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原　著

ダニアレルゲンワクチン標準化に関する日本アレルギー学会タスクフォース報告

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【背景】 1990年代に日本アレルギー学会はスギ花粉アレルゲン標準化を行った．本報はダニアレルゲン標準化について報告する．

【方法】 アレルゲン力価は51名のダニIgE抗体陽性の日本人成人の皮内反応試験により決定した．エキスのIgE結合力価は10名分の患者プール血清を用いた競合ELISAにより，主要アレルゲン含量はサンドイッチELISAにより決定し，両者の相関性を解析した．

【結果】 学会標準品とそのアレルゲン力価を決定した．IgE結合力価はグループ1（Der 1），グループ2アレルゲン含量，その合計値のそれぞれと相関し，主要アレルゲン含量測定が代替試験となりうることが示された．

【結論】 1）学会標準品を選定し，以下を決定した：2）学会標準品のアレルゲン力価およびDer 1含量はそれぞれ100000JAU/mlおよび38.5μg/mlである．3）Der 1含量測定を代替***in vitro***試験とする．4）製造者はDer 1含量が22.2–66.7μg/mlのエキスの力価を100000JAU/mlと表示できる．

Key words: house dust mite allergen standardization —— intradermal testing —— *in vivo* allergenic potency —— major allergen content —— surrogate *in vitro* assay

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Abbreviations: **AU** "allergy unit", **BAU** "bioequivalent allergy unit", **CBER** "Center for Biologics Evaluation and Research", **Der 1** "Der p 1 and Der f 1", **Der 2** "Der p 2 and Der f 2", **DF** "*Dermatophagoides farinae*", **DP** "*Dermatophagoides pteronyssinus*", **FDA** "Food and Drug Administration", **HDM** "house dust mite", **JAU** "Japanese allergy unit", **JSA** "Japanese Society of Allergology", **UAS** "universal allergen standard"

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緒 言

アレルゲン特異的免疫療法については1911年にNoonによる最初の報告があり，以来，一般診療として施行されている[1)-6)]．治療と診断に使用されるアレルゲンワクチン（またはアレルゲンエキス）は，製造方法や製造ロットによって品質が異なるため，標準化が必要である[2)-4)7)-9)]．アメリカ合衆国では，アレルゲン標準化はアレルギー患者の皮内反応試験に基づき行われているが，製造ロットの力価は適切な代替 *in vitro* 試験（固相化した標準アレルゲンエキスへの患者プール血清中IgEの結合に対する阻害活性や特定のアレルゲンの含量の測定）によって決定される[7)]．欧州では，製品は製造業者の社内標準品を用いて標準化され，製造業者独自の単位によって力価表示される[8)]．日本では，日本アレルギー学会（JSA）がスギ花粉アレルゲンワクチンの標準化を行った[10)11)]．JSA標準品スギ花粉エキスのアレルゲン力価は皮内反応試験によって決定され，主要アレルゲンのひとつであるCry j 1の含量測定がJSA標準品以外のスギ花粉エキスの力価を決定するための代替 *in vitro* 試験として採用されている．

室内塵ダニ（house dust mite；以下は単にダニと記す）はアレルギー性鼻炎，喘息，結膜炎，アトピー性皮膚炎といったアレルギー疾患におけるアレルゲンの主要な供給源である[12)13)]．しかしながら，日本人の血清中ダニ特異的IgE陽性者の試験に基づいたダニアレルゲン標準化は行われていない．本報告はJSAのアレルゲン・免疫療法委員会の設置したダニアレルゲン標準化タスクフォースによって作成された．本研究においてタスクフォースは，JSA標準品ダニエキスを選定し，日本人のダニ感作者の皮内反応試験によりそのアレルゲン力価をJAU（Japanese allergy unit）単位で決定するとともに，日本におけるダニアレルゲン標準化に適した代替 *in vitro* 試験を選定した．

方 法

1) ダニアレルゲンエキス

各ダニアレルゲンエキス（以下は単にダニエキスと記す）は2種のダニ，すなわちヤケヒョウヒダニ（*Dermatophagoides pteronyssinus*；DP）とコナヒョウヒダニ（*Dermatophagoides farinae*；DF）に由来するエキスの等体積を混合して調製した．アメリカ合衆国食品医薬品局（Food and Drug Administration；FDA）標準品エキスであるE11-DP（10000AU/ml）およびE10-DF（10000AU/ml）は，FDAのLaboratory of Immunobiochemistry，Division of Bacterial，Parasitic and Allergenic Products，Office of Vaccines Research and Review，Center for Biologics Evaluation and Research（生物学的製剤評価研究センター；CBER）より入手した．皮下注射免疫療法用の市販製剤（DP：lot#B 3117094およびDF：lot#F21G6279，10000AU/ml）はHollister-Stier社（Spokane，WA，USA）より購入した．アルファベットで示した5種類のエキス（Extracts A-E）はALK-Abelló AS社（Hørsholm，Denmark）またはStallergenes SA社（Antony，France）から提供を受けた．タスクフォースは上述以外のエキスをJSA標準ダニエキスとして選択した．これらのダニエキスのグループ1アレルゲン含量の測定およびIgE結合相対力価の測定は相模原病院（Laboratory 1）および麻布大学（Laboratory 2）の2つの研究室で行われた．グループ2アレルゲン総含量の測定はLaboratory 1でのみ行われた．

2) 対象者

本研究の対象の組み入れ基準を，ImmunoCAP試験（ThermoFisher Scientific社，Uppsala，Sweden）において血清中のDPまたはDF特異的IgE抗体が陽性で0.70 kUa/L（クラス2）以上を示した20歳以上50歳以下の日本人成人であることとした．以下を除外基準とした．(a) 皮内反応試験を実施する前腕部に皮膚試験に影響を与えるような皮膚疾患を有する者；(b) 皮内反応試験実施日前に以下の薬剤を使用している者：(1) 検査部位への外用剤（1日以内），(2) 経口抗ヒスタミン薬，経口α刺激薬，経口β刺激薬，検査部位への外用ステロイド薬または外用免疫抑制薬（7日以内），(3) 抗ヒスタミン作用をもつ三環系抗うつ薬またはフェノチアジン系抗精神薬（14日以内），(4) 非選択的β遮断薬（21日以内），(5) 全身性免疫抑制薬（30日以内），(6) 抗体薬（90日以内）；(c) 検査実施日に妊娠している者または妊娠している可能性のある者；

(d) 検査実施前にダニエキスまたはハウスダストエキスの免疫療法を受けたことがある者；(e) 重症喘息の者；(f) アドレナリンに対する過敏症の既往のある者；(g)臨床研究の実施に影響するような心臓，肝臓，腎臓，血液疾患の合併症ならびに感染症を有すると判断される者；(h) 研究実施者によって臨床研究への参画を不適当と判断された者.

皮内反応試験および血液採取は，千葉大学大学院医学研究院耳鼻咽喉科・頭頸部腫瘍学，日本医科大学耳鼻咽喉科，埼玉医科大学呼吸器内科，山梨大学大学院医学工学総合研究部耳鼻咽喉科・頭頸部外科において，各施設の倫理委員会の承認を経て実施された．前もって対象者全員から署名による同意を得ると共に，倫理委員会の定めた書式と方法を用いて匿名性を保持した.

3) 皮内反応試験

まずJSA標準品ダニエキスの候補となるエキスを選定し，それを用いて皮内反応試験を実施した．候補エキスを0.005%日局ポリソルベート80を添加した診断用アレルゲン皮内エキス対照液トリイ® (鳥居薬品株式会社，東京)により希釈し，3倍希釈系列のエキスを作製した．希釈倍率3^7倍(2187倍)～3^{19}倍(1.162×10^9倍)を作製した．診断用アレルゲン皮内エキス対照液トリイを陰性対照として用いた.

過去に記載された方法に従って皮内反応試験を実施した[10)11)]．方法は，閾値判定基準を除いて概ねFDA方式に基づいている．以下に簡単に記す．ツベルクリン用シリンジ(1ml)を用いて，各薬剤の低濃度から順に高濃度へと前腕部へ20μlを皮内投与した．閾値判定は石崎の判定基準に従った[14)]．すなわち，投与15分後に投与部位の膨疹径9mm以上または発赤20mm以上を陽性と判定し，初めて陽性判定となった希釈倍率を閾値希釈倍率とした.

アレルゲン力価の決定については，まず3を底とする閾値希釈倍率の対数を閾値と定め，全対象者における閾値の平均値を求めた．そして，スギ花粉エキス標準化[10)11)]のときと同様に，その値が9以上11未満，11以上13未満，13以上15未満であれば，そのエキスの力価をそれぞれ1000JAU/ml，10000JAU/ml，100000JAU/mlと設定するものとした．この方法では，アレルゲン力価が最大で3^2倍（9倍）異なる複数エキスが同一の力価と判定される可能性がある.

4) ダニグループ1主要アレルゲン含量の測定

モノクローナル抗体を用いたサンドイッチELISAによるダニエキス中のグループ1アレルゲン（Der p 1およびDer f 1）含量測定は，以前に報告された方法に従って行った[15)]．ELISA用スタンダード抗原として，92-*Dp*および92-*Df*[15)16)]と名付けられたダニエキスをそれぞれ用いた．92-*Dp*および92-*Df*は過去にDPおよびDFのダニ虫体からそれぞれ調製されたものであり，その主要アレルゲン含量が既知である[15)16)]．本測定系の妥当性を検証する目的で，他の方法，すなわちIndoor Biotechnologies Inc.社(Charlottesville, VA, USA）およびニチニチ製薬株式会社（三重）から購入したELISAキットを用いたグループ1アレルゲン含量の測定を行った.

5) ダニグループ2主要アレルゲン含量の測定

Der p 2およびDer f 2の含量を合計したグループ2アレルゲン総含量（Der 2）の測定は以前に報告された方法に従って行った[17)18)]．簡単に記すと，Der 2のサンドイッチELISAによる測定を，ウサギポリクローナル抗体と，ELISA用スタンダード抗原としてダニエキス92-*Dp*と92-*Df*[15)16)]の混合液を用いて行った.

6) IgE結合相対力価の測定に使用した血清

ImmunoCAP試験（ThermoFisher社）において血清中のDPまたはDF特異的IgE抗体が陽性で17.5 kUa/L（クラス4）以上を示した19名の患者のうちよりランダムに選択した10名から20mlを採血した．血液の遠心後，血清を分離回収し−80℃にて保存した．この10名分の血清を等量混合し，プール血清を作製した.

7) IgE結合相対力価の測定

アレルゲン特異的IgEの結合阻害試験は，以前に報告された方法に従って，競合ELISAにより行った[19)]．以下に簡単に記す．ELISAプレートをJSA標準品ダニエキスまたはExtract Cを用いてコートした．プール血清（50倍希釈）を，阻害剤としての被験エキス段階希釈液と等体積比で混合し（最終血清希釈倍率100倍），30分間の室温静置の後にプレートのウェルへ添加した．アレルゲン特異的IgEの結合は酵素標識抗ヒトIgE抗体および蛍光基質によって検出した．最大蛍光強度（エキス添加なし）の50%蛍光強度を与えるエ

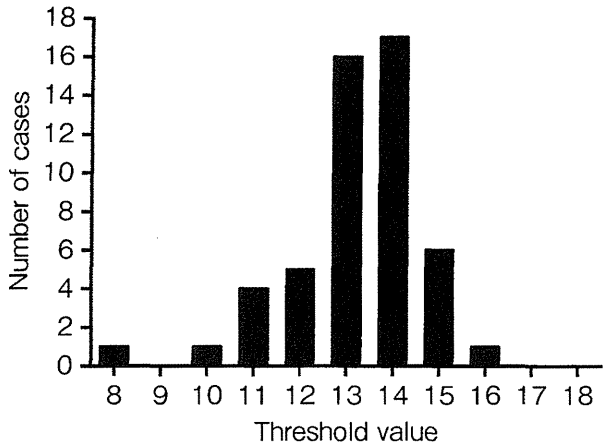

Fig. 1. Distribution of the threshold values that induced positive reactions in the intradermal testing of 51 Japanese adults with positive serum specific IgE to house dust mite allergens. Prepared dilution factors of the Japanese Society of Allergology reference house dust mite extract were from 3^7 (2187) to 3^{19} (1.162×10^9). A total of 20 μl of each diluted extract was administered intradermally into the forearm from low to high concentrations. The diameters for wheal or erythematous skin reaction were measured 15 min after the injection. Threshold value: the exponent of the maximum dilution factor that can induce positive reaction in each subject.

キス希釈倍率の，被験エキスとJSA標準品ダニエキスとの間の比として，被験エキスの相対力価を算出した．

8) 統計解析

主要アレルゲン含量とIgE結合相対力価の対数変換後に，両者の相関性についてピアソンの相関係数を算出した．$p<0.05$の場合に統計的有意差有りと判定した．

結　果

1) JSA標準品ダニエキスの選定

皮内反応試験を行うことが可能であり，かつ日本国内で承認される予定があるエキスを，JSA標準品候補エキスとして選択した．後述のように，この候補エキスは適切なアレルゲン力価および主要アレルゲン含量（グループ1およびグループ2アレルゲン）を保持していた．よって，この候補エキスをJSA標準品ダニエキスとすることを決定した．

2) JSA標準品ダニエキスのアレルゲン力価の測定

52名のダニ特異的IgE陽性の日本人成人を皮内反応試験の対象とした．性別は男性24名，女性28名．年齢（平均±SD）は32.9±6.5歳．原疾患は42名（80.7%）が通年性アレルギー性鼻炎で，そのうち5名に喘息の合併，4名にスギ花粉症の合併を認めた．その他，喘息症例4名，咳喘息1名であった．1名は閾値の確認ができないため，解析から除外した．51名の皮内反応の閾値の度数分布を**Fig. 1**に示す．閾値の希釈倍率指数の平均値（±SD）は13.22（±1.43）であり，これは100000 JAU/mlのアレルゲン力価に相当した．

3) JSA標準品ダニエキスの主要アレルゲン含量

ダニエキス92-*Dp*および92-*Df*は，過去にLaboratory 1において調製され，その主要アレルゲン（Der p 1, Der p 2, Der f 1, およびDer f 2）の各含量が既に決定されている[15)16)]．その92-*Dp*および92-*Df*をELISA用スタンダード抗原としたサンドイッチELISAにより，JSA標準品ダニエキスのダニグループ1およびグループ2主要アレルゲン含量を測定した（**Table 1**）．グループ1アレルゲン測定に関し，2施設で同様の結果が得られた．JSA標準品ダニエキスのDer p 1, Der f 1, および両者の合計含量（Der 1）の，2施設で得られた値の幾何平均はそれぞれ25.6μg/ml，12.9μg/ml，および38.5μg/mlであった．Laboratory 1で得られたDer p 2とDer f 2の合計含量（Der 2）の値は55.5μg/mlであった．

4) Der p 1およびDer f 1含量の測定系の妥当性の検証

本研究におけるDer p 1およびDer f 1含量測定の妥当性を，他のELISA系を用いて検証した（**Table 2**）．2種類の市販のELISAキットを用いて同様の結果を得た．Indoor社製のキットを用いた場合，約20%低い測定値が得られた．このキットが採用しているELISA用スタンダード抗原は“universal” allergen standard（UAS）と名付けられており，欧州連合のCREATE projectによって

Table 1 House dust mite major allergen content in the Japanese Society of Allergology reference house dust mite extract.

Institute	Concentration (μg/ml)			
	Group 1 allergens			Group 2 allergens
	Der p 1	Der f 1	Der 1	Der 2
Laboratory 1	28.2	14.1	42.3	55.5
Laboratory 2	23.2	11.8	35.0	N.D.
Geometric mean	25.6	12.9	38.5	

Der p 1, Der f 1, and Der 2 were measured by sandwich ELISA in two institutes. Der 1, combined total of Der p 1 and Der f 1; Der 2, combined total of Der p 2 and Der f 2; Laboratory 1, Sagamihara National Hospital; Laboratory 2, Azabu University; N.D., not determined.

Table 2 Comparison of house dust mite group 1 allergen contents in the Japanese Society of Allergology reference house dust mite extract determined by sandwich ELISAs using different antibodies and standards.

Institute	ELISA		Concentration (μg/ml)		
	Antibodies[1]	Standard[2]	Der p 1	Der f 1	Der 1
Lab1	Lab1	92-*Dp*/92-*Df*	28.2	14.1	42.3
	Lab1	UAS	22.1	9.69	31.8
	Indoor	92-*Dp*/92-*Df*	27.4	14.6	42.0
	Indoor	UAS	22.3	11.7	34.0
	Nichinichi	Nichinichi	24.3	13.6	37.9
Lab2	Lab1	92-*Dp*/92-*Df*	23.2	11.8	35.0
	Indoor	92-*Dp*/92-*Df*	22.1	14.0	36.1

Der p 1 and Der f 1 were measured by sandwich ELISA in two institutes. [1]Capture and detection antibodies used in ELISA. [2]Standards with defined concentrations used in ELISA. Der 1, combined total of Der p 1 and Der f 1; Lab1, Sagamihara National Hospital; Lab2, Azabu University; UAS, universal allergen standard; Indoor, Indoor Biotechnologies, Ltd.; Nichinichi, Nichinichi Pharmaceuticals, Inc.

開発されたものである[20)21)]. Indoor社キット添付抗体（**Table 2**, Indoor）あるいはLaboratory 1で開発された抗体（**Table 2**, Lab1）のいずれを使用した場合でも，ELISA用スタンダード抗原としてUASを使用（**Table 2**, UAS）すると92-*Dp*と92-*Df*の使用時（**Table 2**, 92-*Dp*/92-*Df*）に比べて低い測定値が得られた．よって，この測定値の小さなずれの原因は，モノクローナル抗体の特異性などの検出系の差異によるものではなく，ELISA用スタンダード抗原として用いた92-*Dp*/92-*Df*とUASの間でDer p 1およびDer f 1濃度の定義が異なるためであることが示唆された．これらの結果より，Laboratory 1において開発されたDer p 1およびDer f 1含量測定系の妥当性が示された．

5）被験ダニエキスの主要アレルゲン含量

JSA標準品ダニエキス以外の7種類のダニエキスの主要アレルゲン含量を測定した（**Table 3**）．5種類のエキス（Extract A-E）はALK社またはStallergenes社より，2種類のエキスはCBER/FDAとHollister-Stier社より入手したものであ

Table 3 House dust mite major allergen contents and *in vitro* total IgE binding potencies of the eight house dust mite extracts determined in Laboratory 1.

Sample	Concentration (μg/ml)[1]					*In vitro* relative potency[2]	
	Der p 1	Der f 1	Der 1	Der 2	Der 1 + Der 2	JSA coating	Extract C coating
JSA	28.2	14.1	42.3	55.5	97.8	1.00	1.00
CBER/FDA	16.7	16.3	33.0	19.6	52.6	0.56	0.51
Hollister-Stier	10.1	2.54	12.6	11.3	23.9	0.35	0.33
Extract A	80.0	54.2	134	254	388	4.89	4.85
Extract B	2.69	0.98	3.67	6.76	10.4	0.14	0.13
Extract C	30.9	52.7	83.6	45.8	129	1.83	2.54
Extract D	3.59	2.50	6.09	2.38	8.47	0.08	0.16
Extract E	2.27	5.45	7.72	3.28	11.0	0.15	0.26

[1]Der p 1, Der f 1, and Der 2 were measured by sandwich ELISA. [2]*In vitro* total IgE binding potency relative to the Japanese Society of Allergology (JSA) reference house dust mite extract was determined on the basis of the results of a competition ELISA that measures the inhibition of allergen-specific IgE binding to plates coated with the JSA reference house dust mite extract (JSA coating) or Extract C (Extract C coating). Der 1, combined total of Der p 1 and Der f 1; Der 2, combined total of Der p 2 and Der f 2. JSA, JSA reference house dust mite extract.

Table 4 House dust mite group 1 allergen contents and *in vitro* total IgE binding potencies of the eight house dust mite extracts determined in Laboratory 2.

Sample	Concentration (μg/ml)			*In vivo* relative potency	
	Der p 1	Der f 1	Der 1	JSA coating[1]	Extract C coating[2]
JSA	23.2	11.8	35.0	1.00	1.00
CBER/FDA	17.6	13.2	30.8	0.71	0.75
Hollister-Stier	9.25	1.79	11.0	0.34	0.34
Extract A	80.5	49.5	130	5.75	5.71
Extract B	2.28	0.93	3.21	0.14	0.14
Extract C	38.8	54.2	93.0	1.47	2.11
Extract D	3.40	4.08	7.48	0.11	0.16
Extract E	2.24	6.87	9.11	0.12	0.20

[1]Der p 1, Der f 1, and Der 2 were measured by sandwich ELISA. [2]*In vitro* total IgE binding potency relative to the Japanese Society of Allergology (JSA) reference house dust mite extract was determined on the basis of the results of a competition ELISA that measures the inhibition of allergen-specific IgE binding to plates coated with the JSA reference house dust mite extract (JSA coating) or Extract C (Extract C coating). Der 1, combined total of Der p 1 and Der f 1; Der 2, combined total of Der p 2 and Der f 2. JSA, JSA reference house dust mite extract.

る．2施設において同様の結果を得た（**Table 3** および **Table 4**, Concentration）．

6) 主要アレルゲン含量と IgE 結合相対力価の相関性

JSA 標準品ダニエキスまたは Extract C を固相化した ELISA プレートへのアレルゲン特異的

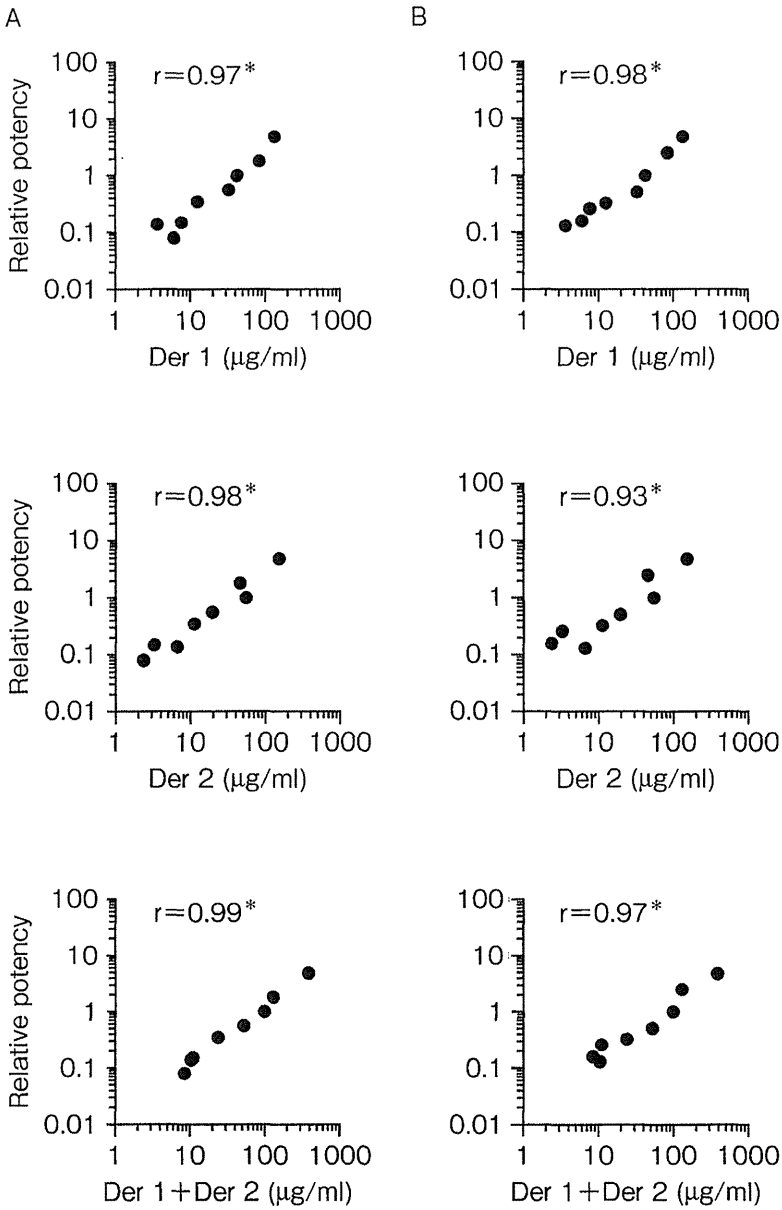

Fig. 2. Correlation between house dust mite major allergen contents and *in vitro* total IgE binding potencies of the eight house dust mite extracts determined in Laboratory 1. The values in Table 3 were used for the analysis. *In vitro* total IgE binding potency relative to the Japanese Society of Allergology (JSA) reference house dust mite extract was determined based on the results of the competition ELISA that measured the inhibition of allergen-specific IgE binding to plates coated with the JSA reference house dust mite extract (A) or Extract C (B). r: Pearson correlation coefficient. *: $p<0.01$.

IgE 結合の阻害を，競合 ELISA により測定した．ImmunoCAP 試験において血清中の DP または DF 特異的 IgE 抗体が陽性で 17.5 kUa/L（クラス 4）以上を示した 19 名の患者のうち 10 名の血清を用いてプール血清を作製し使用した．ダニエキスの総 IgE 結合力価の JSA 標準品ダニエキスに対する相対値を決定した（**Table 3** および **Table 4**, *In vitro* relative potency）．固相化したエキスが

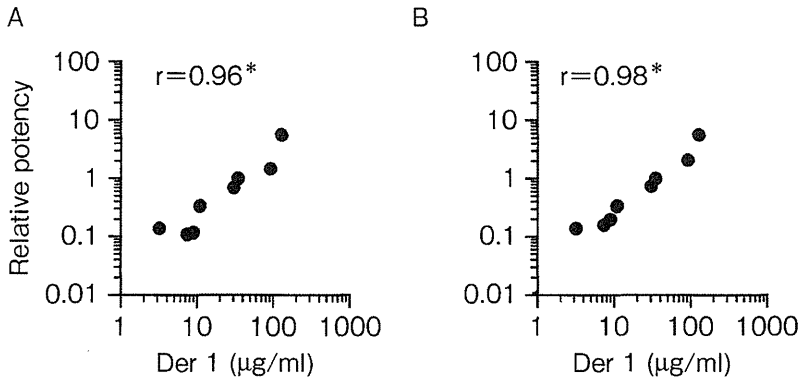

Fig. 3. Correlation between house dust mite group 1 allergen contents and *in vitro* total IgE binding potencies of the eight house dust mite extracts determined in Laboratory 2. The values in Table 4 were used for the analysis. *In vitro* total allergenic potency relative to the Japanese Society of Allergology (JSA) reference house dust mite extract was determined based on the results of the competition ELISA thet measured the inhibition of allergen-specific IgE binding to plates coated with the JSA reference house dust mite extract (A) or Extract C (B). r: Pearson correlation coefficient. *: $p<0.01$.

JSA 標準品ダニエキスである場合と Extract C である場合とで，同様の結果が得られた．2 施設において同様の結果を得た．

主要アレルゲン含量と IgE 結合相対力価の相関性を解析したところ，IgE 結合相対力価と Der 1（**Fig. 2** および **Fig. 3**），Der 2，およびその合計含量（Der 1＋Der 2）（**Fig. 2**）とのピアソンの相関係数は統計的有意差をもって 0.9 以上と算出され，いずれとも高い相関が得られた．

考　察

1990 年代に JSA はスギ花粉アレルゲンワクチンの標準化を行った[10)11)]．JSA 標準品スギ花粉エキスは 10000JAU/ml のアレルゲン力価を示した．JAU 単位[10)11)]は FDA による bioequivalent allergy unit（BAU）および allergy unit（AU）[7)22)-24)]と同様の皮内反応試験によって決定されるが，反応閾値の判定基準は JAU と BAU/AU の間では異なり，注射体積は JAU では 20μl であるのに対し BAU および AU では 50μl である．JSA は Cry j 1 含量測定を JSA 標準品以外のスギ花粉エキスの表示力価を決定するための代替 *in vitro* 試験として選定し，Cry j 1 12.5μg/ml が 10000JAU/ml に対応すると定義した．さらに，Cry j 1 含量が 12.5μg/ml を中心とした 3 倍範囲内（7.3–21μg/ml）であれば 10000JAU/ml と力価表示することができると決定した[10)11)]．本研究では，JSA 標準品ダニエキスを選定し，そのアレルゲン力価をスギ花粉アレルゲン標準化と同じ方法によって JAU 単位を用いて決定した．さらに，8 種類のダニエキスを用いて，IgE 結合阻害 ELISA に基づく *in vitro* 総力価と主要アレルゲン含量の間の相関性を解析した．

本研究において，51 名のダニ特異的 IgE 陽性の日本人成人の皮内反応試験の結果に基づき，JSA 標準品ダニエキスの候補としたエキスのアレルゲン力価は 100000JAU/ml と決定された．この候補エキスは適切なアレルゲン力価（**Fig. 1**）および主要アレルゲン含量（**Table 1**）を保持していたので，これを JSA 標準品ダニエキスとして選定した．製造業者やロットが異なるエキスの力価表示のための代替 *in vitro* 試験は，アレルギー患者プール血清中 IgE の標準品アレルゲンエキスへの結合の阻害活性またはアレルゲンワクチン中の特定のアレルゲンの含量の測定によって行うことができる．アメリカ合衆国では，ダニとカビについては IgE 結合阻害試験によって決定される IgE 結合相対力価を，ブタクサ（short ragweed）とネコについ

ては特定のアレルゲン含量（それぞれAmb a 1とFel d 1）を，ハチ毒については酵素活性（ヒアルロニダーゼとホスホリパーゼ）を，FDAが代替*in vitro*試験として採用している[7]．主要アレルゲン含量とIgE結合相対活性が相関することを報告している文献が存在する[25)-27)]．20種類以上のグループのダニアレルゲンの中で，グループ1およびグループ2アレルゲンが主要アレルゲンであると考えられている（ただし，これらには及ばないが他のダニアレルゲンも重要とする報告もある）[12)13)]．本研究においてIgE結合相対力価と各主要アレルゲン含量の間に高い相関関係が認められた（**Fig. 2**および**Fig. 3**）．ダニ主要アレルゲンの測定がIgE結合相対力価測定と同様に代替*in vitro*試験として適切であることがわかった．

代替*in vitro*試験としての主要アレルゲン含量測定は，いくつかの利点を持つ．主要アレルゲン含量は相対値ではなく絶対値として決定することが可能であり，その測定には患者によって力価が変動する血清を必要としない．2種のダニに由来するグループ1アレルゲン（Der p 1とDer f 1）は，モノクローナル抗体を用いてダニの種特異的に正確に測定することが可能な複数のELISA系が既に確立されており，そのようなELISA系を市販品（「方法」参照）として入手することもできる．しかし，グループ2アレルゲン（Der p 2とDer f 2）については種特異的に測定することが可能なELISA系は入手しやすい状況にはない．本研究において，ポリクローナル抗体を用いたELISAによって決定したDer p 2とDer f 2の合計含量（Der 2）は近似値である．よって，本タスクフォースはDer p 1とDer f 1の合計含量（Der 1）の測定を代替*in vitro*試験として選定することとした．JSA標準品ダニエキスのアレルゲン活性およびDer 1含量をそれぞれ100000JAU/mlおよび38.5 μg/mlと決定したが，さらに，前回のスギ花粉アレルゲン標準化と同様に[10)11)]，Der 1含量が38.5 μg/mlを中心とした3倍範囲内（22.2–66.7μg/ml）であれば，製造業者は100000JAU/mlと力価表示することができると決定した．

Der p 1およびDer f 1測定系の妥当性を検証した（**Table 2**）．ダニエキス92-*Dp*および92-*Df*のグループ1およびグループ2アレルゲンのそれぞれの含量は，以前にLaboratory 1において決定された[15)16)]．92-*Dp*および92-*Df*の主要アレルゲン含量を決定するときにELISA用スタンダード抗原として用いた精製グループ1およびグループ2アレルゲンの濃度は，280 nmでの吸光度に基づいて決定されている．UASは8種類の精製アレルゲン（Der p 1, Der f 1, Der p 2, Fel d 1, Can f 1, Rat n 1, Mus m 1, およびBla g 2）から構成され，それらの濃度はアミノ酸分析に基づいて決定されている[20)21)]．Laboratory 1で開発されたELISA系と比較してIndoor Biotechnologies社製キットによって低い測定値（20% 減）が得られたが（**Table 2**），これはELISA用スタンダード抗原として用いたアレルゲンの元々の濃度決定手段の相違が原因ではないか，と推定された．

結論として，本タスクフォースは本研究において下記の事項の実施または決定を行った．

(1) JSA標準品ダニエキスを選定し，そのアレルゲン力価を皮内反応試験により決定した．

(2) IgE結合力価と主要アレルゲン含量が相関することを示し，Der 1含量測定を他のダニエキスの力価表示のための代替*in vitro*試験とすること（Der 1含量38.5μg/mlが100000JAU/mlに相当すると定義）を決定した．

(3) 製造業者はDer 1含量が22.2–66.7μg/mlであるダニエキスの力価を100000JAU/mlと表示することができる（3倍の許容幅），と定めた．

Der 1含量は，例えば本報告に記載した市販品キット（「方法」参照）を用いたサンドイッチELISAなどの，適切な方法による測定が許容される．JSA標準品ダニエキスはダニアレルゲンワクチン製造業者によるDer p 1含量およびDer f 1含量の測定のためのELISA用スタンダード抗原として有用であり，JSAはJSA標準品ダニエキスの保管と供給を計画中である．

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JAPANESE SOCIETY OF ALLERGOLOGY TASK FORCE REPORT ON STANDARDIZATION OF HOUSE DUST MITE ALLERGEN VACCINES

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Background: In the 1990s, the Japanese Society of Allergology (JSA) standardized Japanese cedar pollen allergen vaccines. In the present study, the task force for house dust mite (HDM) allergen standardization of the Committee for Allergens and Immunotherapy of JSA reports the standardization of HDM allergen vaccines in Japan.

Methods: *In vivo* allergenic potency was determined by intradermal testing of 51 Japanese adults with positive serum specific IgE to HDM allergens. *In vitro* total IgE binding potency was analyzed by the competitive ELISA using a pooled serum, with sera obtained from 10 allergic patients. Concentrations of HDM group 1 (Der 1) and group 2 major allergens in eight HDM allergen extracts were measured by sandwich ELISAs. Correlation between the *in vitro* total IgE binding potency and major allergen levels was analyzed.

Results: We selected a JSA reference HDM extract and determined its *in vivo* allergenic potency. The *in vitro* total IgE binding potency significantly correlated with Der 1 content, group 2 allergen content, and their combined amount, indicating that measurement of major allergen contents can be used as a surrogate *in vitro* assay.

Conclusions: The task force determined the *in vivo* allergenic potency (100000JAU/ml) and Der 1 content (38.5μg/ml) of the JSA reference HDM extract, selected the measurement of Der 1 content as the surrogate *in vitro* assay, and decided that manufacturers can label a HDM allergen extract as having a titer of 100000JAU/ml if it contains 22.2–66.7μg/ml of Der 1.

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Interleukin-25 and mucosal T cells in noneosinophilic and eosinophilic chronic rhinosinusitis

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ABSTRACT

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a heterogeneous disease of uncertain pathogenesis. Memory T cells acquire additional functions during the secondary response and play important roles in chronic inflammation.

Objective: To investigate characteristics of tissue memory $CD4^+$ T cells obtained from patients with noneosinophilic CRSwNP (NECRS) and eosinophilic CRSwNP (ECRS) by focusing on the influence of interleukin (IL)-25.

Methods: Pro-allergic cytokines in tissue homogenates were measured using enzyme-linked immunosorbent assays. NP mononuclear cells and $CD4^+$ T cells were isolated from NPs from patients with CRSwNP. Cytokine expression and $CD4^+$ T-cell subpopulations were analyzed using enzyme-linked immunosorbent assay, flow cytometry, and real-time polymerase chain reaction.

Results: The IL-25 level in NPs increased in patients with ECRS. IL-5 and IL-9 mRNA levels expressed by tissue $CD4^+$ T cells were significantly elevated in patients with ECRS. Most infiltrating $CD4^+$ T cells in ECRS and NECRS expressed CD45RO; however, regardless of the atopic status, high IL-17RB levels were detected in $CD4^+$ T cells from patients with ECRS. IL-17RB mRNA levels expressed by tissue $CD4^+$ T cells significantly correlated with the number of eosinophils in NPs. Elevation of IL-5 and IL-9 production was found in NP mononuclear cells from patients with ECRS, but not in those from patients with NECRS, by stimulation with IL-25 under T-cell receptor stimulation.

Conclusion: Interleukin-25 and a subpopulation of tissue T-helper type 2 and 9 cells that express increased IL-17RB levels could contribute to infiltration of eosinophils in NPs and could have produced the pathologic difference between NECRS and ECRS.

Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous, commonly occurring, chronic inflammatory disease of the sinus. Clinically, CRS is classified into 2 types based on the presence of nasal polyps (NPs). CRS without NPs (CRSsNP) involves T-helper cell type (T_H) 1-skewed inflammation based on histologic and immunologic studies.[1,2] The prognosis is generally satisfactory. In contrast, CRS with NPs (CRSwNP) is characterized by T_H2-skewed inflammation. Surgery is often required to treat NPs and relapse is expected. However, recent studies have described a type of CRSwNP in East Asia that is not characterized by eosinophilic inflammation and exhibits T_H1-skewed inflammation in NPs.[3–5] Noneosinophilic CRSwNP (NECRS) differs from eosinophilic CRSwNP (ECRS) in the comorbidity rate of asthma and postoperative recurrence.[3,6,7] In Western countries, approximately 80% of NPs are eosinophilic, but the mechanism underlying eosinophilic inflammation remains unclear.[4,8] Therefore, comparing these types of CRSwNP in East Asia could be useful for determining the pathogenesis of CRSwNP.

Various types of T cells play important roles in the regulation of chronic inflammation. $CD4^+$ T-cell lineages, such as T_H1, T_H2, and T_H17, and induced regulatory T cells, are defined by their cytokine

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production patterns.[9] Although immunologic analyses of T-helper cytokines present in NPs have been performed, detailed analyses of tissue $CD4^+$ T-cell populations in patients with ECRS or NECRS are lacking. Interleukin (IL)-9–producing $CD4^+$ T cells represent a distinct T-helper subset that has not been extensively studied in CRS.[10,11] The authors previously published data showing an increase in T_H2 cells in NPs obtained from patients with ECRS compared with NPs from those with NECRS but did not study T_H9.[12] T_H9 cells might increase eosinophilic, allergic inflammation. These cells express the transcription factor PU.1, a factor required for T_H9 development, and mouse models have demonstrated that IL-9 enhances allergic airway inflammation and remodeling.[11]

Memory T cells are localized in peripheral tissues and are activated in an antigen-specific manner, but memory T_H2 cells also contribute to innate immunity in allergy.[13] IL-25 is an epithelium-derived cytokine that causes eosinophilic inflammation by T_H2 or T_H9 cells by its receptor IL-17RB.[14–17] IL-25 production by airway epithelial cells is induced in response to various antigens and pathogens, and it causes T_H2-skewed inflammation in a T_H2-specific, antigen-independent manner. In nasal disease, allergic rhinitis is induced by specific allergen exposure and IgE-mediated inflammation. Local allergic reaction might be involved in the pathogenesis of allergic rhinitis and CRSwNP, but the putative allergen, if it exists, has not been discovered in most CRS cases, excluding superantigens or allergic fungal rhinosinusitis.[5,18,19] In patients with asthma and those with eosinophilic granulomatosis with polyangiitis, IL-25 levels are elevated in the respiratory tract and peripheral blood, respectively.[20,21] However, the role of IL-25 in CRS has not been established.

Thus, to investigate the mechanisms of eosinophilic inflammation in CRSwNP, the present study examined IL-25 expression and the characteristics of NP tissue $CD4^+$ T cells including T_H9.

Methods

Patients

Patients with CRSwNP were recruited from Chiba University Hospital, Kimitsu Chuo Hospital, and the Chiba Otolaryngology Surgical Center (Chiba, Japan). All patients met the criteria for CRSwNP defined by the European position paper on rhinosinusitis and NPs.[1] Patients' characteristics are presented in Table 1. All patients signed informed consent forms, and the study was approved by the ethics committees of the Chiba University Graduate School of Medicine and each of the participating hospitals.

All patients underwent computed tomographic (CT) scanning to confirm the diagnosis of CRS and were assigned scores according to the CT scoring system of Lund and Mackay.[22] NP tissues were obtained during endoscopic sinus surgery. Inferior nasal turbinates for use as controls were obtained during turbinectomy from patients with no sinusitis based on CT scan. The diagnosis of asthma was based on a history of wheezing responsive to bronchodilator and confirmatory spirometry demonstrating reversibility. Aspirin-exacerbated respiratory disease was based on a history of respiratory symptoms after ingestion of aspirin or other nonsteroidal anti-inflammatory drugs. Patients were not administered systemic corticosteroids for at least 4 weeks before surgery. Atopic status was evaluated based on clinical history and the presence of IgE specific for house dust mite, Japanese cedar pollen, orchard grass pollen, ragweed pollen, and *Candida, Aspergillus, Alternaria, Penicillium,* and *Cladosporium* species detected using the ImmunoCAP method (Phadia AB, Uppsala, Sweden). CRSwNP was classified as ECRS when the tissue eosinophil count was higher than 70 per high-power field (HPF), with reference to the results of Nakayama et al.[6] The number of tissue eosinophils in an HPF ×400 was counted in a blinded manner by 2 observers.

Immunohistochemistry

Formaldehyde-fixed NPs and control samples were embedded in paraffin. Sections were rehydrated, and antigen retrieval was performed using protease digestion for 5 minutes. The sections were treated with 0.3% H_2O_2, blocked, and then incubated overnight with an anti-IgE antibody diluted to 1:50 (Santa Cruz Biotechnology, Inc, Santa Cruz, California) or controls. This was followed by incubation with a secondary antibody, an antimouse immunoglobulin conjugated to a peroxidase-labeled polymer (En Vision; Dako, Carpinteria, California) for 30 minutes, visualization with 3,3′-diaminobenzidine tetrahydrochloride for 5 minutes, and counterstaining with hematoxylin. The number of tissue IgE^+ cells per HPF was counted in a blinded manner and is presented as the sum of 10 HPFs. For immunofluorescence, samples were fixed in 4% paraformaldehyde and equilibrated in a final concentration of 20% sucrose in phosphate buffered saline. Frozen sections were boiled in Histo VT One (Nacalai Tesque, Kyoto, Japan) for antigen retrieval and blocked by 3% bovine serum albumin and human FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). The sections were incubated overnight with anti–IL-25 (1:200; Lifespan Biosciences, Seattle, Washington), anti–IL-17RB (1:200; Lifespan Biosciences), anti-CD3 (1:100; Abcam, Cambridge, Massachusetts), anti–mast cell tryptase (1:200; Abcam), anti–eosinophil cationic protein (ECP; 1:100; Santa Cruz Biotechnology), or anti–platelet endothelial cell adhesion molecule-1 (PECAM-1; 1:100; Santa Cruz

Table 1
Characteristics of patients[a]

	Control	CRSwNP (n = 69)					
		Nonatopic vs atopic			Noneosinophilic vs eosinophilic		
		Nonatopic	Atopic	*P* value	NECRS	ECRS	*P* value
Patients, n	16	24	45		33	36	
Age (y), median	37	60	52		54	53	
Men/women, n	11/5	13/11	33/12		22/11	24/12	
Asthma (early onset, age <16 y), n	2	1	4	.65	2	3	1.0
Asthma (late onset, age ≥16 y), n	1	7	11	.78	3	15	.0025
Aspirin intolerance, n	0	2	4	1.0	0	6	.026
Atopic status, n	14	0	45		20	25	.46
Blood total IgE level, IU/mL	160	267	516	.020	302	548	.12
Tissue IgE^+ cell count/10 HPFs	35	27	41	.44	7	63	<.001
Blood eosinophil count/mm^3	327	331	398	.25	222	514	<.001
Tissue eosinophil count/HPF	11	110	105	.67	14	186	<.001

Abbreviations: CRSwNP, chronic rhinosinusitis with nasal polyps; ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; HPF, high-power field; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps.
[a]Inferior turbinates were used as control tissue. Statistical analysis was performed by the Mann-Whitney *U* test and the Fisher exact test.

Table 2
Polymerase chain reaction primers used

Primer set	Sense primer, 5′–3′	Antisense primer, 5′–3′
IFN-γ	GAGTGTGGAGACCATCAAGGAAG	TGCTTTGCGTTGGACATTCAAGTC
IL-5	AGCTGCCTACGTGTATGCCA	GCAGTGCCAAGGTCTCTTTCA
IL-9	GACCAGTTGTCTCTGTTTGGGC	TTTCACCCGACTGAAAATCAGTGG
IL-10	TCTCCGAGATGCCTTCAGCAGA	TCAGACAAGGCTTGGCAACCCA
IL-17	CGGACTGTGATGGTCAACCTGA	GAGCATTGATGCAGCCCAAGTTC
T-bet	ATTGCCGTGACTGCCTACCAGA	GGAATTGACAGTTGGGTCCAGG
GATA-3	GCGGGCTCTATCACAAAATGA	GCTCTCCTGGCTGCAGACAGC
PU.1	GACACGGATCTATACCAACGCC	CCGTGAAGTTGTTCTCGGCGAA
Foxp3	GCCCTTGGACAAGGACCCGATG	CATTTGCCAGCAGTGGGTAGGA
RORC	TTTTCCGAGGATGAGATTGC	CTTTCCACATGCTGGCTACA
IL-17RB	ACAAACGCGAGCTTCAGTGGTG	ATGCAGTCGCTGCCACAAGTAG

Abbreviations: IFN-γ, interferon-γ; IL, interleukin.

Biotechnology). This was followed by incubation with Alexa Fluor 488- or 555-labeled secondary antibodies. The same concentrations of isotype controls were used. Nuclei were counterstained with TOPRO3 iodide (Invitrogen, Carlsbad, California). Analyses were conducted using a confocal laser microscope (LSM710; Carl Zeiss, Inc, Oberkochen, Germany).

Preparation of Nasal Polyp Mononuclear Cells and Tissue Homogenates

Nasal polyp mononuclear cells (NPMCs) were obtained as previously described.[12] In brief, freshly obtained NPs were immediately minced and incubated in RPMI-1640 medium that contained 1 mg/mL of collagenase, 0.5 mg/mL of hyaluronidase, and 0.2 mg/mL of *DNaseI* (Sigma-Aldrich, St Louis, Missouri). After incubation, NPMCs were separated using a Ficoll-Hypaque technique. Peripheral blood mononuclear cells (PBMCs) were purified from whole blood.

Nasal polyp tissues from patients with NECRS (n = 18) and ECRS (n = 22) and inferior turbinates from control subjects (n = 16) were weighed and minced. For every 100 mg of tissue, 1 mL of phosphate buffered saline supplemented with aprotinin and leupeptin (Roche, Mannheim, Germany) was added. Tissues were homogenized on ice and centrifuged. The supernatants were stored at −20°C.

Cytokine Assays

Cytokine levels in tissue homogenates and culture supernatants were measured using an enzyme-linked immunosorbent assay. IL-5, IL-13, and interferon-γ (IFN-γ) levels were measured as described previously.[23] IL-9 and IL-25 levels were measured using an enzyme-linked immunosorbent assay development kit (Peprotech, Rocky Hill, New Jersey).

Isolation of $CD4^+$ T Cells

The NECRS (nonatopic, n = 7; atopic, n = 6) and ECRS (nonatopic, n = 7; atopic, n = 13) samples from which sufficient NPMCs could be obtained ($>2 \times 10^7$) were used for cell sorting. $CD3^+CD4^+$ T cells were sorted using a FACSAria II (BD, Franklin Lakes, New Jersey).

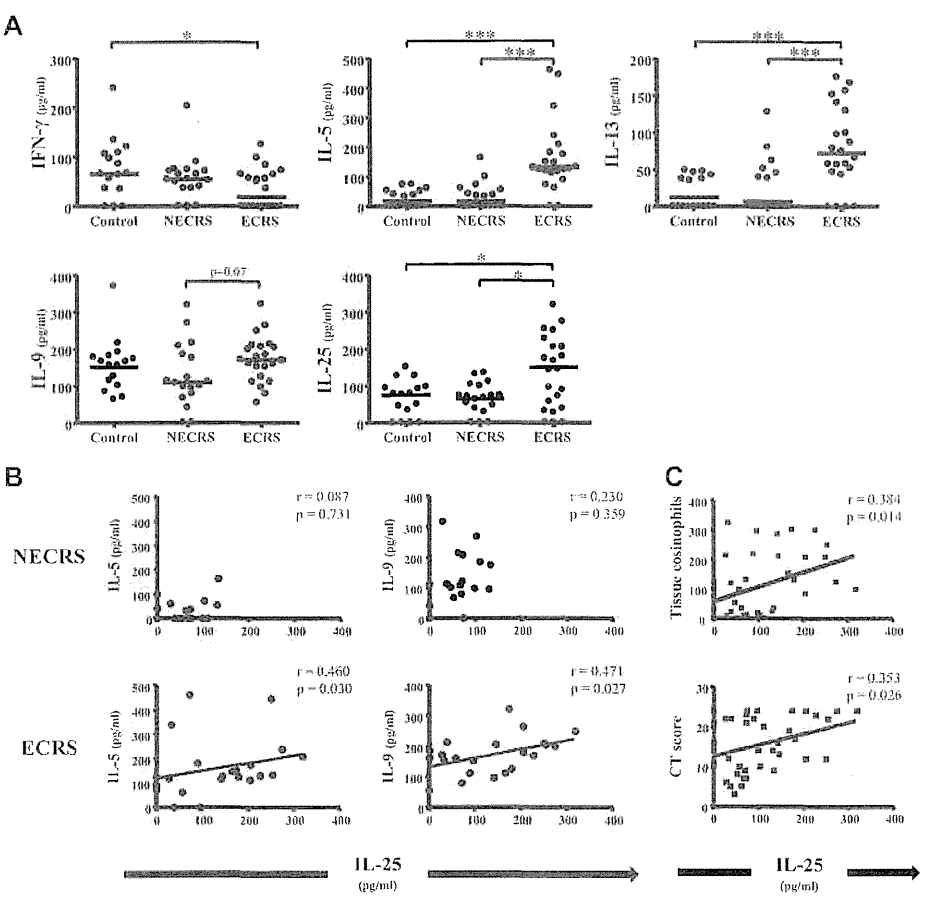

Figure 1. *(A)* Measurement of cytokines in tissue homogenates of inferior turbinates from controls and nasal polyps from patients with noneosinophilic (NECRS) and eosinophilic (ECRS) chronic rhinosinusitis with nasal polyps. *(B)* Correlation of expression levels of interleukin (IL)-25 with IL-5 and IL-9 in nasal polyps. *(C)* Correlation of expression levels of IL-25 in nasal polyps with the number of eosinophils and the Lund-Mackay computed tomographic score. $^*P < .05$, $^{***}P < .001$. IFN-γ, interferon-γ.

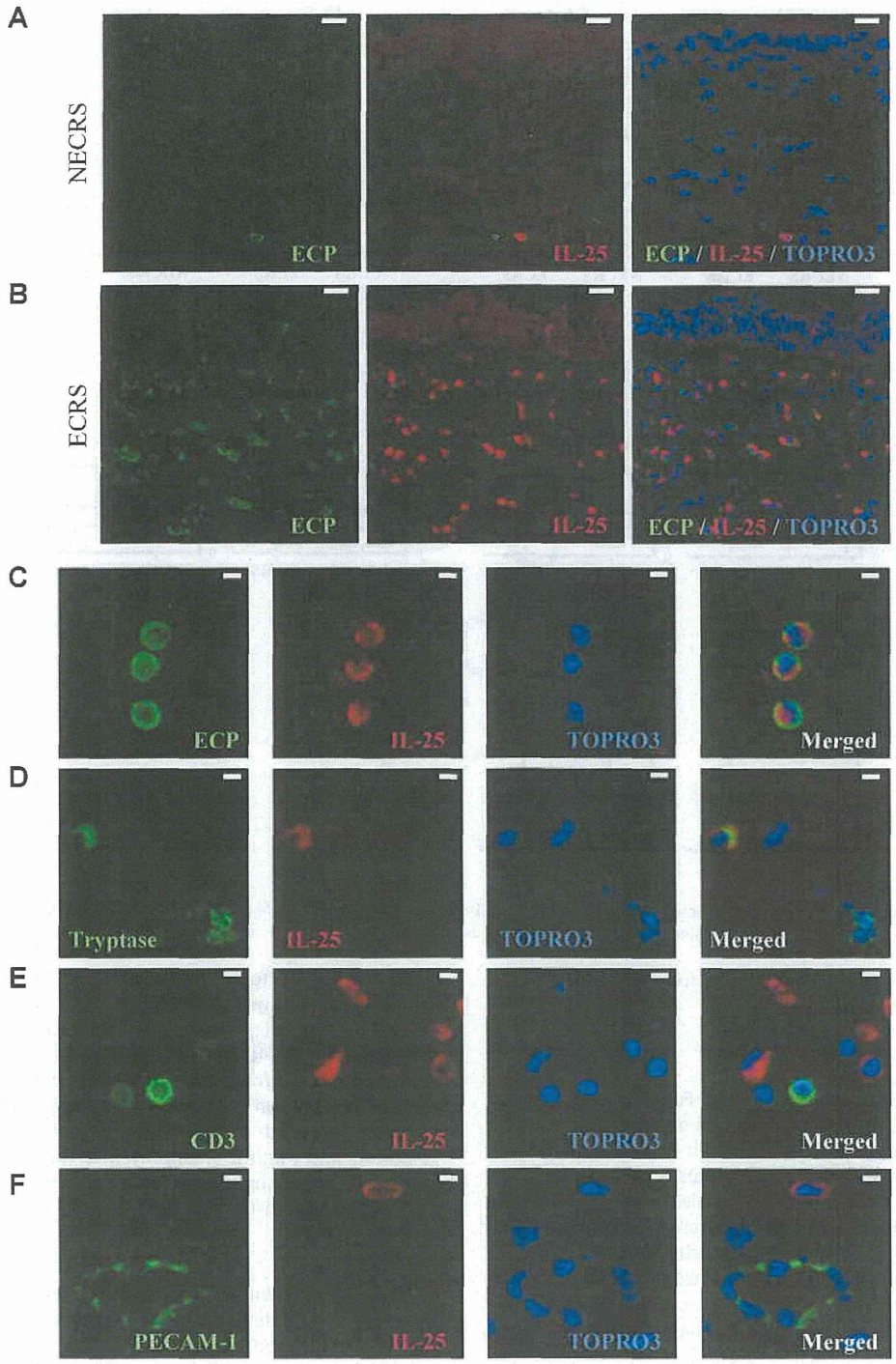

Figure 2. Immunofluorescence analysis of interleukin (IL)-25 expression in nasal polyps. *(A, B)* IL-25 and eosinophil cationic protein (ECP) staining of nasal polyps (original magnification ×40). Scale bar = 20 μm. IL-25 and *(C)* ECP, *(D)* tryptase, *(E)* CD3, and *(F)* platelet endothelial cell adhesion molecule-1 (PECAM-1) (original magnification ×160). Scale bar = 5 μm. ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from sorted fresh $CD4^+$ T cells (Figures 3, 5A) and cultured NPMCs (Figure 6A) using TRIzol reagent (Invitrogen). The cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California). Semiquantitative real-time polymerase chain reaction was performed using the SYBR Green method. The mRNA levels encoding IFN-γ, IL-5, IL-9, IL-10, IL-17, T-bet, GATA-3, PU.1, FOXP3, RORC, and IL-17RB were determined, and relative gene expression was calculated using the comparative *CT* method. The mRNA isolated from $CD4^+$ T cells of sinus mucosal tissues from a CRSsNP sample and NPMCs stimulated with CD3–CD28 and without IL-25 were used for calibration. The mRNA levels for β-actin (*ACTB*) were used to normalize the mRNA levels of the other transcripts. Primer

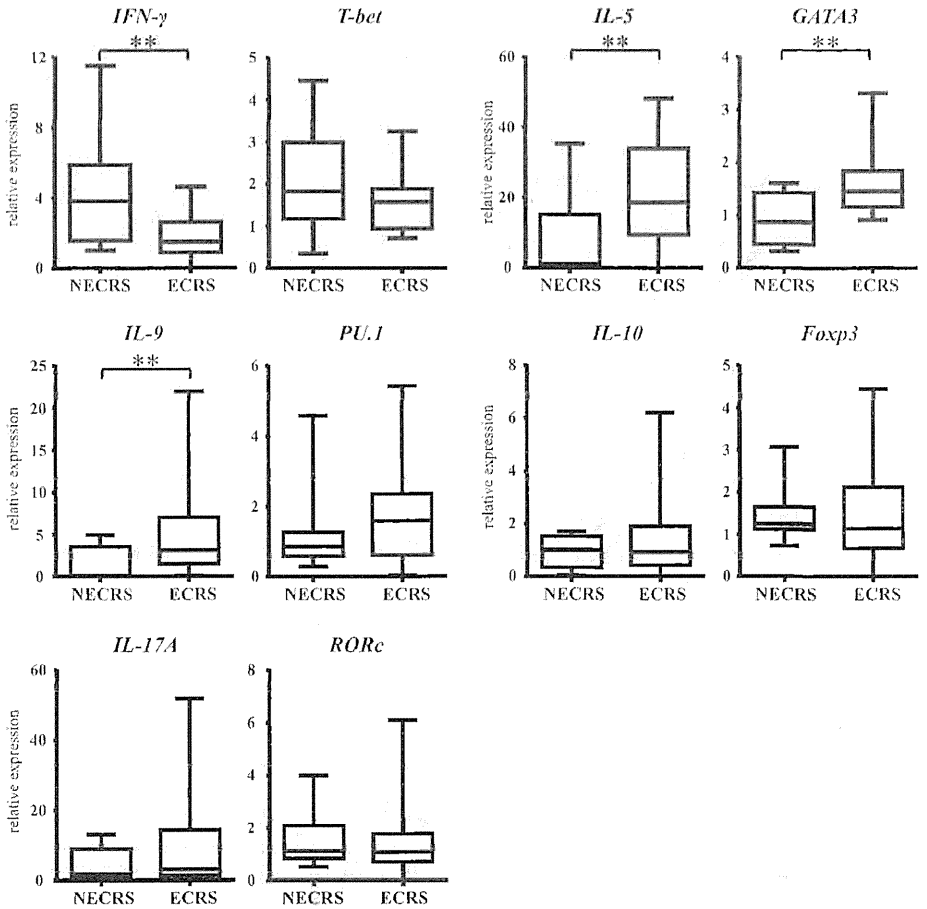

Figure 3. Levels of mRNAs encoding the cytokine and transcription factor in tissue $CD4^+$ T cells isolated from nasal polyps. $^{**}P < .01$. ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; IFN-γ, interferon- γ; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps.

and probe sets (Table 2) were purchased from Eurofins Operon MWG (Ebersberg, Germany).

Flow Cytometry

Samples were incubated with human FcR Blocking Reagent (Miltenyi Biotec) for 30 minutes and stained on ice for 30 minutes. For intracellular staining, cells were fixed with 4% paraformaldehyde for 10 minutes, made permeable for 10 minutes, and blocked with 3% bovine serum albumin for 30 minutes. Samples were stained with a combination using antibodies as follows: fluorescein isothiocyanate and anti-CD3 (UCHT1; BD); phycoerythrin and anti-CD45RO (UCHL1; BD); anti–IL-9 (MH9D1; eBioscience, San Diego, California); peridinin chlorophyll protein complex, cyanine 5.5, and anti-CD4 (RPA-T4; BioLegend, San Diego, California); antigen-presenting cell and anti-CD3 (UCHT1; BD); anti-CD69 (FN50; eBioscience); anti–IFN-γ (4S.B3; BioLegend); and anti–IL-5 (TRFK5; BioLegend). Staining with these antibodies was performed according to the manufacturers' protocols. For IL-17RB staining, NPMCs were stained with anti–IL-17RB (97C691; Lifespan Biosciences; final concentration 5 μg/mL) or isotype control, followed by a secondary phycoerythrin and antimouse IgG1 antibody. Flow cytometry was performed using a FACScalibur flow cytometer (BD) and data were analyzed using FlowJo software (Tree Star, San Carlos, California).

Cell Culture

The NPMCs obtained from patients with NECRS (n = 8) and those with ECRS (n = 10) were cultured (1×10^6 cells/mL) at 37°C for 60 hours. For cytokine production, cells were stimulated with or without plate-bound anti-CD3 (OKT3; 2 μg/mL) plus anti-CD28 (CD28.2; 10 μg/mL; eBioscience) antibodies in the presence or absence of IL-25 (10 or 100 ng/mL; R&D Systems, Minneapolis, Minnesota). For intracellular staining, cells were cultured with or without stimulation with anti-CD3–CD28 antibody-coated plates and re-stimulated with 25 ng/mL of phorbol-12-myristate-13-acetate and 1 μg/mL of ionomycin in the presence of 2 μmol/L of monensin for the final 4 hours. For IL-17RB staining, cells were first cultured on an anti-CD3 antibody-coated plate for 48 hours.

Statistics

Values are shown in dot plots as the median or in bar charts as mean ± standard deviation. In box-and-whiskers plots, the central line of the box represents the median. Minimum ± maximum (whisker) and 25th ± 75th percentile (box) are presented. Statistical analysis was performed using the Mann-Whitney *U* test and the Fisher exact test for comparing data between groups and the Wilcoxon signed-rank test for data within a group. Correlation analysis was performed using the Spearman rank correlation coefficient. *P* values less than .05 were considered significant.

Results

Relations between Atopic Status and Tissue Eosinophil Infiltration in Patients with CRSwNP

Patients with CRSwNP were divided into groups based on atopic status and tissue eosinophil infiltration (Table 1). Total IgE levels in

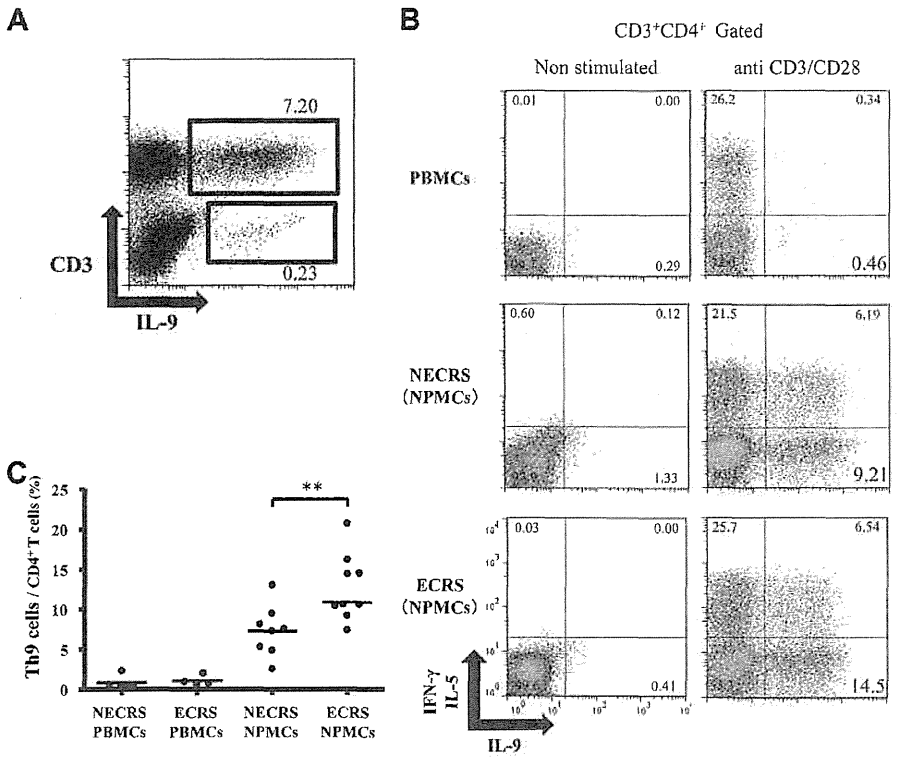

Figure 4. Flow cytometric analysis of interleukin (IL)-9–producing cells. *(A)* Intracellular staining of IL-9 in nasal polyp mononuclear cells (NPMCs) from patients with eosinophilic chronic rhinosinusitis with nasal polyps (ECRS). The percentage of positive cells is shown. *(B)* T-helper type 9 (T_H9) cells were defined as IFN-γ^- IL-5^- IL-9^+ cells after gating on $CD3^+$ $CD4^+$ cells. *(C)* Proportion of T_H9 cells in $CD4^+$ T cells. $^{**}P < .01$. IFN-γ, interferon- γ; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps; PBMC, peripheral blood mononuclear cell.

blood were higher in the atopic group than in the nonatopic group ($P = .02$), whereas the number of local IgE-positive cells in NPs was larger in patients with ECRS than in those with NECRS ($P < .001$). Late-onset asthma and aspirin-intolerant asthma (aspirin-exacerbated respiratory disease) were more prevalent in patients with ECRS compared with those with NECRS, whereas these comorbidities occurred similarly in patients with CRSwNP and nonatopic and atopic status.

Cytokine Levels in NP Tissue Homogenates

The IFN-γ, IL-5, IL-13, IL-9, and IL-25 levels in NPs were measured. The IFN-γ level significantly decreased in NP tissue homogenates from patients with ECRS compared with controls ($P < .05$; Fig 1A). In contrast, IL-5 and IL-13 levels were significantly elevated in patients with ECRS compared with controls ($P < .001$, $P < .001$) and NECRS ($P < .001$, $P < .001$). IL-9 levels were higher in patients with ECRS than in those with NECRS, but the difference was not significant ($P = .07$). IL-25 levels were significantly higher in patients with ECRS than in controls ($P < .05$) and those with NECRS ($P < .05$). IL-25 levels were significantly correlated with levels of IL-5 ($r = 0.460$, $P = .030$) and IL-9 ($r = 0.471$, $P = .027$) in ECRS samples (Fig 1B). IL-25 levels in NPs also were correlated with the numbers of eosinophils ($r = 0.384$, $P = .014$) and CT scores ($r = 0.353$, $P = .026$; n = 40; Fig 1C).

Source of IL-25 in NPs

Nasal polyp tissues were doubly stained with antibodies against IL-25 and ECP, mast cell tryptase, CD3, or PECAM-1. IL-25 was moderately expressed in epithelial cells obtained from patients with NECRS (Fig 2A) and those with ECRS (Fig 2B). There were a few $IL\text{-}25^+$ $tryptase^+$ cells, but most $IL\text{-}25^+$ cells were $IL\text{-}25^+$ ECP^+ in the submucosa (Fig 2B, C, D). $IL\text{-}25^+$ $CD3^+$ and $IL\text{-}25^+$ $PECAM\text{-}1^+$ cells were not detected (Fig 2E, F). In these figures, the epithelium is shown in the upper region. Representative results for 10 patients are shown.

Characteristics of Tissue $CD4^+$ T Cells

The levels of mRNAs encoding cytokines and major transcription factors of tissue $CD4^+$ T cells from patients with NECRS (n = 13) and ECRS (n = 20) were determined using real-time polymerase chain reaction (Fig 3). There was no significant difference among groups in the expression levels of mRNAs encoding T-bet, PU.1, IL-10, FOXP3, IL-17, and RORC. *IFNG* mRNA was significantly upregulated in patients with NECRS ($P < .01$). In contrast, *IL5*, *GATA3*, and *IL9* mRNA levels were significantly upregulated in $CD4^+$ T cells from patients with ECRS ($P < .01$). These mRNAs levels did not differ significantly between the atopic and nonatopic groups. The mRNA levels in $CD4^+$ T cells isolated from inferior nasal turbinates could not be determined because the surgical samples were smaller than those for NPs and sufficient numbers of cells could not be obtained for sorting.

T_H9 Cell Numbers in NPs

The authors previously reported that the numbers of T_H2 cells in NPs were increased more in patients with ECRS than in patients with NECRS[12]; thus, NPs were examined for the presence of T_H9 cells. By flow cytometric analysis, most IL-9–producing cells in NPMCs expressed CD3 (Fig 4A). T_H9 cells are defined as IFN-γ^-IL-$5^-$$IL\text{-}9^+$ $CD3^+CD4^+$ cells and only a few T_H9 cells were detected in PBMCs, whereas a significant number of T_H9 cells was observed in NPMCs and their rate to $CD4^+$ T cells was high (Fig 4B). T_H9 cells in PBMCs from patients with NECRS (n = 5) or ECRS (n = 4) or NPMCs

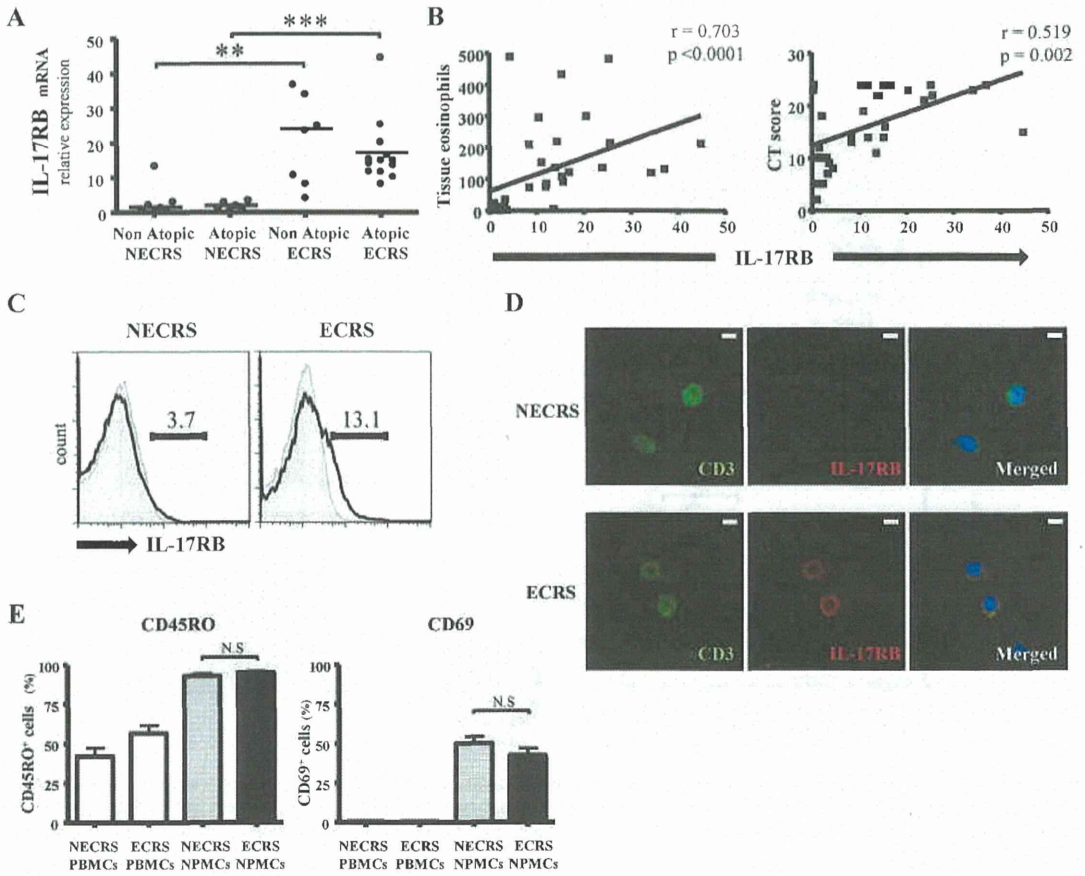

Figure 5. Expression of interleukin (IL)-17RB in tissue $CD4^+$ T cells. *(A)* Real-time polymerase chain reaction analysis of IL-17RB expression in sorted tissue $CD4^+$ T cells. *(B)* Correlation of the IL-17 RB mRNA level in tissue $CD4^+$ T cells with the number of tissue eosinophils and computed tomographic (CT) score. *(C)* Flow cytometric analysis of IL-17RB expression. The figure presents the percentage of IL-17RB–expressing cells gated on $CD3^+CD4^+$ cells. The gray histogram represents isotype control staining. *(D)* Immunofluorescence analysis of IL-17RB. Scale bar = 5 μm. The results of flow cytometry and immunofluorescence represent 4 to 6 patients. *(E)* Flow cytometric analysis of CD45RO and CD69 expression by $CD3^+CD4^+$ T cells. $^{**}P < .01$, $^{***}P < .001$. ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; IFN-γ, interferon- γ; NECRS, non-eosinophilic chronic rhinosinusitis with nasal polyps; NPMC, nasal polyp mononuclear cell; NS, not significant; PBMC, peripheral blood mononuclear cell.

from patients with NECRS (n = 8) or ECRS (n = 9) were measured (Fig 4C). The T_H9 proportion in $CD4^+$ T cells of NPMCs was significantly higher in patients with ECRS than in those with NECRS ($P < .01$).

Tissue Memory $CD4^+$ T Cells from Patients with ECRS Express IL-17RB

The level of mRNA encoding IL-17RB was significantly higher in patients without atopy ($P < .01$) and with atopy ($P < .001$) and ECRS than in those with NECRS (Fig 5A). *IL5* ($r = 0.490$, $P = .004$), *GATA3* ($r = 0.482$, $P = .005$), and *IL9* ($r = 0.449$, $P = .009$) mRNAs in tissue $CD4^+$ T cells significantly correlated with *IL17RB* mRNA levels. Moreover, *IL17RB* mRNA levels in tissue $CD4^+$ T cells were significantly correlated with the number of eosinophils in NPs ($r = 0.703$, $P < .0001$) and CT scores ($r = 0.519$, $P = .002$; Fig 5B). There was no significant correlation with *IL17RB* levels and expression levels of mRNAs encoding IFN-γ, T-bet, PU.1, IL-10, FOXP3, IL-17, and RORC.

Flow cytometric (Fig 5C) and immunofluorescence (Fig 5D) analyses detected increased IL-17RB expression in patients with ECRS. Approximately 95% of $CD4^+$ T cells in NPMCs expressed CD45RO (PBMCs: NECRS, n = 9; ECRS, n = 7; NPMCs: NECRS, n = 15; ECRS, n = 13), and approximately 50% of $CD4^+$ T cells expressed CD69 (Fig 5E). Neither of these expression levels significantly differed between patients with ECRS and those with NECRS.

Influence of IL-25 on Cytokine Production by NPMCs

The levels of mRNAs encoding cytokines and major transcription factors of NPMCs stimulated with CD3–CD28 in the presence or absence of IL-25 (100 ng/mL) were determined using real-time polymerase chain reaction (NECRS, n = 6; ECRS, n = 8; Fig 6A). *IL5* and *GATA3* mRNAs were significantly upregulated by IL-25 stimulation in patients with ECRS ($P < .05$ for the 2 comparisons) but not in patients with NECRS.

CD3–CD28 stimulation of NPMCs induced significant IFN-γ, IL-5, and IL-9 protein levels in the 2 groups (NECRS, n = 8; ECRS, n = 10); however, the IL-5 level was higher in NPMCs from patients with ECRS than in those from patients with NECRS ($P < .01$; Fig 6B). IL-25 stimulation with anti-CD3–CD28 increased IL-5 ($P < .01$) and IL-9 ($P < .01$) production dose-dependently in NPMCs from patients with ECRS, but the same enhancement was not observed in NPMCs from patients with NECRS. IL-25 stimulation (100 ng/mL) with anti-CD3–CD28 increased IL-9 production in NPMCs from patients with ECRS ($P < .01$). There was no significant difference in the proportion of $CD4^+$ T cells in NPMCs between patients with ECRS (11.4 ± 3.1%) and those with NECRS (10.5 ± 3.6%).

Discussion

Most $CD4^+$ T cells infiltrating NPs were memory T cells with different features in CRSwNP disease types classified according to

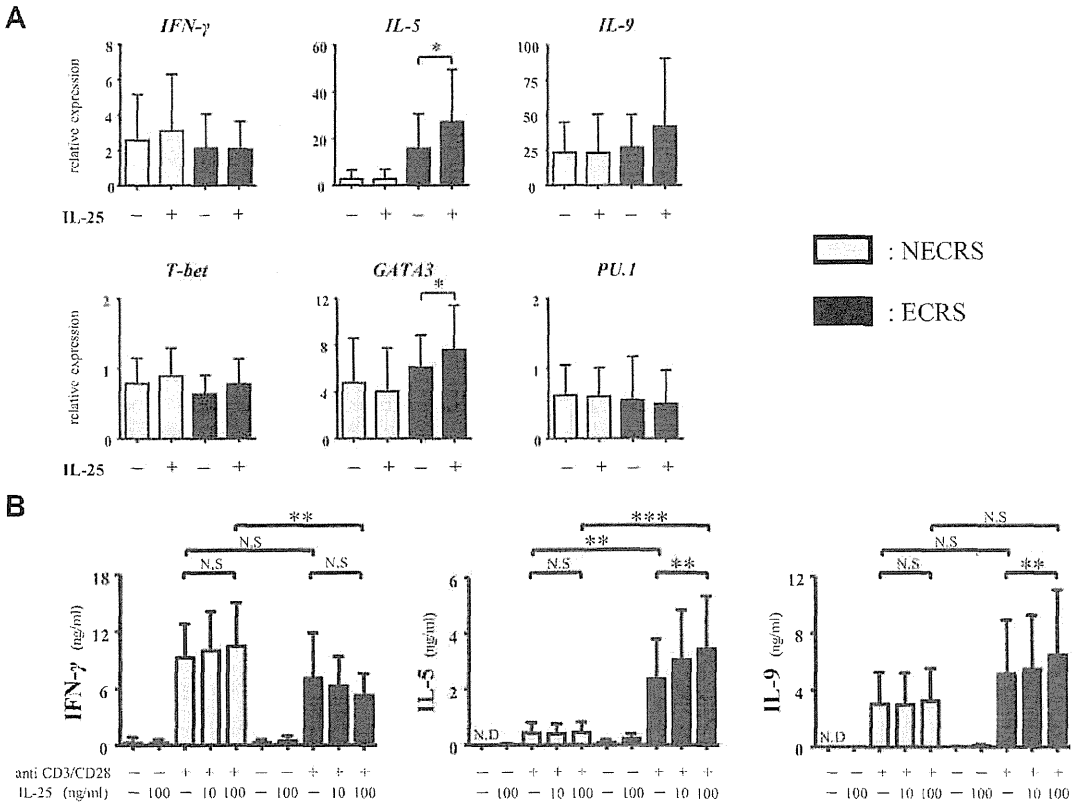

Figure 6. Influence of interleukin (IL)-25 on interferon-γ (IFN-γ), IL-5, and IL-9 production by nasal polyp mononuclear cells (NPMCs). *(A)* Expression levels of genes encoding IFN-γ, IL-5, IL-9, T-bet, GATA-3 and PU.1 in NPMCs. *(B)* IFN-γ, IL-5 and IL-9 protein levels in the supernatants. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; ND, not detected; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps; NS, not significant.

eosinophil infiltration. The *IFNG* mRNA level was elevated in $CD4^+$ T cells obtained from tissues of patients with NECRS (Fig 3). In contrast, *IL5* and *IL9* mRNA levels were elevated in patients with ECRS, which also were characterized by increased IL-17RB expression (Fig 5). Moreover, IL-17RB expression in tissue $CD4^+$ T cells significantly correlated with the number of eosinophils in NPs and CT score. IL-25 levels were increased in NPs obtained from patients with ECRS (Figs 1, 2) and enhanced the production of T_H2- and T_H9-specific cytokines induced by T-cell receptor stimulation of NPMCs obtained from patients with ECRS but not those from patients with NECRS (Fig 6). For tissue T_H2 and T_H9 cells, the functions mediated through IL-25 differed between cells obtained from patients with ECRS and those with NECRS. The present results indicated that IL-25 in NPs and IL-17RB expressing tissue memory $CD4^+$ T cells were associated with eosinophil infiltration in CRSwNP.

The elevated *IL5* and *GATA3* mRNA levels in tissue $CD4^+$ T cells and the IL-5 level in NPs of patients with ECRS indicate that T_H2 cells play important roles in infiltration of eosinophils in NPs. Interestingly, the proportion of T_H9 cells detected in NPMCs (10%) was larger than that in PBMCs (1%; Fig 4). Because some T_H9 cells might be reprogrammed cells from T_H2 cells by transforming growth factor-β for plasticity of the T-cell subset,[24] this result suggests that T_H9 cells were localized in the site as a consequence of a secondary immune response. In addition, the population of T_H9 cells in patients with ECRS was larger than that in patients with NECRS, which was consistent with the *IL9* mRNA levels in $CD4^+$ T cells. IL-9 was first purified and characterized as a T-cell growth factor and is a potent stimulus for mast cell development and its FcεRI upregulation.[25–27] IL-9 induces goblet cell hyperplasia and upregulates mucus expression in airway epithelial cells,[28,29] although further studies will be required to determine the role of IL-9 in NPs.

In contrast, there was no difference in the numbers of regulatory T cells and T_H17 cells in tissue $CD4^+$ T cells between patients with ECRS and those with NECRS (Fig 3). FOXP3 expression decreased in patients with CRSwNP compared with the expression of factors associated with regulatory T cells in patients with CRSsNP or CRSwNP.[4,30] However, because the source of IL-10 or FOXP3 was not addressed in those studies, a role for regulatory T cells was not established. Moreover, there were no differences in the populations of T_H17 cells in patients with NECRS or ECRS, which agrees with another study by Jiang et al.[31] In contrast, an increased *IFNG* mRNA level characterizes tissue $CD4^+$ T cells in patients with NECRS. These results indicate that NECRS involves T_H1-skewed inflammation and that tissue T_H1–T_H2 balance is linked to the pathogenesis of the CRSwNP subtype.

Memory T cells are localized in peripheral tissue and defend against secondary infection.[32,33] Memory $CD4^+$ T cells also acquire additional functions during the secondary response and are subdivided based on a homing function or expression of surface molecules in addition to subsets of cytokine production.[34,35] The authors propose that T_H2 cells with such an additional functions have pathogenic potential in eosinophilic inflammation.[36] In a study of mouse allergy models, Islam et al[37] proposed that IL-5 derived from IL-17RB- and CCR8-expressing T_H2 cells amplified local secondary allergic immune responses. Endo et al[38] showed that IL-5 produced by $CD62L^{low}CXCR3^{low}$ T_H2 cells plays a critical role in allergic disease. In the present study, tissue memory $CD4^+$ T cells from patients with ECRS were characterized by high IL-17RB expression levels. IL-5 and IL-9 production was observed in