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医療技術実用化総合研究事業（臨床研究・治験推進研究事業）

分担研究報告書

難治性SLEに対するボルテゾミブ療法の有効性・安全性検証試験

ループス腎炎のポドサイトにおけるCD86分子を介した蛋白尿発現メカニズムに関する研究

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研究要旨：ループス腎炎におけるポドサイトは糸球体基底膜の上に存在し、蛋白尿のバリア機能を有している。以前、プロテアーゼ阻害薬であるボルテゾミブのループス腎炎抑制効果が報告された(Nature Medicine 14, 748 - 755 (2008))が、形質細胞制御が主なメカニズムであり、ポドサイトへの影響については明らかにされていない。そこで今回、我々は健常人およびループス腎炎患者から分離したIgGを用いて、ヒトのポドサイトと免疫担当細胞の関与について検討した。健常人およびループス腎炎IgGとポドサイトとをインキュベートし、その機能変化を調べた。Microarrayによる Gene Ontology 解析ではポドサイトのアクチンフィラメント再構成やCD86, CD80などの免疫細胞の活性化に関与するsignal pathwayの亢進がループス腎炎IgGでみられた。ポドサイトにも免疫活性を有し、ボルテゾミブが形質細胞だけではなく、ポドサイトに直接、機能変化をもたらす可能性が示唆された。

A. 研究目的

SLE患者の中で、腎障害は症例の約40～75%に認められ、重要な予後規定因子である。しかし、これまでのところループス腎炎における蛋白尿発現のメカニズムについては明らかにされていない。ループス腎炎におけるポドサイトはバリア機能を有し、その足突起の構造変化と蛋白尿発現との関連が示唆されている。最近、生物学的製剤アバタセプト(CTLA-Ig製剤)がT細胞のみならず、ポドサイトにも直接的な効果を示すことが報告されている(N Engl J Med. 2013 Dec 19;369(25):2416-23.)。一方で、プロテアーゼ阻害薬であるボルテゾミブのループス腎炎抑制効果が報告された(Nature Medicine 14, 748 - 755 (2008))が、形質細胞制御が主な

メカニズムであり、ポドサイトへの影響については明らかにされていない。

研究方法

今回、我々は健常人およびループス腎炎患者から分離したIgGを用いて、ヒトのポドサイトのcell line (AB8/13)における影響について検討した。健常人およびループス腎炎患者の血清からIgG purification kitを用いてIgGを分離し、IgGをポドサイト とともに24-48時間インキュベートして、Microarrayによる網羅的遺伝子解析を行った。さらにSLEのモデルマウスであるMRL/*lpr*マウスの腎組織を用いて、ポドサイトにおけるCD86発現を*in situ* hybridizationにて検討を行った。

(倫理面への配慮)

本研究は「全身性エリテマトーデスの病態を多角的に解析する臨床研究」、「自己免疫疾患における T 細胞分化シグナル制御機構の解明」および「難治性全身性エリテマトーデスに対するボルテゾミブの有効性・安全性探索試験に対する追加クロスオーバー試験」というテーマで長崎大学病院倫理委員会および長崎大学先導生命科学研究支援センター動物実験施設より承認を得て、十分にインフォームドコンセントを行い検体採取している。匿名化された検体を用い、情報管理を厳重に行っているため倫理面での問題はない。

H. 研究結果

蛍光抗体法にて健常人およびループス腎炎患者由来の IgG は neonatel Fc receptor(FcRn)を介してポドサイトの細胞質に局在することが確認された。Microarray による Gene Ontology 解析ではポドサイトのアクチンフィラメント再構成や CD86, CD80 などの免疫細胞の活性化に関与する signal pathway の亢進がループス腎炎 IgG でみられた。また SLE のモデルマウスである MRL/*lpr* マウスの腎組織ではコントロールである MRL/*MPJ* マウスに比して、ポドサイトマーカーである nephrin mRNA 中の CD86 mRNA の発現の増強を認めた。

考察

ループス腎炎由来の IgG はポドサイトにおけるアクチンフィラメントの再構成をもたらし、細胞骨格変化に関与し、病的状態にみられる足突起の癒合などをもたらす可能性がある。また、ループス腎炎におけるポドサイトでは抗原提示細胞と同様に CD86 分子の発現増強がみられ、co-stimulatory pathway を介した免疫活性を有し、ボルテゾミブが形質細胞だけではなく、ポドサイトにも直接、機能変化をもたらす可能性が示唆された。

E．結論

ボルテゾミブの SLE、ループス腎炎の病態制御には形質細胞だけでなく、多彩な細胞をターゲットとする multiple pathway を介した抑制効果が期待される。

F. 研究発表

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G. 知的財産権の出願・登録状況
（予定を含む）

7. 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)

8. 実用新案登録

該当無し

9. その他

該当無し

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	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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一瀬邦弘,古賀智裕,川上 純	特集：キナーゼ阻害によるリウマチ性疾患の治療―現在と未来―.	分子リウマチ	7(4)	23-27

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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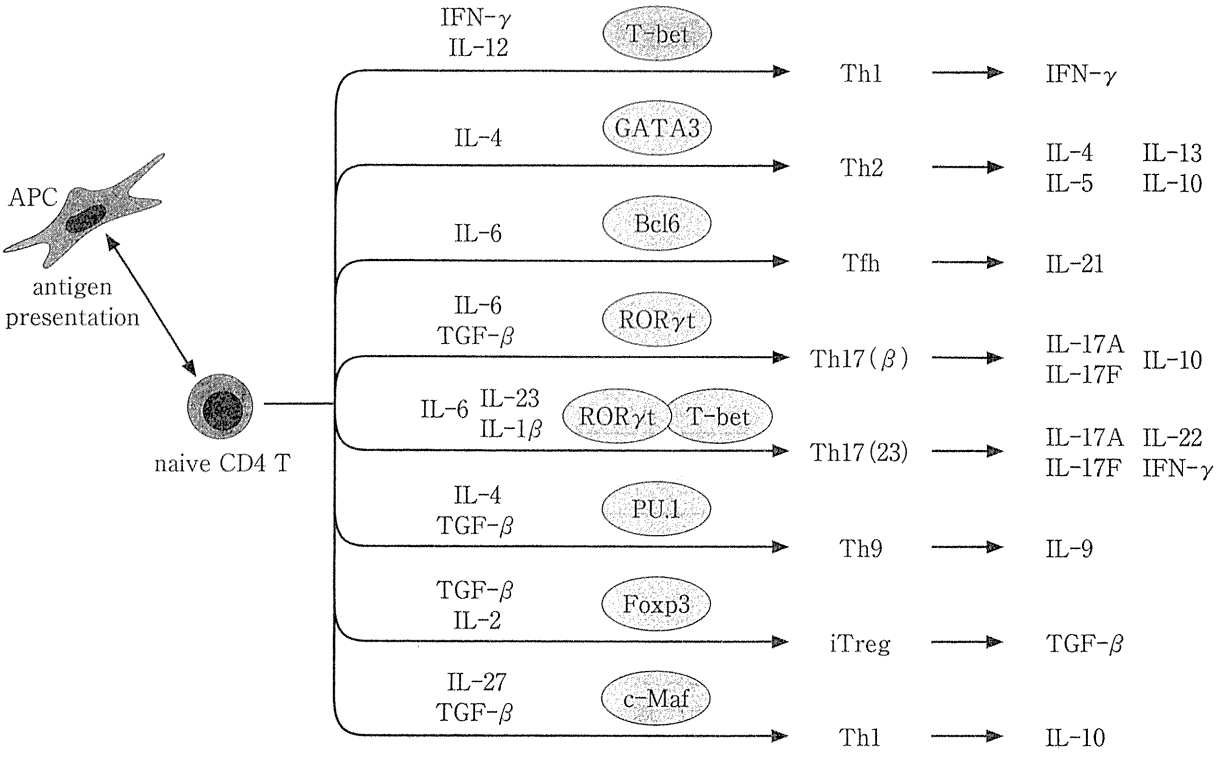

図1 Th17細胞の分化に必要なサイトカインと転写因子（文献[6]より引用）

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

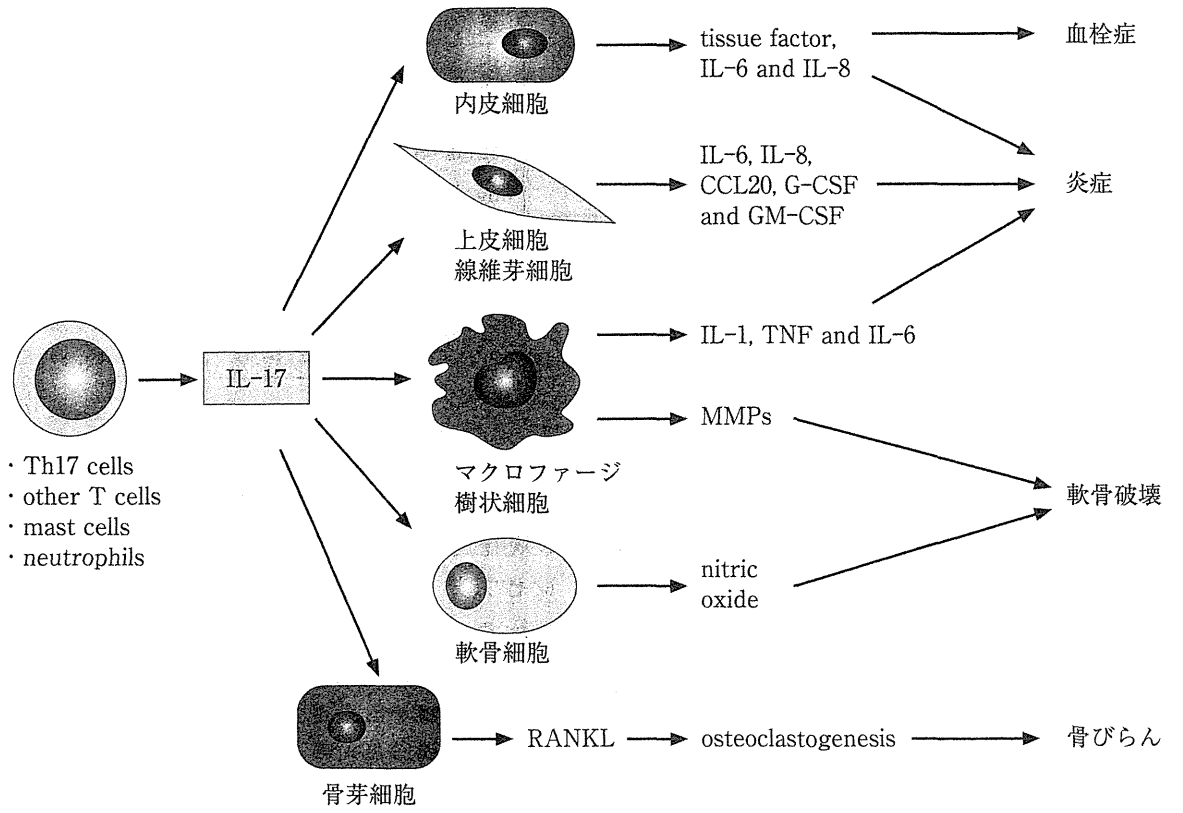

図2 IL-17による炎症や関節破壊のメカニズム(文献[11]より改変)

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

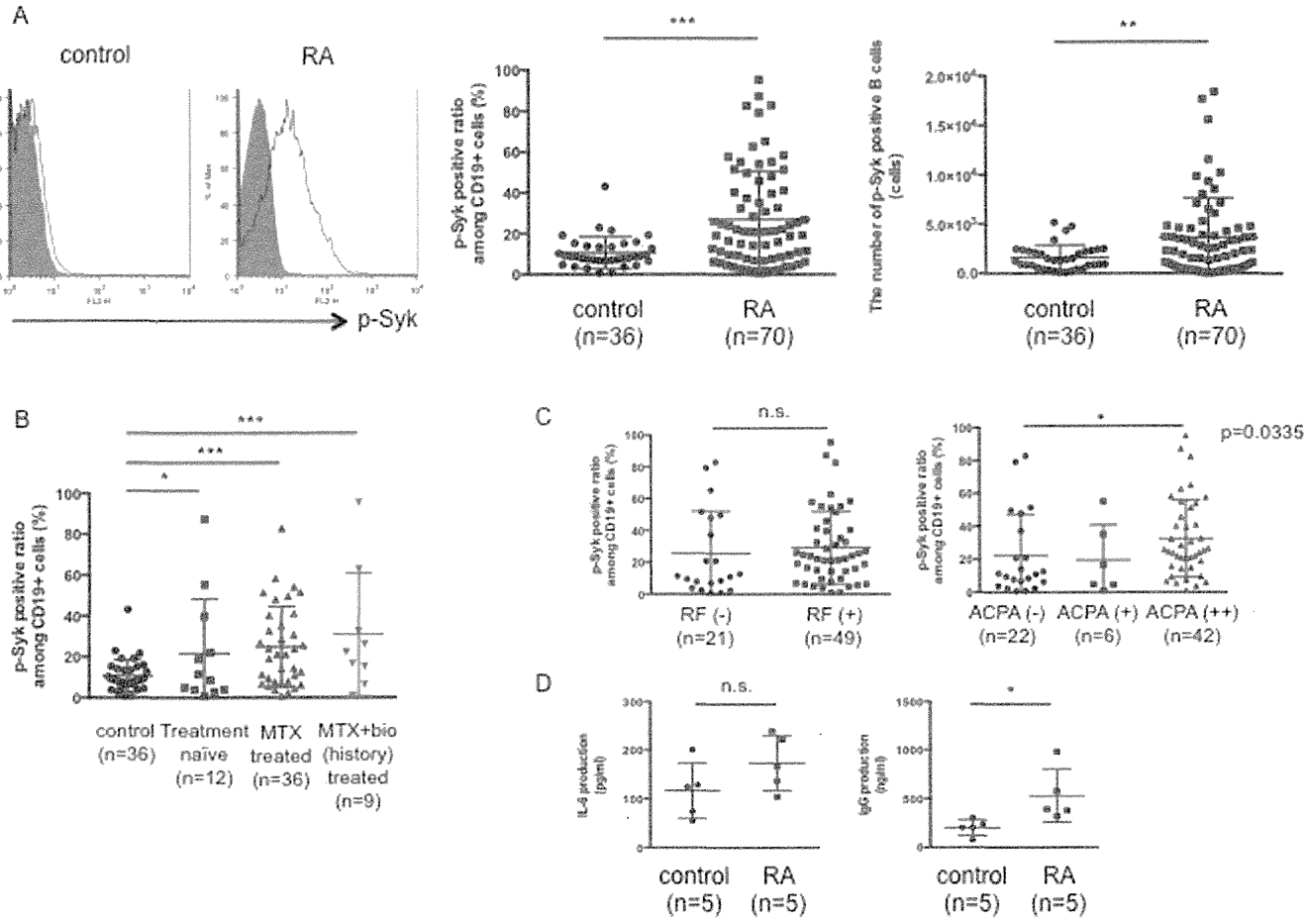

Figure 1. Phosphorylation of Syk in CD19+ B cells of healthy donors (controls) and patients with rheumatoid arthritis (RA). **A,** Representative histograms showing Syk phosphorylation in peripheral blood B cells from 70 RA patients and 36 healthy control subjects (left), and the ratio of pSyk-positive 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.

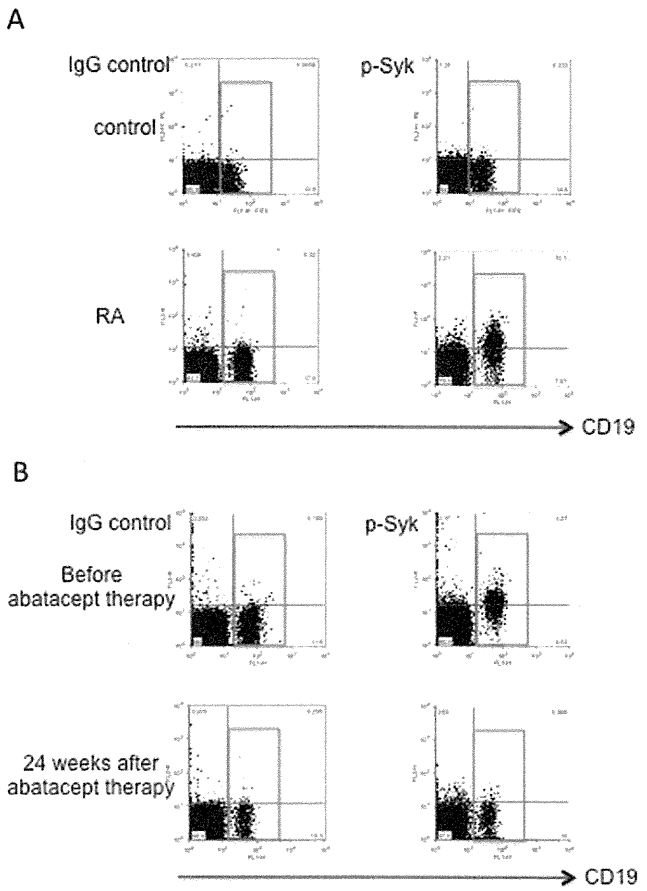

Figure 2. Phosphorylation of Syk in CD19+ B cells from the peripheral blood of healthy subjects (controls) and patients with rheumatoid arthritis (RA) and in RA patients before and after treatment with abatacept. **A,** Expression of pSyk on CD19+ B cells was assessed by flow cytometry in the peripheral blood mononuclear cells (PBMCs) of controls and RA patients. **B,** Levels of pSyk in CD19+ B cells were compared in RA patients before and 24 weeks after treatment with abatacept. In **A** and **B,** the lymphocyte region of PBMCs was gated for expression of pSyk on CD19+ B cells (indicated by gray-shaded boxed areas), in comparison to that in IgG control experiments.

and analyzed by flow cytometry to determine the levels of Syk phosphorylation in peripheral blood CD19+ B cells. The control subjects and RA patients were matched for sex but not age. Analysis of the effect of age on Syk phosphorylation showed that the level of pSyk in B cells was not correlated with age in either the control subjects or the RA patients (in controls [mean ± SD age 38.7 ± 9.6 years], Spearman's $r^2 = 0.0625$, $P = 0.23$; in RA patients, Spearman's $r^2 = 0.0008$, $P = 0.82$). Although the expression level of Syk in B cells was not different between the groups (results not shown), the level of Syk phosphorylation in B cells was significantly higher in RA patients compared to healthy controls (mean ± SD percentage of pSyk-positive B cells among CD19+ B cells, 27.7 ± 23.2% in RA patients versus 11.9 ± 8.2% in controls; $P = 0.0019$, by Student's *t*-test) (Figures 1A and 2A).

We estimated the absolute numbers of total B cells and pSyk-positive B cells in the RA patients and healthy controls. Although there was no significant difference in the percentage and absolute number of total B cells between RA patients and healthy controls (mean ± SD percentage of total B cells relative to number of lymphocytes, 11.2 ± 6.2% in RA patients versus 13.3 ± 6.6% in controls [$P = 0.08$, by Student's *t*-test]; absolute number of CD19+ cells, 12,844 ± 7,120 in RA patients versus 15,199 ± 7,482 in controls [$P = 0.09$, by Student's *t*-test]), the absolute number of pSyk-positive B cells was significantly higher in RA patients than in controls (mean ± SD 3,639 ± 4,021 versus 1,608 ± 1,285; $P = 0.0137$, by Student's *t*-test) (Figure 1A). In addition, the proportions of B cell subsets classified into CD19+CD27− naive B cells and CD19+CD27+ memory B cells were comparable between the RA patients and healthy controls (mean ± SD percentage of CD19+CD27− naive B cells, 83.0 ± 6.2% in RA patients versus 84.9 ± 5.0% in controls [$P = 0.24$, by Student's *t*-test]; percentage of CD19+

Table 2. Relationship between clinical characteristics and the ratio of pSyk-positive cells among CD19+ B cells in patients with rheumatoid arthritis*

	Spearman's rho†
Age	0.0961
Disease duration (mean months)	0.0320
Prednisolone (or equivalent) (mg/day)	0.0915
Methotrexate (mg/week)	−0.1516
Tender joint count (28 total)	0.0045
Swollen joint count (28 total)	0.1821
DAS28-CRP	0.0217
DAS28-ESR	0.1135
CDAI	0.0637
SDAI	0.0501
CRP level (mg/dl)	−0.0513
ESR (mm/hour)	0.1221
MMP-3 level (ng/ml)	0.2019
IgG level (mg/dl)	0.0311
Presence of ANAs	−0.0798

* DAS28-CRP = Disease Activity Score in 28 joints using C-reactive protein level; ESR = erythrocyte sedimentation rate; CDAI = Clinical Disease Activity Index; SDAI = Simplified Disease Activity Index; MMP-3 = matrix metalloproteinase 3; ANAs = antinuclear antibodies.
† Values are Spearman's rank correlation coefficients for each characteristic in relation to the percentage of pSyk-positive CD19+ B cells. None of the values were significant.

CD27+ memory B cells, 17.0 ± 6.0% in RA patients versus 15.1 ± 5.0% in controls [P = 0.20, by Student's t-test]). These results suggest that pSyk expression is up-regulated in RA patients compared to healthy control subjects irrespective of the proportions of B cell subsets.

We next investigated differences in pSyk levels among 3 groups of RA patients: treatment-naive RA patients (n = 12), MTX-treated RA patients (n = 36), and MTX + biologics (history)–treated RA patients (n = 9). RA patients who had been treated with other disease-modifying antirheumatic drugs and/or corticosteroids were excluded from the analysis. The expression levels of pSyk in all 3 groups of RA patients were significantly higher than those in the control group (mean ± SD percentage of pSyk-positive cells among CD19+ cells, 21.6 ± 7.7% in treatment-naive RA patients, 24.8 ± 3.3% in MTX-treated RA patients, and 30.8 ± 10.0% in MTX + biologics–treated RA patients versus 10.7 ± 1.3% in controls; P = 0.0036, by Student's t-test). There was no significant difference in the pSyk level among each of the 3 RA treatment groups (Figure 1B).

We then assessed the correlation between patient background characteristics and Syk phosphorylation in B cells (Table 2). Syk phosphorylation levels in B cells were not correlated with indices of RA disease activity, such as the tender joint count, swollen joint count, CRP level, ESR, MMP-3 level, DAS28-CRP, DAS28-ESR, CDAI, and SDAI. There was also no correlation with age, sex, duration of disease, use or dosage of steroids, or use or dosage of oral MTX. Interestingly, Syk phosphorylation was significantly higher in B cells of patients strongly positive for ACPAs (mean ± SD percentage of pSyk staining among CD19+ B cells, 22.2 ± 24.9% in RA patients negative for ACPAs and 19.5 ± 21.5% in RA patients positive for ACPAs versus 32.6 ± 23.5% in RA patients strongly positive for ACPAs; P = 0.0335, by Kruskal-Wallis test) (Figure 1C and Table 3).

We also investigated whether the up-regulation of pSyk expression in the B cells of RA patients was associated with enhanced B cell activation. CD19+ B cells were purified from the peripheral blood of the RA patients and healthy control subjects and then cultured in a stimulus-free medium for 3 or 5 days for assessment of IL-6 or IgG production, respectively. The levels of IL-6 tended to be more pronounced in the B cells of RA patients compared to healthy controls, but the difference was not statistically significant (mean ± SD IL-6 concentration, 174.0 ± 56.8 pg/ml in RA patients versus 116.6 ± 57.1 pg/ml in controls; P = 0.136, by Wilcoxon's rank sum test) (Figure 1D). IgG production by B cells was significantly higher in RA patients (mean ± SD 529.5 ± 270.7 ng/ml) compared to controls (204.1 ± 83.2 ng/ml; P = 0.033, by Wilcoxon's rank sum test) (Figure 1D). The results of these in vitro studies add support to the notion that pSyk expression in B cells is correlated with the production of autoantibodies in RA patients.

Table 3. Relationship between levels of Syk phosphorylation and subsets of clinical characteristics in patients with rheumatoid arthritis (RA)*

	pSyk, %†
Sex	
Male	27.4 ± 24.5
Female	32.6 ± 22.0
RA disease stage	
I	38.1 ± 28.6
II	24.6 ± 22.3
III	18.7 ± 11.4
IV	32.4 ± 27.8
RA functional class	
I	24.7 ± 19.9
II	28.4 ± 26.0
III	28.4 ± 16.1
RF	
Negative	25.4 ± 26.7
Positive	29.3 ± 23.0
ACPAs‡	
Negative	22.2 ± 24.9
Positive	19.5 ± 21.5
Strongly positive	32.6 ± 23.5

* RF = rheumatoid factor; ACPAs = anti–citrullinated protein antibodies.
† Values are the mean ± SD percentage of pSyk staining among CD19+ B cells.
‡ P = 0.0335 between groups.

Inhibition of Syk phosphorylation in B cells of RA patients following treatment with abatacept. The results presented thus far suggest that the phosphorylation of Syk in RA B cells is involved in the production of ACPAs. Autoantibody production by B cells requires the involvement of T cells, and treatment with abatacept can inhibit the activation of T cells. Based on this background, we hypothesized that abatacept inhibits Syk phosphorylation in B cells. For this purpose, we investigated the effect of abatacept on Syk phosphorylation by comparing it with the effect of TNF inhibitors (infliximab n = 10, golimumab n = 3, etanercept n = 1, adalimumab n = 1). Biologics-naive RA patients were selected for this analysis. Abatacept (n = 12) or a TNF inhibitor (n = 15) was administered to patients with MTX-resistant RA, and the change in B cell Syk phosphorylation after the treatment was investigated.

We found that the posttreatment clinical background was not significantly different between patients

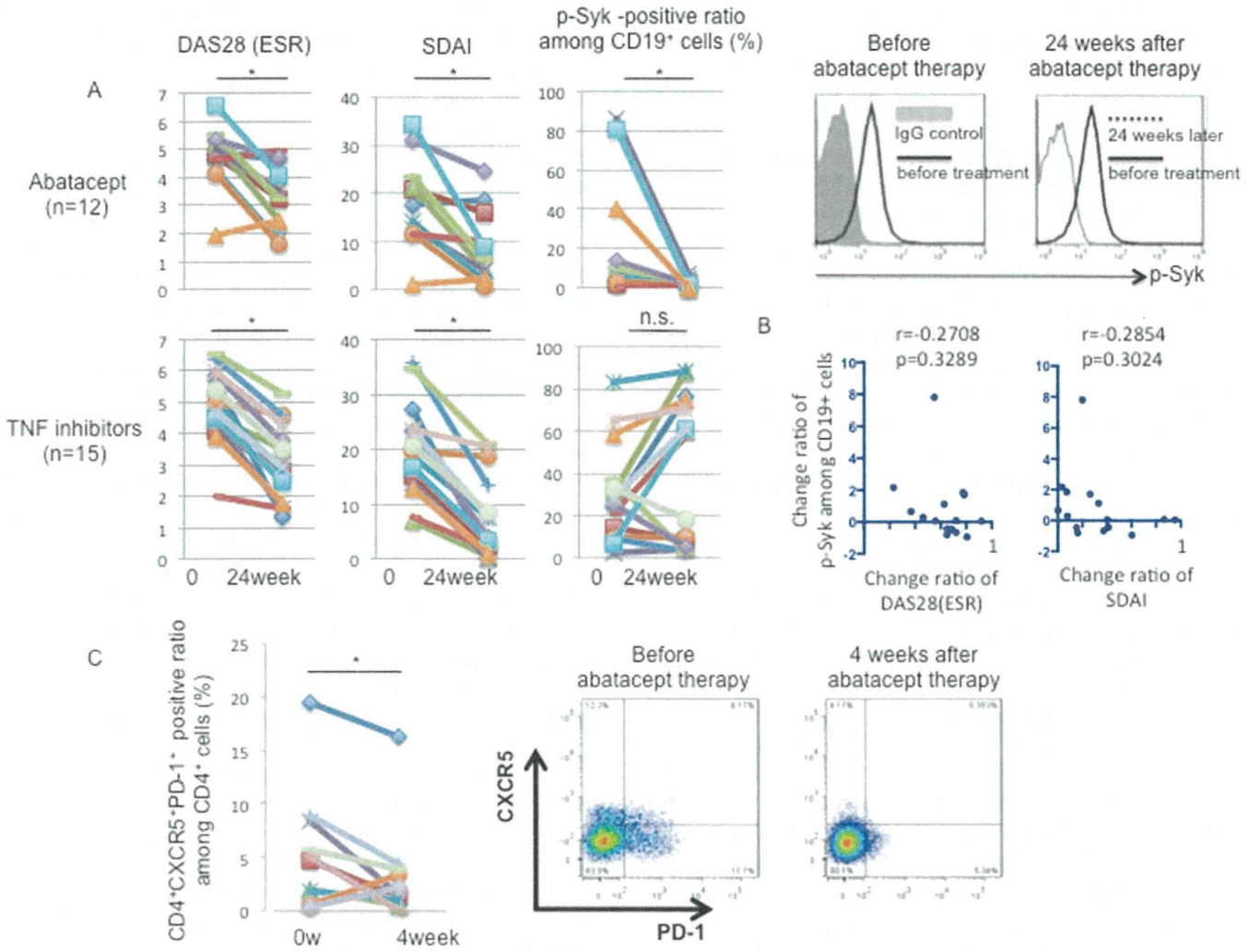

Figure 3. Changes in Syk phosphorylation in B cells and effects on follicular T helper (Tfh) cells among CD4+ T cells before and after treatment of rheumatoid arthritis (RA) patients with abatacept or tumor necrosis factor (TNF) inhibitors. **A,** Left, Changes in the Disease Activity Score in 28 joints using the erythrocyte sedimentation rate (DAS28-ESR), the Simplified Disease Activity Index (SDAI), and the ratio of pSyk-positive cells among CD19+ B cells were assessed in RA patients before and 24 weeks after treatment with abatacept or TNF inhibitors. Right, Changes in the levels of pSyk in CD19+ B cells were assessed in RA patients before and after treatment with abatacept. IgG served as control. **B,** The correlation between change in the ratio of pSyk-positive cells among CD19+ cells and change in the DAS28-ESR and SDAI was assessed in RA patients treated with TNF inhibitors. Change in the ratio was calculated as (value after treatment − value before treatment)/value before treatment. **C,** Left, Changes in the ratio of CD4+CXCR5+PD-1+ cells (Tfh cells) among CD4+ cells were assessed in RA patients before and 4 weeks after treatment with abatacept. Right, Representative flow cytometry data are shown. Colored bars and symbols in **A** and **C** represent individual patients. $* = P < 0.05$. NS = not significant; PD-1 = programmed death 1.

treated with abatacept and those treated with TNF inhibitors (results not shown), including the levels of ACPAs (mean ± SD 85.4 ± 91.9 units/ml in abatacept-treated RA patients versus 77.7 ± 89.6 units/ml in TNF inhibitor–treated RA patients). During the period from week 0 to week 24 after treatment, the DAS28 decreased from a mean ± SD 4.8 ± 1.1 to 3.4 ± 1.1 in the abatacept treatment group ($P = 0.002$, by Wilcoxon's matched-pairs signed-rank test) and from 4.9 ± 1.1 to 3.1 ± 1.2 in the patients treated with TNF inhibitors ($P < 0.001$, by Wilcoxon's matched-pairs signed-rank test). Furthermore, in patients treated with abatacept and those treated with TNF inhibitors, the SDAI decreased from 19.4 ± 10.1 to 9.6 ± 8.4 ($P = 0.0069$, by Wilcoxon's matched-pairs signed-rank test) and from 20.0 ± 8.5 to 7.4 ± 7.5 ($P < 0.001$, by Wilcoxon's matched-pairs