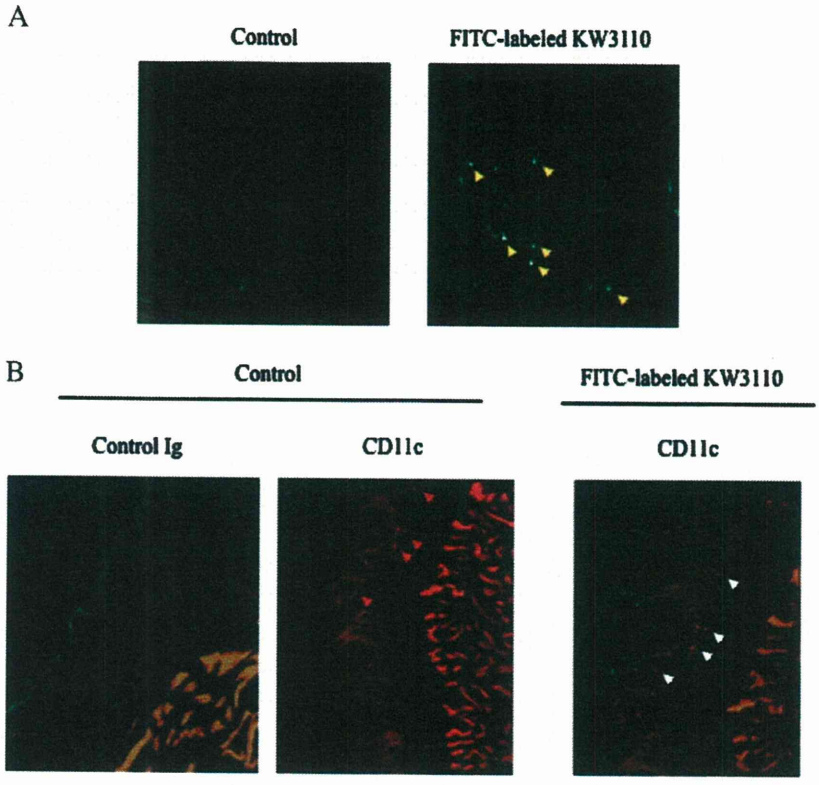

Figure 2 FITC-labeled KW3110 bacteria were detected in the oral mucosa 1 h after the sublingual administration, by confocal microscopy (A, yellow arrow). Many fragmented and some intact forms of KW3110 were observed to be phagocytosed by DCs in the double staining with anti-CD11c (B, white arrow). Magnification is 100× for all images.

(eBioscience) with PE-conjugated Abs to MHC Class II IA+IE (M5/114.15), CD80 (16-10A-1), CD86 (GL-1), B7RP-1 (HK5.3), PD-L2 (122), OX40L (RM134L), 4-1BBL (TKS-1) (all eBioscience), PD-L1 (9G2), or APC-conjugated CCR-7 (4B12) (BioLegend). Data were analyzed using the Cellquest software (Becton Dickinson).

2.13. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. Data are shown as mean ± SEM or SD.

3. Results

3.1. KW3110 enhanced CCR-7 and PD-L2 expression

After incubation for 24 h with LPS, L-92, or KW3110, the expression of MHC class II^{high}, CD40, CD80, CD86, B7RP-1 and PD-L1 similarly increased; however, that of CCR-7 and PD-L2 was enhanced only by KW3110 in OVA-stimulated DCs, and not by LPS or L-92 (data not shown). Administration of *Lactobacillus* without OVA stimulation induced a low level of the surface markers, and the concentration of *Lactobacillus* and LPS required for expression of the highest levels of these markers with OVA stimulation was determined to be 10 $\mu g.mL^{-1}$ in a preliminary analysis.

3.2. KW3110 was phagocytosed in the intact form by DCs

As shown in Figure 1A, the DCs phagocytosed KW3110 bacteria in the intact form, and 52% of cultured $CD11c^+$ DCs showed uptake of FITC-labeled KW3110 over 24 h based on FACS analysis (Figure 1B). In contrast, only a few L-92 bacteria were phagocytosed, and even the uptake of FITC was very low.

3.3. KW 3110 decreased cytokine production from Th2 cells

In co-culture study of DCs and Th2-skewed $CD4^+$ T cells from DO11.10 Tg mice, KW3110 decreased IL-5 and IL-13 production (from 0.210 ± 0.13 pg/mL to 0.041 ± 0.06 pg/mL for IL-5, and from 0.206 ± 0.35 pg/mL to 0.082 ± 0.19 pg/mL for IL-13, respectively); however, L-92 did not have this influence. IL-4 production was low, with no difference between various stimulations. Treatment of KW3110-treated DCs with anti-PD-L2 antibodies abrogated the inhibition of IL-5 and IL-13 production. The level of PD-L2 on DCs was the same with stimulation by OVA alone and with L-92 in OVA-stimulated DCs. In the response provoked by OVA alone, the PD-L2 antibody did not affect IL-5 or IL-13 production.

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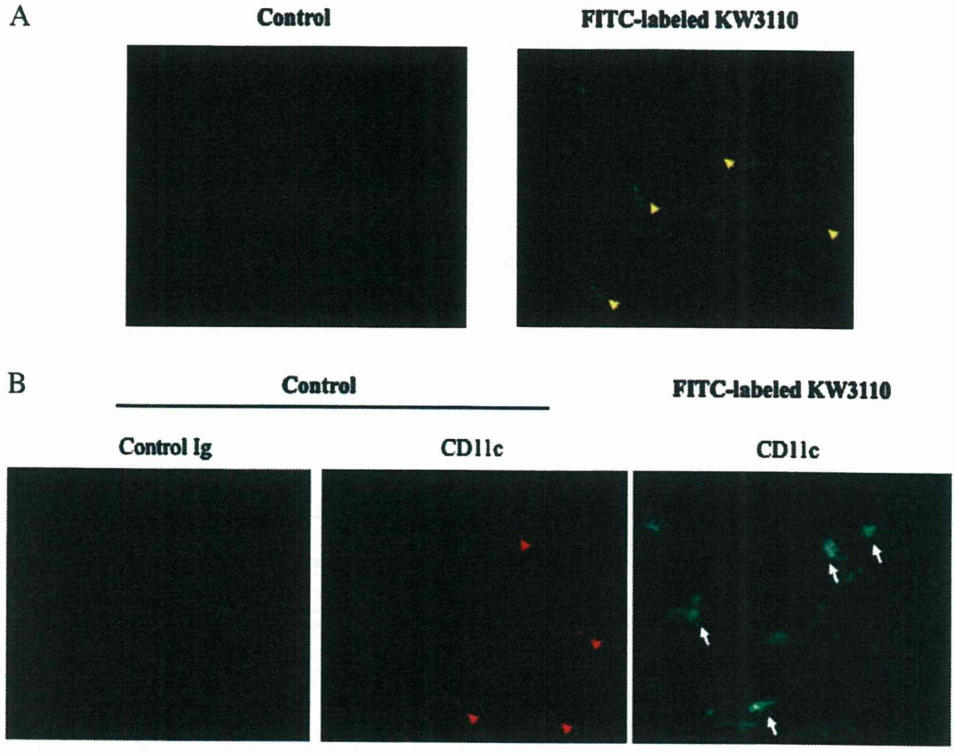

Figure 3 At 3 h after sublingual administration of FITC-labeled KW3110 stimulated with OVA, KW3110 bacteria were detected in CLNs by confocal microscopy (A, yellow arrow). CD11c-positive cells were identified by treatment with biotin-labeled anti-CD11c$^+$ (B, red arrow). Many fragmented and some intact KW3110 (CD11c$^+$ FITC$^+$ cells) bacteria were phagocytosed by DCs, as identified by double staining (B, white arrow), but were not found in the CLNs of control mice that did not receive KW3110. Magnification is 400× for all images.

3.4. Sublingually administered KW3110 migrated to CLNs

FITC-labeled KW3110 was detected in the oral mucosa 1 h after the sublingual administration, by confocal microscopy (Figure 2A). The presence of CD11c-positive cells was shown by treatment with biotin-labeled anti-CD11c. Double staining with anti-CD11c showed that many fragmented and some intact KW3110 (CD11c$^+$ FITC$^+$ cells) organisms were phagocytosed by DCs (Figure 2B). Similarly fragmented and some intact forms of KW3110 were phagocytosed by DCs in CLNs 3 h after administration (Figure 3). Twelve hours later, these KW3110 were rarely detected in the oral mucosa and the CLNs.

3.5. KW3110 attenuated the nasal symptoms and serum IgE level in the OVA sensitized mice through PD-L2 expression

After OVA nasal provocation, the frequency of sneezing and nasal rubbing events decreased significantly in mice that received sublingual administration of KW3110 at 0.5 mg/day compared with those that received PBS (Table 1A). This response was dose-dependent and was stronger in mice receiving 0.5 mg.mL^{-1} KW3110 compared to those receiving a 0.05 mg.mL^{-1} dose. The response reached a plateau at a dose of 0.5 mg.mL^{-1} and there was no significant difference between mice receiving 0.5 and 5 mg.mL^{-1} KW3110 (data not shown). The frequency of sneezing and nasal rubbing events was not decreased in OVA-sensitized mice receiving sublingual L-92 or 0.5 mg of intragastric KW3110. Nasal symptoms were decreased to some extent at a ten-fold higher intragastric dose (5 mg.day^{-1}) of KW3110, but the effect was less marked than for sublingual administration at 0.5 mg/day (data not shown). Serum OVA-specific IgE was significantly reduced in mice receiving sublingual KW3110 but not in others, including those receiving intragastric KW3110 at 5 mg.day^{-1}. Treatment of OVA-sensitized mice with anti-PD-L2 mAb before KW3110 sublingual administration abrogated the effects of KW3110 (Table 1B).

3.6. Sublingually administered KW3110 decreased Th2 cytokine production and the number of IgE-secreting cells in CLNs of sensitized mice

Cytokine production from CD4$^+$ T cells in CLNs is shown in Table 2B. Of the Th2 cytokines examined, the IL-4, IL-5, and IL-13 levels were reduced in CD4$^+$ T cells from mice that received KW3110 sublingually, but not in those from mice that received intragastric KW3110. Reduction of IFN-γ or enhancement of IL-10 was not observed in CD4$^+$ T cells from any mice.

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Table 1A Influence of intragastric administration via a gavage tube or sublingual administration of KW3110 or L-92 on nasal symptoms and serum OVA-specific IgE and on cytokine production in CD4+ T cells in CLNs.

		Sneezing		Nasal rubbing		Serum anti-OVA IgE	
		Counts		Counts		(ng /ml)	
Nasal symptoms and serum IgE							
Gavage tube (0.5 mg)	PBS	61±15	n.s.	48±08	n.s.	425±015	n.s.
(n=18 in each group)	KW3110	51±13	n.s.	54±08	n.s.	478±029	n.s.
	L92	68±23		44±06		468±087	
Sublingual. (0.5mg)	PBS	55±06	*	48±10	*	568±054	*
(n=16 in each group)	KW3110	23±05		20±03	n.s.	278±054	
	L92	50±15	*	35±05		457±084	*
Non immunized mice (control)		10±04	**	15±11	**	n.d.	

Values are presented as the mean±SD. *P<0.05; n.s., not significant; n.d., not detectable. **P<0.01, compared with data from other immunized group.

Table 1B Influence of intragastric administration via a gavage tube or sublingual administration of KW3110 or L-92 on nasal symptoms and serum OVA-specific IgE and on cytokine production in CD4+ T cells in CLNs.

		IL-4		IL-5		IL-13		IFN-Ω		IL-10	
		(ng /ml)		(ng /ml)		(ng /ml)		(ng /ml)		(ng /ml)	
Cytokine production in CD4+ T cells derived from cervical lymph nodes											
Gavage tube (0.5 mg)	PBS	0.48±0.07	n.s.	2.55±0.56	n.s.	3.47±0.97	n.s.	23.08±2.98	n.s.	0.23±0.04	n.s.
(n = 18 in each group)	KW3110	0.51±0.18	n.s.	2.22±0.58	n.s.	3.89±0.18	n.s.	29.25±6.24	n.s.	0.18±0.04	n.s.
	L92	0.39±0.18		2.93±0.43		4.07±0.58		12.53±3.14		0.28±0.05	
Sublingual. (0.5 mg)	PBS	0.52±0.08	*	2.47±0.48	*	4.62±0.08	*	24.62±4.08	n.s.	0.12±0.06	n.s.
(n = 16 in each group)	KW3110	0.22±0.13		1.09±0.23		1.57±0.13		22.16±0.13		0.15±0.03	
	L92	0.36±0.03	*	2.38±0.17	*	3.17±0.71	*	28.23±3.46	n.s.	0.14 ± 0.02	n.s.
Non-immunized mice (control)		n.d.		n.d.		** n.d.		n.d.		n.d.	

Values are presented as the mean±SD. *P<0.05; n.s., not significant; n.d., not detectable.

The number of IgE-secreting cells was higher in CLNs than in BM cells, ILNs, and the spleen from OVA-sensitized mice (Figure 4A). This number was significantly decreased in CLNs from mice that received submucosal administration of KW3110. Treatment with anti-PD-L2 antibody abrogated the reduction of Th2 cells (Tables 2A and 2B) and IgE-secreting cells (Figure 4B).

4. Discussion

DCs play a crucial role in the induction of primary T-cell-dependent immune responses [20]. Upon uptake of antigens on the mucosal surface, immature DCs in the oral mucosa are thought to migrate to the regional CLNs [21]. This suggests that the oral mucosa provides a potential route for vaccine administration and thus the interaction of oral DCs with T cells in regional lymph nodes is of interest [22,23]. In this study, we showed that *Lactobacillus* spp. administered sublingually might migrate to the CLNs and influence the maturation of DCs induced by uptake of antigen, but that the patterns differ significantly among strains.

KW3110 strongly enhanced the expression of CCR-7 and PD-L2 on DCs, compared to L-92. Sublingual administration of low doses of KW3110 in OVA-sensitized mice *in vivo* decreased IgE production and nasal symptoms induced by nasal OVA provocation, whereas the same dose of the L-92 strain or intragastric administration of KW3110 had no effect. Th2 cytokine expression and IgE synthesis in CLNs (the major area of antigen presentation and induction of effector cells in the OVA-sensitized mice) were strongly suppressed by sublingual KW3110 but not by L-92 treatment. Pre-treatment with neutralizing antibodies to PD-L2 abrogated the anti-allergic effects of sublingual KW3110. Earlier studies have shown that CCR-7 expression accelerates migration of DCs to the regional lymph nodes [24]. It is also reported that PD-L2 expression on DCs is associated with inhibition of Th2 cytokine production [18,24,25]. Matsumoto et al. showed that PD-L2 was highly expressed on pulmonary DCs and macrophages of the allergic sensitized mice, and administration of anti-PD-L2 antibody enhanced airway hyperreactivity and production of Th2 cytokines [18]. These observations suggest that DCs with high CCR-7 expression that phagocytose KW3110 migrate to the CLNs and subsequently suppress production of Th2 cytokines and IgE, mainly through expression of PD-L2.

The anti-allergic actions of L-92 observed in previous studies were brought about by induction of Treg through TLR2 [13]. However, L-92 may not stimulate DCs directly to induce CCR-7 or PD-L2, and may not effectively activate DCs in the oral submucosa when administered sublingually, due to poor

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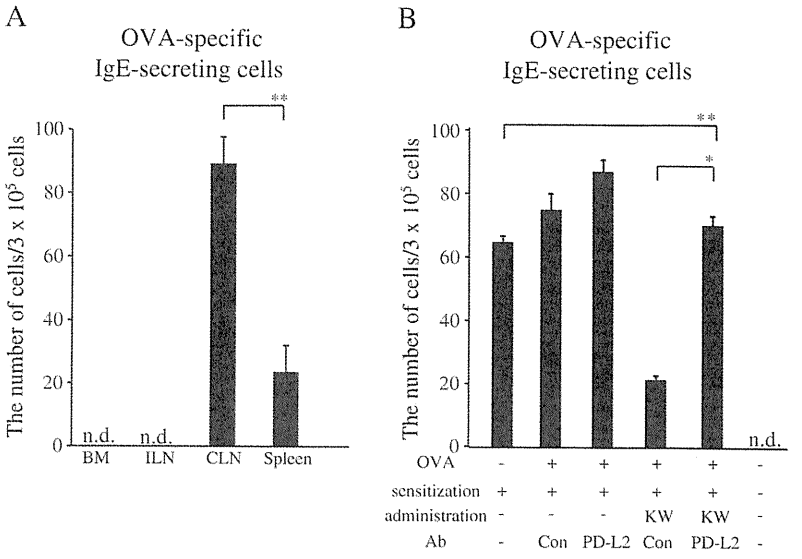

Figure 4 Inhibition of Ag-specific IgE production by KW3110. The number of OVA-specific IgE-secreting cells among cells from lymphoid organs and BM was analyzed by ELISPOT (A). Anti-PD-L2 treatment before oral submucosal administration of KW3110 blocked the reduction of IgE production in cells derived from CLNs (B).

phagocytosis. *In vitro*, DCs phagocytose a higher number of KW3110 organisms than L-92, as shown in this study. This may be because the L-92 strain is twice as large as the KW3110 strain. It is unclear if regulatory T cells were induced by sublingual administration of KW3110, because IL-10 induction or suppression of IFN-γ was not observed in the CLNs. Although the different responses among the strains of *Lactobacillus* may reflect different surface compositions and interactions with different TLRs, the roles of TLR2 or TLR9 in the immune responses induced by KW3110 are unclear [12].

KW3110 is used widely as an additive in yoghurt, beverages, and other foods, and its safety has been certified. However, in our previous study of patients with Japanese cedar pollinosis, 12 weeks intake of 50 mg day^{-1} of heat-killed KW3110, which was commenced 4 weeks before pollen dispersal, had only a limited clinical effect [26]. The KW3110 in that study was administered in capsule form; this is digested and dissolved by gastric juice or digestive enzymes, but the metabolites are unknown. In the present study, there was no benefit from intragastric administration of KW3110, but direct administration of the same dose of intact KW3110 to the oral mucosa did have an effect, with improvement in symptoms and reduced Th2 cytokine production.

5. Conclusions

Our results suggest that sublingual administration of KW3110 may reduce allergic inflammation, but the optimum dose and administration protocol require clarification. A recent review of randomized controlled studies of sublingual immunotherapy (SLIT) for allergic rhinitis suggests that this approach is safe and may be effective as an alternative route of administration [27]. Improved efficacy of SLIT could be achieved by adjuvant therapy with sublingual administration of KW3110, since the anti-allergic effects of sublingual administration were enhanced by simultaneous stimulation with an antigen in this study. A clinical study in patients with allergic rhinitis will be performed to examine this hypothesis, and may yield further information on the potential of *Lactobacillus* therapy.

Table 2A Effect of anti-PD-L2 antibody treatment after KW3110 sublingual administration.

Antigen	Administration	Neutralizing Ab	Sneezing Counts		Nasal rubbing Counts		Serum anti-OVA IgE (ng/ml)	
Nasal symptoms and serum IgE								
–	–	–	61±9		68±11		651±051	
OVA	–	Control Ig	52±12	n.s.	67±07	n.s.	695±037	n.s.
OVA	–	PD-L2	64±13	n.s.	70±19	n.s.	677±017	n.s.
OVA	KW3110	Control Ig	18±05	*	24±08	*	202±058	*
OVA	KW3110	PD-L2	49±07	n.s.	54±17	n.s.	623±015	n.s.
Non-immunized mice (control)			01±02	**	05±03	**	n.d.	

Values are presented as the mean ± SD. Number of mice in each group was 18. *P<0.05; n.s., not significant; n.d., not detectable. **P<0.01, compared with data from other immunized group.

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Table 2B Effect of anti-PD-L2 antibody treatment after KW3110 sublingual administration.

Antigen	Stimulation	Neutralizing Ab	IL-4 (ng/ml)		IL-5 (ng/ml)		IL-13 (ng/ml)	
Cytokine production in CD4+ T cells derived from cervical lymph nodes								
–	–	–	0.41±0.13	n.s.	2.94±0.14	n.s.	4.16±0.14	n.s.
OVA	–	Control Ig	0.34±0.05	n.s.	2.83±0.58	n.s.	3.15±0.25	n.s.
OVA	–	PD-L2	0.49±0.19	n.s.	2.80±0.32	n.s.	3.49±0.36	n.s.
OVA	KW3110	Control Ig	0.14±0.04	*	0.78±0.07	*	1.08±0.09	*
OVA	KW3110	PD-L2	0.48±0.09		2.56±0.27		3.17±0.14	
Non immunized mice (control)			n.d.		n.d.		n.d.	

Values are presented as the mean±SD. Number of mice in each group was 18. *P<0.05; n.s., not significant; n.d., not detectable.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

[1] J. Bousquet, P. van Cauwenberge, N. Khaltaev, Allergic rhinitis and its impact on asthma, J. Allergy Clin. Immunol. 108 (2001) S147–S334.
[2] E. Valovirta, S.E. Myrseth, S. Palkonen, The voice of the patients: allergic rhinitis is not a trivial disease, Curr. Opin. Allergy Clin. Immunol. 8 (2008) 1–9.
[3] P.S. Norman, Immunotherapy: 1999–2004, J. Allergy Clin. Immunol. 113 (2004) 1013–1023.
[4] R.F. Lockey, G.L. Nicoara-Kasti, D.S. Theodoropoulos, S.C. Bukantz, Systemic reactions and fatalities associated with allergen immunotherapy, Ann. Allergy Asthma Immunol. 87 (2001) 47–55.
[5] S.L. Prescott, B. Bjorkstén, Probiotics for the prevention or treatment of allergic diseases, J. Allergy Clin. Immunol. 120 (2007) 255–262.
[6] E. Pohjavuori, M. Viljanen, R. Korpela, M. Kuitunen, M. Tiittanen, O. Vaarala, E. Savilahti, *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy, J. Allergy Clin. Immunol. 114 (2004) 131–136.
[7] G.I. Betsi, E. Papadavid, M.E. Falagas, Probiotics for the treatment or prevention of atopic dermatitis: a review of the evidence from randomized controlled trials, Am. J. Clin. Dermatol. 9 (2) (2008) 93–103.
[8] J.Z. Xiao, S. Kondo, N. Yanagisawa, N. Takahashi, T. Odamaki, N. Iwabuchi, K. Miyaji, K. Iwatsuki, H. Togashi, K. Enomoto, T. Enomoto, Probiotics in the treatment of Japanese cedar pollinosis: a double-blind placebo-controlled trial, Clin. Exp. Allergy 36 (2006) 1425–1435.
[9] H. Johannsen, S.L. Prescott, Practical prebiotics, probiotics and synbiotics for allergists: how useful are they? Clin. Exp. Allergy 39 (2009) 1801–1814.
[10] L. Van Overtvelt, H. Moussu, S. Horiot, S. Samson, V. Lombardi, L. Mascarell, A. van de Moer, R. Bourdet-Sicard, P. Moingeon, Lactic acid bacteria as adjuvants for sublingual allergy vaccines, Vaccine 28 (2010) 2986–2992.
[11] D. Fujiwara, S. Inoue, H. Wakabayashi, T. Fujii, The anti-allergic effects of lactic acid bacteria are strain dependent and mediated by effects on both Th1/Th2 cytokine expression and balance, Int. Arch. Allergy Immunol. 135 (2004) 205–215.
[12] S. Ichikawa, R. Fujii, D. Fujiwara, Y. Komiyama, T. Kaisho, M. Sakaguchi, Y. Konishi, MyD88 but not TLR2, 4 or 9 is essential for IL-12 induction by lactic acid bacteria, Biosci. Biotechnol. Biochem. 71 (2007) 3026–3032.
[13] G. Weiss, S. Rasmussen, L.H. Zeuthen, B.N. Nielsen, H. Jarmer, L. Jespersen, H. Frøkiaer, *Lactobacillus* acidophilus induces virus immune defence genes in murine dendritic cells by a Toll-like receptor-2-dependent mechanism, Immunology 131 (2010) 268–281.
[14] A. Torii, S. Torii, S. Fujiwara, H. Tanaka, N. Inagaki, H. Nagai, *Lactobacillus acidophilus* strain L-92 regulates the production of Th1 cytokine as well as Th2 cytokines, Allergol. Int. 56 (2007) 293–301.
[15] C. Scheicher, M. Mehlig, R. Zecher, K. Reske, Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor, J. Immunol. Methods 154 (1992) 253–264.
[16] K. Shida, J. Kiyoshima-Shibata, M. Nagaoka, K. Watanabe, M. Nanno, Induction of interleukin-12 by *Lactobacillus* strains having a rigid cell wall resistant to intracellular digestion, J. Dairy Sci. 89 (2006) 3306–3317.
[17] M.Y. Kimura, H. Hosokawa, M. Yamashita, A. Hasegawa, C. Iwamura, H. Watarai, M. Taniguchi, T. Takagi, S. Ishii, T. Nakayama, Regulation of T helper type 2 cell differentiation by murine Schnurri-2, J. Exp. Med. 201 (2005) 397–408.
[18] K. Matsumoto, H. Inoue, T. Nakano, M. Tsuda, Y. Yoshiura, S. Fukuyama, F. Tsushima, T. Hoshino, H. Aizawa, H. Akiba, D. Pardoll, N. Hara, H. Yagita, M. Azuma, Y. Nakanishi, B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism, J. Immunol. 172 (2004) 2530–2541.
[19] K. Takamura, S. Fukuyama, T. Nagatake, D.Y. Kim, A. Kawamura, H. Kawauchi, H. Kiyono, Regulatory role of lymphoid chemokine CCL19 and CCL21 in the control of allergic rhinitis, J. Immunol. 179 (2007) 5897–5906.
[20] B.N. Lambrecht, Dendritic cells and the regulation of the allergic immune response, Allergy 60 (2005) 271–282.
[21] J.H. Song, J.I. Kim, H.J. Kwon, D.H. Shim, N. Parajuli, N. Cuburu, C. Czerkinsky, M.N. Kweon, CCR7-CCL19/CCL21-

regulated dendritic cells are responsible for effectiveness of sublingual vaccination, J. Immunol. 182 (2009) 6851–6860.
[22] J.P. Allam, B. Niederhagen, M. Bücheler, T. Appel, H. Betten, T. Bieber, S. Bergé, N. Novak, Comparative analysis of nasal and oral mucosa dendritic cells, Allergy 61 (2006) 166–172.
[23] U.H. von Andrian, T.R. Mempel, Homing and cellular traffic in lymph nodes, Nat. Rev. Immunol. 3 (2003) 867–878.
[24] Y. Latchman, C.R. Wood, T. Chernova, et al., PD-L2 is a second ligand for PD-1 and inhibits T cell activation, Nat. Immunol. 2 (2001) 261–268.
[25] E. Oflazoglu, D.A. Swart, P. Anders-Bartholo, H.K. Jessup, A.M. Norment, W.A. Lawrence, K. Brasel, J.E. Tocker, T. Horan, A.A. Welcher, D.R. Fitzpatrick, Paradoxical role of programmed death-1 ligand 2 in Th2 immune responses in vitro and in a mouse asthma model in vivo, Eur. J. Immunol. 34 (2004) 3326–3336.
[26] S. Yonekura, Y. Okamoto, T. Okawa, M. Hisamitsu, H. Chazono, K. Kobayashi, D. Sakurai, S. Horiguchi, T. Hanazawa, Effects of daily intake of *Lactobacillus* paracasei strain KW3110 on Japanese cedar pollinosis, Allergy Asthma Proc. 30 (2009) 397–405.
[27] P. Moingeon, T. Batard, R. Fadel, F. Frati, J. Sieber, L. Van Overtvelt, Immune mechanisms of allergen-specific sublingual immunotherapy, Allergy 61 (2006) 151–165.

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