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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

発明の名称：中枢神経ループス(NPSLE)診断用バイオマーカー、出願番号：特願 2013-055543、公開番号：特開2014-181967、発明者：一瀬邦弘、大山要、川上純、黒田直敬、中嶋秀樹、岸川直哉、馬場雅子、出願日：平成25年3月18日(2013.3.18)、公開日：平成26年9月29日(2014.9.29)

2. 実用新案登録

なし

3. その他

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

【書籍】

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
一瀬邦弘, 川上 純	関節リウマチの発症要因と発症メカニズム　Th17細胞	該当無し	最新関節リウマチ学―寛解・治癒を目指した研究と最新治療―	日本臨牀	大阪市	2014	53-58
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雑誌【和文】

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
岩本 直樹, 川上 純	【自己免疫性血液疾患:診断と治療の進歩】 病態の基礎 自己抗体の産生機序.	日本内科学会雑誌	103(7)	1564-1569	2014
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浦田幸朋、石井智徳、張替秀郎、佐々木毅、その他12名	東北地方におけるB型肝炎再活性化前向き研究について	最新医学	68(3)	395-402	2013

Ⅳ. 研究成果の刊行物、別冊

発症メカニズム

Th17細胞

T helper 17 cell

一瀬邦弘　　川上　純

Key words：関節リウマチ(RA)，Th17細胞，IL-17A，抗IL-17抗体

はじめに

関節リウマチ(RA)の主たる免疫担当細胞としてCD4 T細胞を介したメカニズムが中心的に議論されている．ヒトの炎症滑膜組織ではCD4 T細胞の浸潤がみられ，実験動物であるII型コラーゲン誘発関節炎モデル(collagen induced arthritis: CIA)においてもCD4 T細胞がII型コラーゲンに感作され活性化され，関節炎をきたすとされる．もともとRAではCD4 T細胞の中でも，Th1型の代表的なサイトカインであるinterferon(IFN)-γやTh1細胞への分化に必須のinterleukin(IL)-12の産生が亢進していることが報告されており[1]，1990年代まではTh1細胞優位の疾患であると考えられていた．しかしながら，IFN-γやIL-12をノックアウトするとマウスのCIAモデルの関節炎が悪化するという現象がみられたことから[2]，従来のTh1/Th2パラダイムによらない新規のT細胞の存在が指摘されていた．そのような状況の中で，RA患者の滑膜に浸潤しているT細胞からIL-17の発現が亢進していることが報告された[3]．更に関節炎などの他の自己免疫動物モデルでもIL-17を産生するCD4T細胞サブセットである，Th17細胞が病態に関与していることが次第に明らかとなり，RAはTh17細胞優位の自己免疫疾患であるという考え方が受容されるようになってきた．

1 Th17細胞

ヒトのIL-17は1995年にT細胞由来のサイトカインとして初めてクローニングされた[4]．2005年にはIL-17を産生する新規のヘルパーCD4 T細胞としてTh1やTh2とも異なるTh17細胞が新たに同定された[5]．IL-17AはTh17細胞系の最も重要な役割を担っている可溶性の催炎症性サイトカインである．IL-17Aはホモ二量体であり，6種類あるIL-17サイトカインファミリーに属する．IL-17AはIL-17サイトカインファミリーの中でIL-17Fと最も高い類似性を示す．IL-17A/IL-17Fヘテロ二量体はIL-17AとIL-17Fの中間の生物活性を有するとされるが，ヒトの自己免疫疾患に対するこのIL-17A/IL-17Fヘテロ二量体への関与は依然として明らかにされていない．IL-17AおよびIL-17Fとも同じ受容体(IL-17RAおよびIL-17RC)に結合する．IL-17FよりもIL-17Aの方が*in vitro*での生物活性が高いのは，個々の受容体サブユニットに対するIL-17AおよびIL-17Fの結合親和性の差によるものと考えられる．これ

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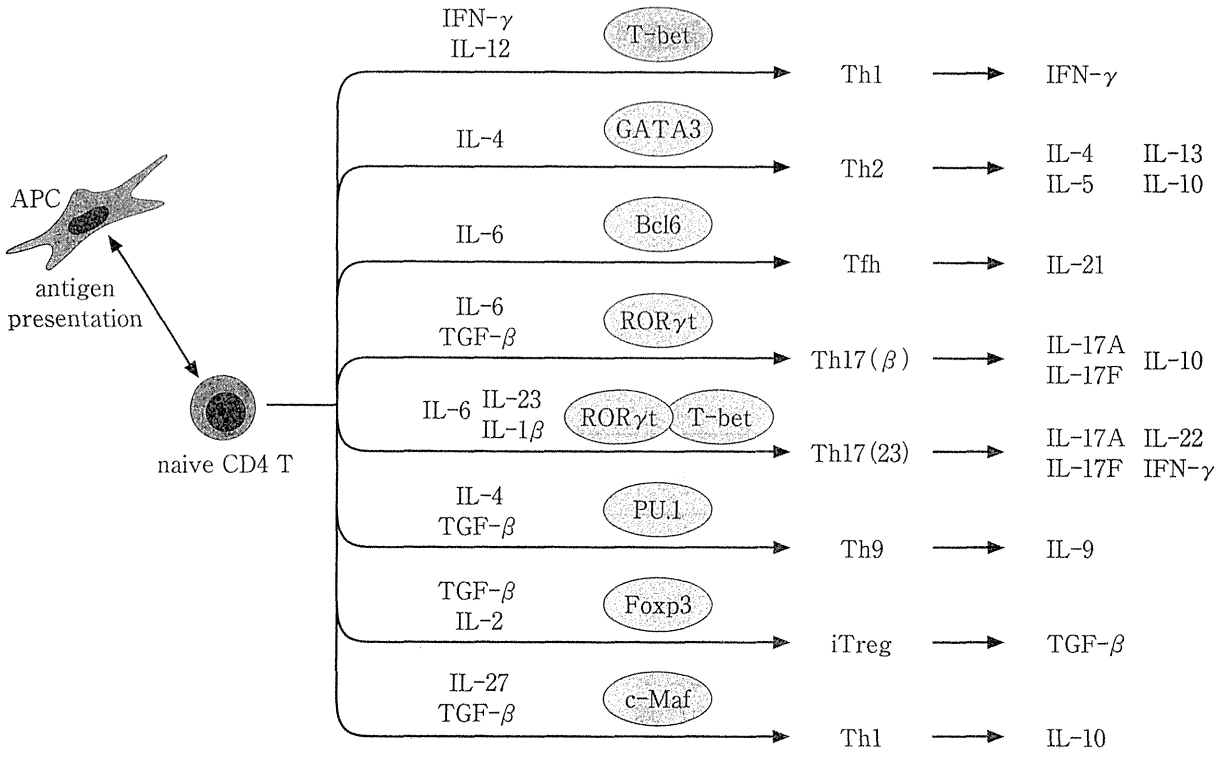

図1　Th17細胞の分化に必要なサイトカインと転写因子（文献[6]より引用）

らの受容体からのシグナル伝達にはAct1およびTRAF6が関与する．IL-17RAは種々の細胞上に普遍的に発現しているが，IL-17RCは造血細胞上の発現が少ない．このようにIL-17受容体は広範囲に発現しているため，IL-17Aは上皮細胞，樹状細胞，マクロファージ，線維芽細胞，骨芽細胞，内皮細胞を含む種々の細胞に作用しうる．

最近では，Th17細胞は大きく2つのサブセットが存在していることが知られるようになり，分化に必要とされるサイトカインや，それぞれが産生するサイトカインやケモカインの種類により分類されている（**図1**）．一つは前述のnaïve CD4T細胞からIL-6とTGF-βにより分化誘導される従来型のTh17[Th17(β)]で，IL-17A，IL-17Fに加えて，高IL-10，chemokine(C-C motif) ligand(CCL) 20を産生し，CC chemokine receptor(CCR) 6を細胞表面に発現している．もう一方はIL-6，IL-23，IL-1βによって分化するTh17[Th17(23)]であり，高IL-22，CCL9を産生し，CXC chemokine receptor (CXCR) 3を細胞表面に発現している[6]．自己免疫疾患モデルでは，Th17(23)細胞の方が高い病態形成能を有し，IL-23はIL-17Aのみ発現するTh17細胞をIL-17A/IFN-γの両方を産生する細胞へと変換することが報告されている[7]．このようにヒトIL-17産生細胞にはヘルパーCD4T細胞のサブセットとしてのTh17とは異なり，IFN-γを同時に産生するものが認められる．大腸炎モデルにおいてはTh細胞から産生されるIL-17AがTh1細胞の分化を直接抑制するため，大腸炎に防御するように働く[8]．しかしながらIL-23はこの大腸炎を増悪させることから，前記のIL-17/IFN-γの両産生細胞が病態悪化に関与していると考えられている[9]．更にヒトIL-17産生細胞の一部は制御性T細胞の転写因子であるFoxp3を発現し，抑制機能を有していることも報告されており[10]，ヒトTh17細胞の機能とその役割については未知の点も多い．

2　IL-17とRA

IL-17による炎症や関節破壊のメカニズムとして以下の点が挙げられる（**図2**）．①IL-17は

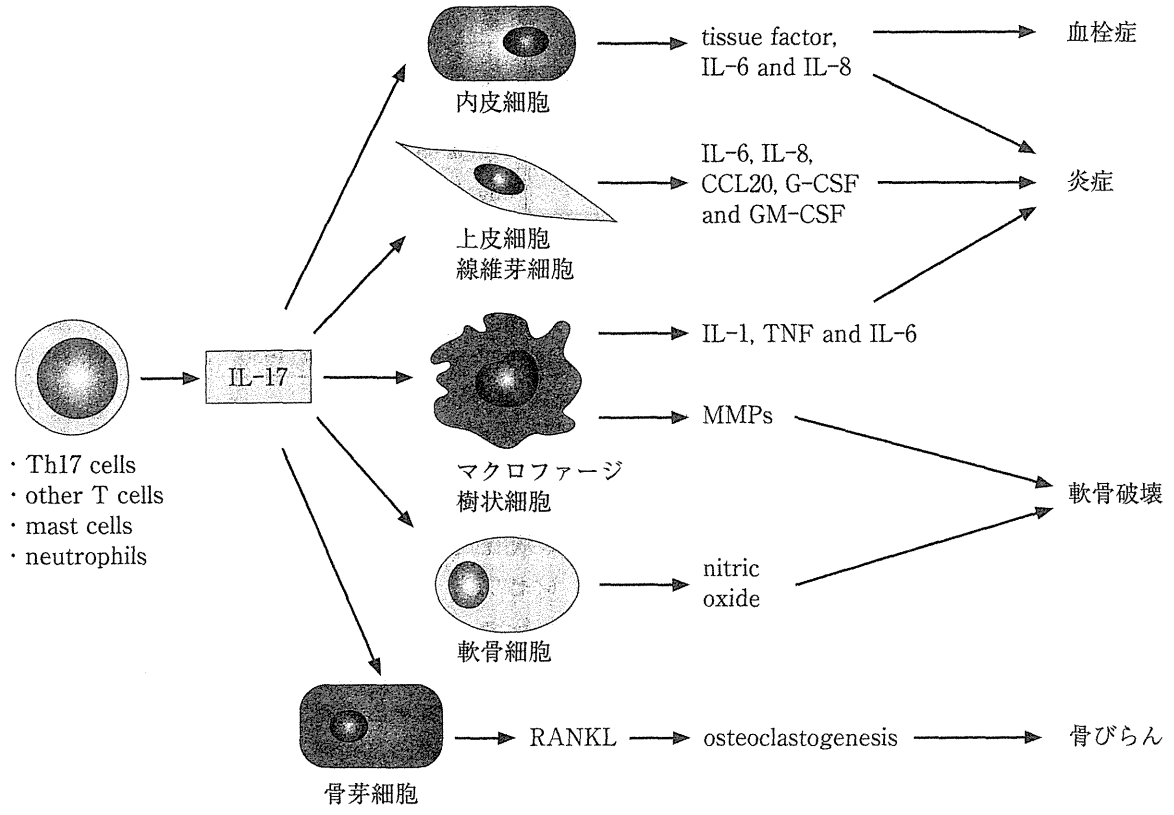

図2 IL-17による炎症や関節破壊のメカニズム(文献[11]より改変)

種々の病態における急性炎症反応に関与する．すなわちIL-17は上皮細胞や線維芽細胞などの間葉系細胞からIL-6やIL-8などのサイトカインやケモカインを放出させ，急性反応物質や組織における炎症細胞の集簇を促す．また，②IL-17は慢性炎症，例えば軟骨破壊にも関与する．IL-17は軟骨細胞や骨芽細胞におけるマトリックス産生を抑制し，関節破壊や組織修復阻害作用を有する．更に，③IL-17はmatrix metalloproteinases(MMPs)の機能と産生を活性化させ，TNF-αとの連動により不可逆性の軟骨破壊をきたすことがマウスモデルで報告されている．④IL-17は骨破壊にも関与している．IL-17は骨芽細胞においてreceptor activator of NF-κB ligand(RANKL)の発現を増加させ，RANKシグナルの活性化を介して破骨細胞への分化を促進する．これらの作用により，IL-17は関節炎を惹起し，それを持続させる慢性炎症作用を有しており，RAの病態形成に重要な役割を果たしていると考えられる[11]．

3 関節炎動物モデルにおけるTh17

これまで動物実験において，complete Freund's adjuvantとともにII型コラーゲンでマウスやラットを免疫し，多発性関節炎を誘導するII型コラーゲン誘導性関節炎(CIA)モデルマウスが主に用いられてきた．長い間，このモデルではTh1型自己免疫反応によって発症すると考えられてきた．Th1細胞の分化誘導を促すIFN-γやIL-12はTh17細胞分化を阻害するが，IFN-γやIL-12ノックアウトマウスではTh17細胞が増大し，関節炎モデルの悪化がみられた．一方でTh17分化を促進させるIL-23をノックアウトすると関節炎発症が抑制された[2]．IL-17を関節内に過剰発現させると著明な炎症，骨びらんや軟骨破壊などの症状を引き起こし，またIL-17ノックアウトマウスや抗IL-17抗体投与でもCIAが軽症化していることから，IL-17が関節炎に関与している可能性は高いと考えられる．CIAモデルマウス以外にもSKGマウ

表1 IL-17またはIL-17R阻害薬を用いた臨床治験

drug	target	phase	status	reference
secukinumab (AIN457)	IL-17A	II	completed	15)
		III	ongoing	15)
ixekizumab (LY2439821)	IL-17A	II	completed	15)
brodalumab (AMG827)	IL-17RA	II	completed	NCT00950989; NCT00771030

スはT細胞刺激伝達系に関与するZAP70の点突然変異により，CD4T細胞の胸腺選択に異常をきたし，自己反応性のCD4T細胞依存性に関節炎を自然発症する．関節炎を発症したSKGマウスのCD4$^+$ T細胞をT細胞欠損ヌードマウスやT/B細胞欠損SCIDマウスに養子移入すれば関節炎を発症するが，IL-17ノックアウトSKGマウスでは関節炎を発症しなかった．更にIL-1 receptor agonist(IL-1RA)ノックアウトマウスはIL-1RAがIL-1に対する内在性の抑制因子であることから，IL-1の高発現を介して，関節炎を自然発症する．このマウスの関節炎では，Th17細胞からのIL-17産生を亢進させることが報告されており[12]，IL-17が関節炎発症に重要な役割をもつことが示されている．その他，自然免疫の活性化を介したTh17細胞分化のメカニズムも近年明らかにされ，Toll like receptor(TLR)や真菌感染に関与するC-type lectin receptorなどの経路も研究されている[11]．

4 ヒトRAにおけるTh17

RA患者の滑膜組織ではIL-17が高発現しており，滑膜細胞におけるIL-17 mRNAの発現が，RA患者における関節破壊の予測因子であることが報告されている[13]．また一方で発症初期のRAでのみIL-17が検出されたとする報告もある[14]．RA患者の滑膜培養細胞と抗IL-17抗体をともにインキュベートすると，IL-6産生が平均54％減少していることが報告され，IL-17阻害がRAのような慢性炎症の治療的側面を担う可能性が示唆された[3]．

5 臨床的応用におけるTh17

現在進行中の臨床治験としてIL-17Aとそのレセプターであるil-17RAに対する抗体治療が行われている(**表1**)．現在のRA治療で頻用されているTNF-α製剤でも30％程度は効果不十分例があり，そのような症例では他のオプションが望まれる．その中でどのような症例がIL-17阻害薬に適合するのかを更に検討する必要がある．ヒトを対象とした初めての臨床試験として抗IL-17Aモノクローナル抗体であるsecukinumabが2005年12月にRA患者を対象として開始された．secukinumabは，高親和性ヒト抗ヒトIL-17Aモノクローナル抗体(アイソタイプ：IgG1/kappa)である．secukinumabはヒトIL-17Aに結合し，*in vitro*および*in vivo*でこのサイトカインの生物活性を中和する．RA患者を対象とした1年間の第II相試験(CAIN457F2201;237人)では，secukinumab 25，75，150または300 mgを月1回皮下投与したところ，16週後に最大56％のACR20反応率が得られ，疾患活動性スコア28(DAS28)はベースラインから最大で1.4ポイント低下した．75 mg群，150 mg群および300 mg群の有効性は同様であり，52週後まで維持された．secukinumabはおおむね忍容性良好であり，安全性は他の生物学的製剤と同様であった．

ヒト化IgG4抗IL-17Aモノクローナル抗体である，ixekizumabはphase II試験で生物学的製剤ナイーブとTNF-IRの患者に投与された．3，10，30，80または180 mgの皮下注射を0，1，2，4，6，8と10週目に投与され，生物学的製

剤ナイーブ群では治療開始12週で用量依存性に良好な治療反応を認めた．TNF-IRコホートでは80，180 mgの高用量が割り付けられたが，治療開始12週におけるACR20反応率はそれぞれ40％，39％であった．

完全ヒト型IgG2抗IL-17RAモノクローナル抗体であるbrodalumabはRA患者40人に対してphase II試験が行われている．生物学的製剤ナイーブの活動性のあるRA患者252人に対しbrodalumabを皮下注射にて70，140または210 mgを0，1，2，4，6，8と10週目に投与した．治療開始後12週におけるACR50はbrodalumab群で10-16％，プラセボ群で13％，またDAS28のベースラインからの変化は両群に差を認めなかった．brodalumabの臨床治験に関しては治療効果が得られなかったと結論づけられた．

現在，少なくとも2つのIL-17経路をターゲットとしたRAに対する臨床試験が行われている[15]．

おわりに

RAにおけるTh17細胞の役割について概説した．ヒトのRAにおけるIL-17阻害による臨床的応用では一定の評価がなされている．しかしながら，RA滑膜組織の免疫組織による検討ではIL-17陽性細胞はT細胞の1％以下と報告されており[3]，またヒトの末梢血におけるIL-17陽性細胞の割合はわずか数％にすぎず，Th17細胞がRAの病態においてどのような役割を果たしているかまだ明らかにはなっていない．ヒトのRAでは動物モデルと異なりヘテロな病態であるため，罹病期間や疾患活動性によってもIL-17の関与は変化すると思われ，結果の解釈にはしばらく時間を要するものと思われる．今後の臨床研究の結果が待たれる．

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Activation of Syk in Peripheral Blood B Cells in Patients With Rheumatoid Arthritis

A Potential Target for Abatacept Therapy

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Objective. **B cells play a pivotal role in the pathogenesis of autoimmune diseases. Although Syk functions as a key molecule in B cell receptor signaling, the pathologic role of Syk in B cells in rheumatoid arthritis (RA) remains unclear. The purpose of this study was to assess the relevance of activation of Syk in B cells to the pathologic development of RA and to the responsiveness of RA patients to treatment with biologics.**

Methods. **Healthy subjects (n = 36) and patients with moderate or severe RA disease activity (n = 70) were studied. The phosphorylation of Syk (pSyk) in peripheral blood B cells was measured by flow cytometry, and its correlation with clinical characteristics and changes after administration of biologic agents was evaluated.**

Results. **Levels of pSyk in peripheral blood B cells were preferentially higher in patients with RA compared to healthy subjects. Patients with significantly higher pSyk levels were strongly positive for anti–citrullinated protein antibodies (ACPAs). High pSyk levels were not correlated with the severity of disease activity. Treatment with abatacept, but not tumor necrosis factor inhibitors, significantly reduced the levels of pSyk in RA peripheral blood B cells. Abatacept also significantly reduced the proportion of follicular helper T (Tfh) cells.**

Conclusion. **Levels of pSyk in peripheral blood B cells were significantly elevated in patients with RA, and these patients also exhibited strong positivity for ACPAs. These data suggest that abatacept seems to inhibit the phosphorylation of Syk in B cells, as well as the development of Tfh cells, thus highlighting the relevance of B cell–T cell interactions as a potential target of abatacept therapy in RA.**

Activated autoreactive B cells produce autoantibodies and inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor α (TNFα). The expression of costimulatory molecules, such as CD40 and CD80, is enhanced on B cells and is involved in the interactive activation with surrounding immunocompetent cells, including T cells. B cells have an antigen-presenting activity, particularly in autoimmune diseases, and are associated with the activation of autoreactive T cells. Therefore, B cells play an important role in the pathogenetic processes of rheumatoid arthritis (RA).

Rituximab, a chimeric anti-CD20 antibody, eliminates B cells through antibody- and complement-dependent cytotoxic activities. The efficacy of rituximab

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has been demonstrated in RA patients with high disease activity (in the Dose-Ranging Assessment: International Clinical Evaluation of Rituximab in Rheumatoid Arthritis [DANCER] trial [1]) and in RA patients resistant to TNF inhibitor therapy (in the Randomized Evaluation of Long-term Efficacy of Rituximab in Rheumatoid Arthritis [REFLEX] trial [2]). Rituximab was approved for the treatment of RA in the US in 2006 and is currently considered the second-line biologic agent, subsequent to TNF inhibitor therapy. In addition to these studies, some clinical studies have demonstrated the efficacy of a humanized anti-CD20 antibody, ocrelizumab, and a fully human anti-CD20 antibody, ofatumumab, in patients with RA resistant to TNF inhibitor therapy, indicating that B cells are an evident therapeutic target for RA.

Syk is a 72-kd nonreceptor tyrosine kinase discovered by Taniguchi et al (3) in 1991. Syk is involved in the signaling pathway through Fc receptors, which are broadly expressed on immunocompetent cells, such as B cells, dendritic cells, mast cells, macrophages, and neutrophils, and on molecules associated with cell adhesion, such as integrin (4,5).

Recently, the importance of Syk in the pathologic processes of RA has been reported. The results of a phase II clinical study of R406, a Syk inhibitor, in patients resistant to treatment with methotrexate (MTX) indicated that phosphorylation of Syk (measured as levels of pSyk) was increased in the synovial tissue of RA patients compared to healthy subjects and patients with osteoarthritis (6–8). Another experimental study using the synovial cells from these patients demonstrated that R406 inhibits TNFα-induced activation of mitogen-activated protein kinases and the expression of the matrix metalloproteinase 3 (MMP-3) gene, thus highlighting the significant role of Syk in synovial fibroblasts of RA patients (9).

In addition, previous studies elucidated the role of Syk in B cells. Syk has important roles in B cell maturation and survival (10,11). The Toll-like receptor 9 (TLR-9) signaling pathway is involved in the activation of B cells and autoantibody production by B cells (12,13). In this regard, we have recently demonstrated that signaling through Syk results in effective signal transduction of TLR-9 by inducing optimal expression of TNF receptor–associated factor 6 (TRAF6), and that this signaling is important for antibody production by B cells (14). Based on these results, we hypothesized that Syk phosphorylation in B cells is involved in the pathologic processes of RA through the production of autoantibodies, such as rheumatoid factor (RF) and anti–citrullinated protein antibodies (ACPAs).

T cells (especially Th1 and Th17 cells) also play a pivotal role in the pathogenesis of RA (15,16). Recently, follicular helper T (Tfh) cells, whose primary task is to drive the formation of B cell responses, have been recognized as a critical regulator of autoimmunity (17,18). We and other investigators have elucidated the mechanism of Tfh cell differentiation (19,20); however, the exact role of this T helper cell subset in RA remains elusive.

Abatacept, a fusion protein containing CTLA-4 and Ig, which is referred to as a T cell–selective costimulatory regulator, inhibits the activation of T cells. However, little is known about the T cell populations targeted by abatacept. The effect of abatacept on antigen-presenting cells has also been reported (21–23). The inhibitory effect of abatacept on T cell–dependent antibody production has been reported in mice and cynomolgus monkeys (24,25). Evidence suggests that abatacept also has an inhibitory effect on bone destruction, by suppressing the production of RF and ACPAs (26). However, the effect of abatacept on human B cells is unknown. Based on these observations, abatacept is predicted to regulate the activation of not only T cells but also B cells, directly and/or indirectly.

In this study, we observed significantly elevated Syk phosphorylation in the peripheral blood B cells of patients with RA compared to healthy subjects, and we demonstrated that the levels of pSyk were significantly high in patients who were strongly positive for ACPAs. Moreover, treatment with abatacept, but not with TNF inhibitors, significantly inhibited Syk phosphorylation in B cells. Interestingly, treatment with abatacept significantly reduced the proportion of Tfh cells, which could be a possible mechanism for the reduction in Syk phosphorylation in B cells. The results suggest that Syk plays an important role in ACPA production by B cells in patients with RA, and that abatacept inhibits both Syk phosphorylation in B cells and the development of Tfh cells.

PATIENTS AND METHODS

Patients. Table 1 summarizes the baseline characteristics of the 70 patients with RA. The healthy control subjects (n = 36) were either staff members of our hospital or healthy subjects who visited our hospital for medical examinations. Patients with RA who were resistant to treatment comprised those whose score of RA disease activity was >3.1 on the Disease Activity Score in 28 joints using erythrocyte sedimentation rate (DAS28-ESR) (27), despite having received treat-

Table 1. Characteristics of the study patients with rheumatoid arthritis (n = 70)*

Age, mean ± SD years	61.4 ± 15.1
Sex, no. female/no. male	60/10
Disease duration, mean ± SD months	91.5 ± 114.4
Prednisolone (or equivalent)	
No. not receiving treatment/total no.	11/70
Dosage, mean ± SD mg/day	3.4 ± 1.9
Methotrexate	
No. not receiving treatment/total no.	53/70
Dosage, mean ± SD mg/week	13.0 ± 3.6
Tender joint count, mean ± SD	8.5 ± 7.3
Swollen joint count, mean ± SD	7.3 ± 6.3
CRP, mean ± SD mg/dl	2.0 ± 3.0
ESR, mean ± SD mm/hour	53.2 ± 33.3
IgG, mean ± SD mg/dl	1,512.5 ± 452.5
RF	
Mean ± SD IU/ml	149.7 ± 407.7
No. negative/no. positive	21/49
ACPA status, no.	
Negative	22
Positive	6
Strongly positive	42
MMP-3, mean ± SD ng/ml	194.8 ± 246.7
DAS28-CRP, mean ± SD	4.7 ± 1.4
DAS28-ESR, mean ± SD	5.5 ± 1.4
CDAI, mean ± SD	26.3 ± 15.0
SDAI, mean ± SD	28.3 ± 16.8
HAQ score, mean ± SD	1.3 ± 0.9
No. not treated with biologics/total no.	57/70

* CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; RF = rheumatoid factor; ACPA = anti–citrullinated protein antibody; MMP-3 = matrix metalloproteinase 3; DAS28-CRP = Disease Activity Score in 28 joints using CRP level; CDAI = Clinical Disease Activity Index; SDAI = Simplified Disease Activity Index; HAQ = Health Assessment Questionnaire.

ment with adequate doses of antirheumatic drugs, mainly MTX, for a minimum of 3 months, and who showed no response or only a moderate response to treatment according to the European League Against Rheumatism (EULAR) improvement criteria (28). The Human Ethics Review Committee of the university reviewed and approved our study, including the collection of peripheral blood samples from healthy adults and patients with RA. Each subject provided a signed participation consent form.

Measurements. The background factors investigated were sex, age, duration of RA, and doses of corticosteroids and MTX. We also evaluated the severity of morning stiffness, number of swollen joints, number of tender joints, and patient's evaluations of pain and overall health by visual analog scales, in addition to global evaluations of health by the attending physician. The laboratory tests included measurements of the C-reactive protein (CRP) level, ESR, IgG, RF, ACPAs, and MMP-3. We consulted the American College of Rheumatology (ACR)/EULAR 2010 classification criteria for RA (29) to select the cutoff values for stratification of ACPA positivity. Low-positive ACPA refers to IU values that are higher than the upper limit of normal (ULN) but ≤3 times the ULN for the laboratory and assay, whereas high-positive ACPA refers to IU values that are >3 times the ULN for the laboratory and assay. The variables investigated included the DAS28 using CRP level (DAS28-CRP), DAS28-ESR, the Clinical Disease Activity Index (CDAI) (30), the Simplified Disease Activity Index (SDAI) (31), the Health Assessment Questionnaire (HAQ) (32), and history of biologics use.

Flow cytometry analysis. Peripheral blood mononuclear cells (PBMCs) from 36 normal healthy volunteers and from 70 patients with RA whose diagnosis met the ACR 1987 revised classification criteria for RA (33) were isolated from the peripheral blood using lymphocyte separation medium (ICN/Cappel Pharmaceuticals). For surface and intracellular staining, 2×10^5 PBMCs, which were acquired after strict deletion of dust by threshold adjustment, were subjected to fluorescence-activated cell sorting analysis. PBMCs were fixed with phosphate buffered saline (PBS) containing 1% formaldehyde and then permeabilized with PBS containing 0.1% saponin. After washing, the PBMCs were resuspended in saponin–PBS and stained with mouse anti-human Syk monoclonal antibodies (mAb) (Abcam) and mouse anti-human pSyk (pY348) mAb (BD PharMingen), followed by washing with saponin–PBS. Phycoerythrin-labeled goat anti-mouse IgG polyclonal antibody (BD PharMingen) was used as a secondary antibody. After washing with saponin–PBS, the PBMCs were stained with fluorescein isothiocyanate–labeled mouse anti-human CD19 antibodies (BD PharMingen).

The rate of pSyk expression in B cells was calculated as the percentage of pSyk-positive CD19+ B cells relative to total CD19+ B cells. We defined pSyk-positive CD19+ B cells as cells in which the intensity of staining was higher than the background staining with IgG control antibody. The proportion of CD19+ B cells (relative to total cells) in healthy donors and RA patients was a mean ± SD 15,199 ± 7,482 cells (7.6 ± 3.7%) and 12,844 ± 7,120 cells (6.6 ± 3.6%), respectively.

Tfh cells were stained with anti-CD4, anti-CXCR5, and anti–programmed death 1 (anti–PD-1) antibodies (BD PharMingen). The proportion of CD4+ cells (relative to total cells) was 20,364 ± 17,727 cells (8.2 ± 7.0%), while that of CD4+CXCR5+PD-1+ cells (relative to total cells) was 1,841 ± 3,940 cells (0.7 ± 1.5%). Stained cells were analyzed on a flow cytometer (FACSCalibur; BD PharMingen). The cells were collected and analyzed with FlowJo software (Tree Star).

In vitro B cell activation analysis. CD19+ B cells were purified from the peripheral blood of the healthy control subjects and RA patients. The cells were cultured in stimulation-free medium for 3 days to assess the production of IL-6 or for 5 days to assess the production of IgG. IL-6 production was determined using a BD Cytometric Bead Array human Flex set (BD PharMingen). Flow cytometry was carried out using a FACSCalibur and CellQuest software (Becton Dickinson). IgG levels in the culture medium were determined using a human IgG enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories).

Statistical analysis. Data are expressed as the mean ± SD. Differences between groups for variables with normal distribution and homoscedasticity were compared using Student's *t*-test. Differences between groups for variables with skewed distribution were compared using Wilcoxon's rank sum test. Analysis of variance followed by the Bonferroni/Dunn post hoc test was used to compare data from 3 groups with normal distribution. The Kruskal-Wallis test followed by the Bonferroni/Dunn post hoc test was used to compare data from

>3 groups with skewed distribution. Correlation analysis was performed using Spearman's correlation coefficients. Baseline and posttreatment values within each sample were compared using Wilcoxon's matched-pairs signed-rank test. *P* values less than 0.05 were considered significant. All analyses were conducted using PASW Statistics software version 18.0 (IBM).

RESULTS

Patient background. This study was conducted in 70 patients with RA who were receiving treatment in our hospital in Japan. The clinical features of the RA patients are described in Table 1. The washout period in patients who had previously received biologics (etanercept, golimumab, adalimumab, tocilizumab, abatacept) was more than 1 month. Infliximab required a 60-day washout.

High Syk phosphorylation in B cells of ACPA-positive RA patients. PBMCs were isolated from 36 healthy donors (as controls) and 70 patients with RA

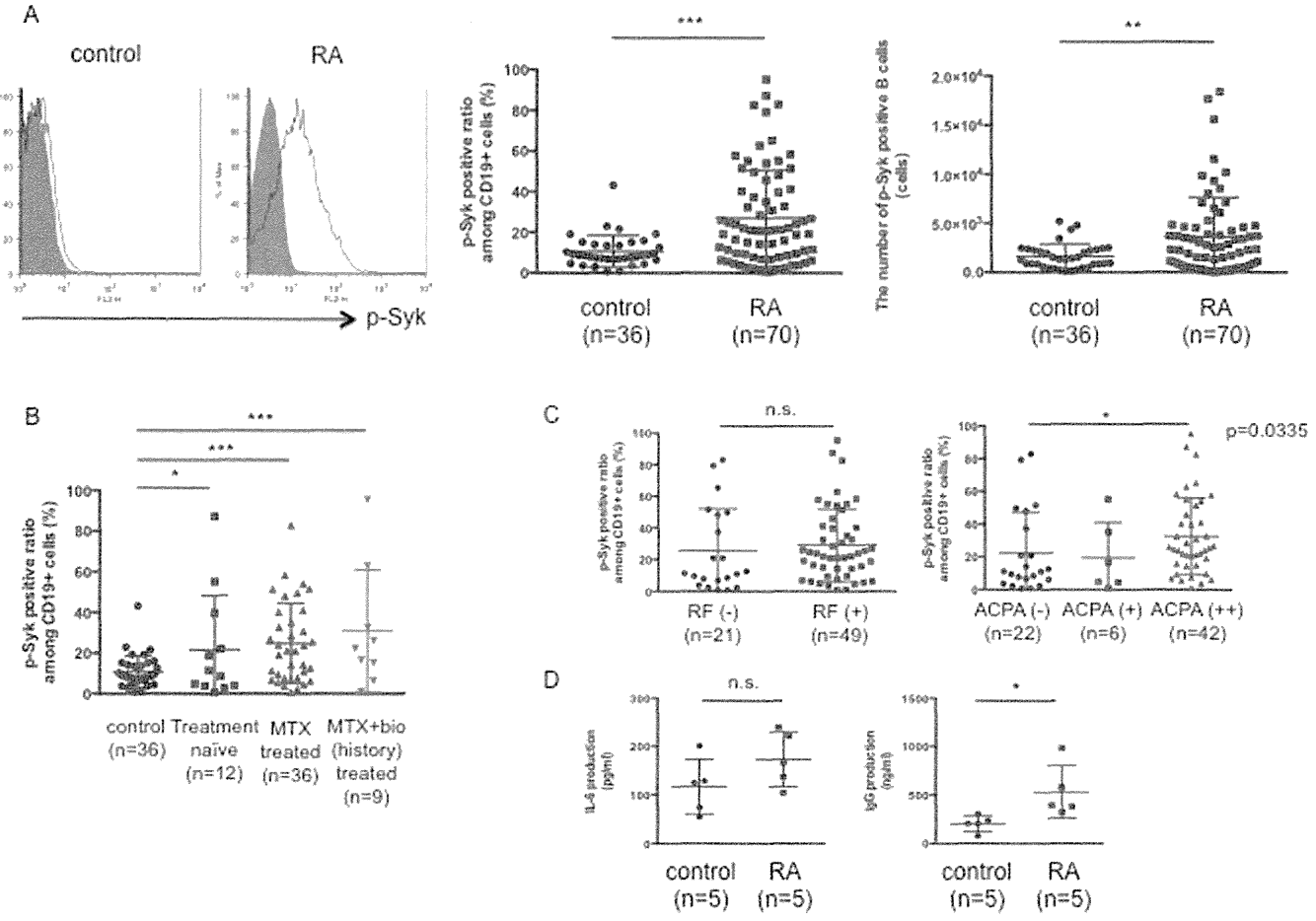

Figure 1. Phosphorylation of Syk in CD19+ B cells of healthy donors (controls) and patients with rheumatoid arthritis (RA). **A,** Representative histograms showing Syk phosphorylation in peripheral blood B cells from 70 RA patients and 36 healthy control subjects (left), and the ratio of pSyk-positive cells among CD19+ B cells (middle) and absolute number of pSyk-positive CD19+ B cells (right) in RA patients compared to healthy controls. **B,** Ratio of pSyk-positive cells among CD19+ B cells in 3 groups of RA patients: treatment-naive (n = 12), methotrexate (MTX)–treated (n = 36), and MTX + biologics (bio) (history)–treated (n = 9). RA patients treated with other disease-modifying antirheumatic drugs and/or corticosteroids were excluded. **C,** Ratio of pSyk-positive cells among CD19+ B cells in RA patients negative for rheumatoid factor (RF) (defined as <15 IU/ml, based on the normal limit at our hospital) or positive for RF (defined as ≥15 IU/ml), and RA patients negative (−), positive (+), or strongly positive (++) for anti–citrullinated protein antibodies (ACPAs) (defined as <4.5 units/ml, 4.5–13.5 units ml, and >13.5 units/ml, respectively, based on the normal limit at our hospital). **D,** Production of interleukin-6 (IL-6) (left) and IgG (right) by CD19+ B cells purified from the peripheral blood of healthy controls and RA patients. B cells were cultured in stimulus-free RPMI medium for 3 days (for IL-6) or 5 days (for IgG). Production of IL-6 in the supernatants was assayed by cytometric bead array, while IgG in the supernatants was quantified by enzyme-linked immunosorbent assay. Symbols represent individual subjects; bars show the mean ± SD. * = $P < 0.05$, ** = $P < 0.01$; *** = $P < 0.001$. NS = not significant.