発表者氏名	論文タイトル名	発表雑誌名	巻	頁	出版年
Akashi N, Matsumoto I, Tanaka Y, Inoue A, Yamamoto K, Tanaka Y, Umeda N, Hayashi T, Goto D, Ito S, Sekiguchi K, Sumida T.	Comparative suppressive effect in models of autoimmune arthritis by tyrosine kinase inhibitors imatinib and nilotinib.	Mod. Rheumatol	in press		
Hikami K, Kawasaki A, Ito I, Koga M, Ito S, Hayashi T, Matsumoto I, Tsutsumi A, Kusaoi M, Takasaki Y, Hashimoto H, Arinami T, Sumida T, Tsuchiya N.	Association of a functional polymorphism in the 3' untranslated region of SPI1 with systemic lupus erythematosus.	Arthritis Rheum.	in press		
Tsuboi H, Wakamatsu E, Iizuka M, Nakamura Y, Sugihara M, Suzuki T, Ogishima H, Hayashi T, Goto D, Ito S, Matsumoto I, Sumida T.	Importance of serine727 phosphorylated STAT1 in IFNgamma-induced signaling and apoptpsis of human salivary gland cells.	International Journal of Rheumatic Diseases	in press		
Kawasaki A, Ito S, Furukawa H, Hayashi T, Goto D, Matsumoto I, Kusaoi M, Ohashi J, Graham R R, Matsuta K, Behrens T W, Tohma S, Takasaki Y, Hashimoto H, Sumida T, Tsuchiya N.	Association of TNIP1 with systemic lupus erythematosus in a Japanese population : a case-control association study.	Arthritis Res. Ther.	12	R174	2010
Iizuka M, Wakamatsu E, Tsuboi H, Nakamura Y, Hayashi T, Matsui M, Goto D, Ito S, Matsumoto I, Sumida T.	M3 muscarinic acetylcholine receptor reactive immune response induces Sjogren's syndrome-like sialoadenitis.	J.Autoimmunity	35	383-389	2010
Horikoshi M, Suzuki T, Sugihara M, Kondo Y, Tsuboi H, Uehara T, Hama M, Takase K, Ohno S, Ishigatsubo Y, Yoshida Y, Sagawa A, Ikeda K, Ota T, Matsumoto I, Ito S, Sumida T.	Comparison of low-field dedicated extremity magnetic resonance imaging with articular ultrasonography in patients with rheumatoid arthritis.	Mod. Rheumatol.	20	556-560	2010
Hayashi T, Ito S, Goto D, Matsumoto I, Sumida T.	Elevated level of serum cystatin-C concentration is a useful predictor for myelosuppression induced by methotrexate for treatment of rheumatoid arthritis.	Mod Rheumatol.	20	548-555	2010
Tsuboi H, Matsumoto I, Wakamatsu E, Nakamura Y, Iizuka M, Hayashi T, Goto D, Ito S, Sumida T.	New epitopes and function of anti-M3 muscarinic acetylcholine receptor antibodies in patients with Sjogren's syndrome.	Clin. Exp. Immunol.	162	53-61	2010
Ogishima H, Ito S, Tsutsumi A, Sugihara M, Goto D, Matsumoto I, Obata-Yasuoka M, Hamada H, Yoshikawa H, Takahashi H, Murashima A, Sumida T.	High dose unfractionated heparin therapy in a pregnant patient with antiphospolipid syndrome: a case report	International Journal of Rheumatic Diseases	13	e32-35	2010
Kawasaki A, Ito I, Ito S, Hayashi T, Goto D, Matsumoto I, Takasaki Y, Hashimoto H, Sumida T, Tsuchiya N.	Association of TNFAIP3 polymorphism with susceptibility to systemic lupus erythematosus in a Japanese population.	Journal of Biomedicine and Biotechnology.	207578	Epub 2010 May 27.	2010
Sumida T, Tsuboi H, Iizuka M, Nakamura Y, Matsumoto I.	Functional role of M3 muscarinic acetylcholine receptor (M3R) reactive T cells and anti-M3R autoantibodies in patients with Sjogren's syndrome.	Autoimmunity Reviews	9	615-617	2010

発表者氏名	論文タイトル名	発表雑誌名	巻	頁	出版年
Suzuki T, Ito S, Handa S, Kose K, Okamoto Y, Minami M, Sugihara M, Horikoshi M, Tsuboi H, Hayashi T, Goto D, Matsumoto I, Sumida T.	New low-field extremity MRI, compacTscan: comparison with whole body 1.5 tesla conventional MRI.	Mod. Rheumatol.	20	331-336	2010
Segawa S, Goto D, Yoshiga Y, Sugihara M, Hayashi T, Chino Y, Matsumoto I, Ito S, Sumida T.	Inhibition of transforming growth factor-β signaling attenuates IL-18 plus IL-2-induced interstitial lung disease in mice.	Clin. Exp. Immunol.	160	394-402	2010
Ito I, Kawasaki A, Ito S, Kondo Y, Sugihara M, Horikoshi M, Hayashi T, Goto D, Matsumoto I, Tsutsumi A, Takasaki Y, Hashimoto H, Matsuta K, Sumida T, Tsuchiya N.	Replication of association between FAM167A(C8orf13)-BLK region and rheumatoid arthritis in a Japanese population.	Ann Rheum Dis.	69	936-937	2010
Chen Q, Muramoto K, Masaaki N, Ding Y, Yang H, Mackey M, Li W, Inoue Y, Ackermann K, Shirota H, Matsumoto I, Spyvee M, Schiller S, Sumida T, Gusovsky F, Lamphier M.	A novel antagonist of the prostaglandin E(2) EP(4) receptor inhibits Th1 differentiation and Th17 expansion and is orally active in arthritis models.	Br J Pharmacol.	160	292-310	2010
Wang Y, Ito S, Chino Y, Goto D, Matsumoto I, Murata H, Tsutsumi A, Hayashi T, Uchida K, Usui J, Yamagata K,Sumida T.	Laser Microdissection-based Analysis of Cytokine Balance in the Kidneys of Patients with Lupus Nephritis.	Clin. Exp. Immunol.	159	1-10	2010
Umeda N, Ito S, Hayashi T, Goto D, Matsumoto I, Sumida T.	A patient with rheumatoid arthritis who had a normal delivery under etanercept treatment.	Intern. Med	49	187-9	2010
松本功	サイトカインのすべて TGF-β	臨床免疫・アレルギー科増刊号	in press		
松本功	薫風吹く膠原病診療—臨床を駆ける進歩の風—シェーグレン症候群	内科	in press		
松本功	K/BxNマウス—血清移入関節炎—	Clinical Calcium	in press		
松本功	関節リウマチにおける自己抗体の病態への関与	リウマチ科	in press		
井上明日香、松本功、住田孝之	TIARPによる炎症性サイトカインの産生抑制	臨床免疫・アレルギー科	in press		
松本功	関節リウマチを疑ったら 診断治療のupdateと鑑別すべき膠原病—シェーグレン症候群—	medicina	in press		
明石直嗣、松本功、住田孝之	関節リウマチ治療におけるイマチニブの可能性	分子リウマチ治療	3	179-182	2010
松本功、井上明日香、田中陽子	関節炎におけるTNFa-induced adipose related protein (TIARP)	リウマチ科	44	354-360	2010
坪井洋人、松尾直美、飯塚麻菜、中村友美、松本功、住田孝之	シェーグレン症候群におけるM3ムスカリン作動性アセチルコリン受容体抗体のエピトープと機能解析	日本臨床免疫学会雑誌	33	222-228	2010

発表者氏名	論文タイトル名	発表雑誌名	巻	頁	出版年
明石直嗣、松本功、住田孝之	RAの治療標的としてのimmunoproteasome	Frontiers in Rheumatology & Clinical Immunology	4	181-184	2010
松本功、井上明日香、田中陽子、明石直嗣、岩波慶一、住田孝之	TNF・誘導分子TIARPと自己免疫疾患	Mebio	27	68-75	2010
住田孝之、坪井洋人、飯塚麻菜、中村友美、松本功	M3R反応性T細胞とシェーグレン症候群	Mebio	27	84-93	2010
松本功	膠原病—病態への新たなアプローチと治療展開—関節リウマチの分子標的と制御	最新医学	65	960-964	2010
松本功	実験的関節炎に対するリボゾームを用いた抗原特異的治療戦略	リウマチ科	43	385-393	2010
飯塚麻菜、若松英、松本功、坪井洋人、中村友美、松井稔、住田孝之	シェーグレン症候群の病態形成におけるM3ムスカリン作動性アセチルコリン受容体に対する自己免疫応答の解明	日本臨床免疫学会雑誌	33	87-91	2010
井上明日香、松本功、住田孝之	自己免疫関節炎におけるTNF・-induced adipose related protein(TIARP)の役割	医学のあゆみ	232	818-819	2010
松本功	関節炎における自己抗体の病因的意義	最新医学	65	280-285	2010
村上洋介, 上阪等	関節炎におけるTREM-1の役割	リウマチ科	44(1)	61-68	2010
村上洋介, 上阪等	【自己免疫疾患の発症における関与分子】自己免疫疾患の発症とTREM-1	臨床免疫・アレルギー科	53(5)	447-454	2010
村上洋介, 上阪等	【疾患の制御　臨床から免疫へ】炎症増強因子としてのTREM-1	日本臨床免疫学会会誌	32(4)	242-248	2009
Nakashioya H, Nakano K, Watanabe N, Miyasaka N, Matsushita S, Kohsaka H.	Therapeutic effect of D1-like dopamine receptor antagonist on collagen-induced arthritis of mice.	Mod Rheumatol	in press		
Toyomoto M, Ishido S, Miyasaka M, Sugimoto H, Kohsaka H.	Anti-arthritic effect of E3 ubiquitin ligase, c-MIR, expression in the joints.	Int Immunol	in press		
Seki I, Suzuki M, Miyasaka, N, Kohsaka H	Expression of CD45 isoforms correlates with differential proliferative responses of peripheral CD4+ and CD8+ T cells.	Immunol Lett	129(1)	39-46	2010
Fujieda Y, Kataoka H, Odani T, Otomo K, Kato M, Fukaya S, Oku K, Horita T, Yasuda S, Atsumi T, Koike T.	Clinical features of reversible posterior leukoencephalopathy syndrome in patients with systemic lupus erythematosus.	Mod Rheumatol	in press		
Yamada H, Atsumi T, Amengual O, Koike T, Furuta I, Ohta K, Kobashi G	Anti-beta2 glycoprotein-I antibody increases the risk of pregnancy-induced hypertension: a case-controlled study.	J Reprod Immunol.	84(1)	95-99	2010
Nakamura A, Shimizu C, Nagai S, Yoshida M, Aoki K, Kondo T, Miyoshi H, Wada N, Tajima T, Terauchi Y, Yoshioka N, Koike T.	Problems in diagnosing atypical Gitelman's syndrome presenting with normomagnesaemia	Clin Endocrinol (Oxf).	72(2)	272-276	2010

発表者氏名	論文タイトル名	発表雑誌名	巻	頁	出版年
Miyoshi H, Souza SC, Endo M, Sawada T, Perfield JW 2nd, Shimizu C, Stancheva Z, Nagai S, Strissel KJ, Yoshioka N, Obin MS, Koike T,Greenberg AS	Perilipin overexpression in mice protects against diet-induced obesity.	J Lipid Res.	51(5)	975-982	2010
Shimane K, Kochi Y, Horita T, Ikari K, Amano H, Hirakata M, Okamoto A, Yamada R, Myouzen K, Suzuki A, Kubo M, Atsumi T, Koike T, Takasaki Y, Momohara S, Yamanaka H, Nakamura Y, Yamamoto K.	The association of a nonsynonymous single-nucleotide polymorphism in TNFAIP3 with systemic lupus erythematosus and rheumatoid arthritis in the Japanese population	Arthritis Rheum.	62(2)	574-579	2010
Kondo T, Yasumoto A, Arita K, Sugita J, Shigematsu A, Okada K, Takahata M, Onozawa M, Kahata K, Takeda Y, Obara M, Yamamoto S, Endo T, Nishio M, Sato N, Tanaka J, Hashino S, Koike T, Asaka M, Imamura M.	Successful treatment of acute myelogenous leukemia with favorable cytogenetics by reduced-intensity stem cell transplantation.	*Int J Hematol.*	91(2)	310-321	2010
Suzuki E, Amengual O, Atsumi T, Oku K, Hashimoto T, Kataoka H, Horita T, Yasuda S, Ieko M, Fukushima K, Koike T	Increased expression of phospholipid scramblase 1 in monocytes from patients with systemic lupus erythematosus.	J Rheumatol.	37(8)	1639-1645	2010
Ieko M, Yoshida M, Naito S, Nakabayashi T, Kanazawa K, Mizukami K, Mukai M, Atsumi T, Koike T	Increase in plasma thrombin-activatable fibrinolysis inhibitor may not contribute to thrombotic tendency in antiphospholipid syndrome because of inhibitory potential of antiphospholipid antibodies toward TAFI activation	Int J Hematol.	91	776-783	2010
Nanjo K, Nagai S, Shimizu C, Tajima T, Kondo T, Miyoshi H, Yoshioka N, Koike T	Identification and functional analysis of novel calcium-sensing receptor gene mutation in familial hypocalciuric hypercalcemia.	Endocrine J.	57(9)	787-792	2010
Takahata M, Hashino S, Okada K, Onozawa M, Kahata K, Sugita J, Shigematsu A, Kondo T, Yamamoto S, Endo T, Nishio M, Ito Y, Tanaka J, Koike T, Asaka M, Imamura M.	Reduced intensity conditioning regimen with fludarabine,busulfan,and low-dose TBI(Flu-BU2-TBI):Clinical efficacy in high-risk patients	Am J Hematol.	84	243-248	2010
Fukae J, Kon Y, Henmi M, Sakamoto F, Narita A, Shimizu M, Tanimura K, Matsuhashi M, Kamishima T, Atsumi T, Koike T.	Change of synovial vascularity in a single finger joint assessed by power Doppler sonography correlated with radiographic change in rheumatoid arthritis:Comparative study of a novel quantitative score with a semiquantitative score.	Arthritis Care Res.	62(5)	657-663	2010
Kamishima T, Fujieda Y, Atsumi T, Mimura R, Koike T, Terae S, Shirato H.	Contrast-Enhanced Whole Body Joint MR Imaging in Patients with Unclassified Arthritis Developing Early Rheumatoid Arthritis in 2 Years:Feasibility Study and Correlation with MR Imaging Findings of the Hands.	Am J Roentgenol.	195	287-292	2010

発表者氏名	論文タイトル名	発表雑誌名	巻	頁	出版年
Chang, Y-J., H. Y. Kim, L. A. Albacker, H.-H. Lee, N. Baumgarth, S. Akira, P. Savage, S. Endo, T. Yamamura, J. Maaskant, N. Kitano, A. Singh, A. Bhatt, G. Besra, P. van den Elzen, B. Appelmelk, R. W. Franck, G. Chen, R. DeKruyff, M. Shimamura, P. Illarionov, and D. Umetsu	Influenza A infection in suckling mice expands a population of NKT cells that protects mice as adults from airway hyperreactivity	J. Clin. Invest.	121	57-69	2010
荒浪利昌, 山村 隆	炎症とT細胞サブセット. 特集抗体療法	治療学	44	11-13	2010
能登大介, 山村 隆	免疫性神経疾患の免疫学	内科	105	756-761	2010
三宅幸子, 山村 隆	NKT細胞と多発性硬化症	Mebio	27	94-101	2010
Myouzen K, Kochi Y, Shimane K, Fujio K, Okamura T, Okada Y, Suzuki A, Atsumi T, Ito S, Takada K, Mimori A, Ikegawa S, Yamada R, Nakamura Y, Yamamoto K	Regulatory polymorphisms in EGR2 are associated with susceptibility to systemic lupus erythematosus	Hum Mol Genet.	19	2313-20	2010
Fujio K, Okamura T, Yamamoto K	The family of IL-10-secreting CD4+ T cells	Adv Immunol.	105	99-130	2010
Okamoto A, Fujio K, Yamamoto K	The future of lupus therapy modulating autoantigen recognition.	Lupus.	19	1474-81	2010
Iwata S, Saito K, Tokunaga M, Yamaoka K, Nawata M, Yukawa S, Hanami K, Fukuyo S, Miyagawa I, Kubo S, Tanaka Y.	Phenotypic changes of lymphocytes in patients with systemic lupus erythematosus who are in longterm remission after B cell depletion therapy with rtuximab.	J Rheumatol	in press		
Tanaka Y, Takeuchi T, Mimori T, Saito K, Nawata M, Kameda H, Nojima T, Miyasaka N, Koike T.	Discontinuation of infliximab after attaining low disease activity in patients with rheumatoid arthritis, RRR (remission induction by remicade in RA) study.	Ann Rheum Dis	69	1286-1291	2010
Sawamukai N, Yukawa s, Saito K, Nakayamada S, Kambayashi T, Tanaka Y.	Mast cell-derived tryptase inhibits apoptosis of human rheumatoid synovial fibroblasts via rho-mediated signaling.	Arthritis Rheum	62	952-959	2010

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
松本　功	免疫グロブリン・補体・免疫複合体	三森経世	文光堂	2010
		リウマチ・膠原病内科 クリニカルスタンダード	東京	30-34
松本　功	多発性筋炎/皮膚筋炎	住田孝之	診断と治療社	2010
		COLOR ATLAS膠原病・リウマチ 改訂第2版	東京	100-105
松本　功	再発性多発軟骨炎	小池隆夫、住田孝之	診断と治療社	2010
		GUIDELINE膠原病・リウマチ 改訂第2版	東京	123-126
Atsumi T, Amengual O, Koike T.	Antiphospholipid syndrome:pathogenesis	Lahita RG	Academic Press	2010
		Systemic Lupus Erythematosus 5th edition	San Diego	945-66
Sakuishi, K., Miyake, S., Yamamura, T.	Role of NK cells and Invariant NKT cells in Multiple Sclerosis	Gramm, U.	Springer-Verlag	2010
		Molelcular Basis of Multiple Sclerosis.The Immune System Series "Results and Problems in Cell Differentiation"	Heidelberg	127-147
石川　昌	環境因子とアレルギー・自己免疫疾患	分子予防環境医学研究会	本の泉社	2010
		分子予防環境医学	東京都	584-591

Ⅴ　平成２２年度班会議プログラム

プログラム

13：00ー13：05　　開会の辞

13：05ー13：15　　厚生労働省　挨拶

13：15ー　　研究発表

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筑波大学大学院人間総合科学研究科疾患制御医学専攻臨床免疫学

住田　孝之

2．　13：35ー13：55

免疫疾患におけるTH1/TH2細胞の役割と制御に関する研究

筑波大学大学院人間総合科学研究科生命システム医学専攻分子発生生物学

高橋　智

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Collagen induced arthritis (CIA)における樹状細胞の役割と機能の解析

慶應義塾大学医学部

小安　重夫

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TNFα誘導性TIARPの自己免疫性関節炎における役割と、RA患者におけるシトルリン化GPI抗体の同定に関する研究

筑波大学大学院人間総合科学研究科疾患制御医学専攻臨床免疫学

松本　功

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TREM1及びそのリガンド修飾による免疫疾患制御法の開発

東京医科歯科大学大学院医歯学総合研究科膠原病・リウマチ内科学

上阪　等

… … …　　コーヒーブレイク　14：55ー15：10　　… … …

6．　15：10ー15：30

自己免疫疾患における免疫担当細胞のシグナル異常とその制御
（関節リウマチにおけるRasGRP4の発現検討）

北海道大学大学院医学研究科　内科学講座・第二内科
小池　隆夫

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関節炎モデルにおけるMR1拘束性MAIT細胞の役割に関する研究

国立精神・神経センター神経研究所免疫研究部
山村　隆

8．　15：50ー16：10

CD4陽性CD25陰性LAG3陽性制御性T細胞の抗体産生抑制能に関する研究

東京大学医学部アレルギーリウマチ内科
藤尾　圭志

9．　16：10ー16：30

自己免疫疾患におけるケモカイン・ケモカインレセプターの役割と制御戦略に関する研究：SLEモデルにおける抗BLC中和抗体の治療効果の検討

東京大学大学院医学系研究科
石川　昌

10．　16：30ー16：50

SLEにおける自己抗体産生機構とB細胞を分子標的とした治療戦略に関する研究

産業医科大学医学部第一内科学講座
田中良哉

16：50ー16：55　　閉会の辞

VI　研究成果刊行物・別刷

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Pathogenic role of immune response to M3 muscarinic acetylcholine receptor in Sjögren's syndrome-like sialoadenitis

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ABSTRACT

The aim of this study was to clarify the role of the immune response to muscarinic type 3 receptor (M3R) in the pathogenesis of Sjögren's syndrome (SS). $M3R^{-/-}$ mice were immunized with murine M3R peptides and their splenocytes were inoculated into $Rag1^{-/-}$ ($M3R^{-/-} \rightarrow Rag1^{-/-}$) mice. $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice had high serum levels of anti-M3R antibodies and low saliva volume. Histological examination showed marked infiltration of mononuclear cells in the salivary glands and immunohistochemistry demonstrated that the majority of these cells were $CD4^+$ T cells with a few B cells and several IFN-γ- and IL-17-producing cells. Apoptotic cells were present in the salivary glands of $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Moreover, transfer of only $CD3^+$ T cells from $M3R^{-/-}$ immunized with M3R peptides into $Rag1^{-/-}$ mice resulted in cell infiltration and destruction of epithelial cells in the salivary glands, suggesting that M3R reactive $CD3^+$ T cells play a pathogenic role in the development of autoimmune sialoadenitis. Our findings support the notion that the immune response to M3R plays a crucial role in the pathogenesis of SS-like autoimmune sialoadenitis.

1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by infiltration of lymphocytes into lacrimal and salivary glands, and clinically by dry eyes and dry mouth [1]. Immunohistochemical studies have shown that most infiltrating lymphocytes around the labial salivary and lachrymal glands are CD4-positive αβ T cells [2]. B cells are also detected in inflammatory lesions and act as antigen-presenting cells (APC) and produce autoantibodies such as anti-Ro/SSA and anti-La/SSB antibodies. Previous studies analyzed autoantigens recognized by T cells infiltrating the labial salivary glands of patients with SS and several candidate autoantigens such as Ro/SSA 52 kDa, [3] α-amylase, [4] heat shock protein, T cell receptor BV6 [5] and M3 muscarinic acetylcholine receptor (M3R) [6,7] have been identified.

M3R is the major muscarinic acetylcholine receptor in the salivary glands. Studies in M3R knockout ($M3R^{-/-}$) mice showed the involvement of these receptors in saliva secretion [8], suggesting that dysfunction of the M3R signaling in salivary glands can lead to a decrease in salivary secretion. The presence and specificity of anti-M3R antibodies in SS have been investigated using enzyme-linked immunosorbent assay (ELISA) or flow cytometry assay [9,10]. Robinson et al. [11] demonstrated that human anti-M3R antibodies reduced salivary secretion in NOD $Ig\mu^{null}$ mice. We also reported previously the presence of M3R reactive T cells in some patients with SS [12]. At this stage, however, it is not clear whether the immune response to M3R plays a role in the onset and progression of SS.

Amagai et al. [13] described a mouse model of pemphigus vulgaris (PV) using the combination of desmoglein 3 knockout ($Dsg3^{-/-}$) mice without Dsg3 and adoptive splenocytes transfer to lymphocyte-free Dsg3-positive $Rag2^{-/-}$ mice ($Dsg3^{-/-} \rightarrow Rag2^{-/-}$). In the $Dsg3^{-/-} \rightarrow Rag2^{-/-}$ mice, the Dsg3 molecule induces a powerful immune response and the cells break self-tolerance and attack Dsg3 autoantigens. The same approach might be applicable to T cell-mediated autoimmune disease as well as antibody-mediated disease such as PV.

Our research interest is the pathogenic role of the immune response against M3R in patients with SS. In the present study, we

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immunized M3R$^{-/-}$ mice with murine M3R peptides and transferred their splenocytes into Rag1$^{-/-}$ mice (M3R$^{-/-}$ → Rag1$^{-/-}$). We succeeded in establishing M3R-induced SS-like sialoadenitis in M3R$^{-/-}$ → Rag1$^{-/-}$ mice as a new murine model of SS. The results also showed that CD3^{+} T cells play a crucial role in the generation of autoimmune sialoadenitis. We also discuss the possible molecular mechanism of M3R-induced autoimmunity in SS.

2. Materials and methods

2.1. Mice

C57BL/6j (B6) mice (M3R$^{+/+}$) were purchased from Charles River Laboratory (Yokohama, Japan). Rag1$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). M3R$^{-/-}$ mice, which were generated from B6 mice, were kindly supplied by Dr. Matsui (Tokyo-Nishi Tokushukai Hospital, Tokyo, Japan). Mice were maintained under specific pathogen-free conditions in the Laboratory Animal Resource Center. All experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals at Tsukuba University.

2.2. Synthesized peptides encoding M3R extracellular regions

Six types of peptides encoding murine M3R extracellular domains: N-terminus 1 (MTLHSNSTTSPLFPNISSSWVHSPSEAGLP, N1), N-teminus 2 (VHSPSEAGLPLGTVSQLDSYNISGTSGNFS, N2), N-terminus 3 (NISQTSGNFSSNDTSSDPLGGHTIWQV, N3), 1st extracellular loop (FTTYIIMNRWALGNLACDLW, 1st), 2nd extracellular loop (QYFVGK RTVPPGECFIQFLSEP, 2nd) and 3rd extracellular loop (VLVNTF CDSCIPKTYWNLGY, 3rd), were synthesized chemically by a solid-phase procedure and purified by high performance liquid chromatography (AnyGen, Korea).

2.3. Immunization of mice

M3R$^{+/+}$ and M3R$^{-/-}$ mice were immunized intradermally at the base of the tail with a mixture of free-form extracellular peptides (each 20 μg) in Incomplete Freund's Adjuvant (IFA, Difco, Detroit, MI) containing 250 μg of inactivated Mycobacterium tuberculosis (H37RA, Difco). Pertussis toxin (500 ng; Sigma–Aldrich, Tokyo, Japan) was injected intraperitoneally on the day of immunization. On day 10, each mouse received another intradermal injection of the same mixture of peptides (each 20 μg) emulsified with IFA containing 250 μg of H37RA (Fig. 1A).

2.4. Analysis of cytokine profiles

On day 20 after the first immunization, the spleen was isolated from each M3R$^{+/+}$ and M3R$^{-/-}$ mouse and homogenized. The red blood cells were removed from the splenocytes by treatment with 0.16 M NH_4Cl solution. Then, the homogenates were adjusted to 1×10^6 cells/ml and incubated with RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1000 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol, together with a mixture of extracellular peptides (each 5 μg/ml) for 72 h under 5% CO_2 at 37 °C. After collection of the supernatant, the levels of IFN-γ, IL-4 and IL-17 were measured by enzyme-linked immunosorbent assay (ELISA) (IFN-γ, IL-4, and IL-17; R&D Systems Minneapolis, MN).

2.5. Adoptive transfer and cell sorting

On day 20 after the first immunization, the spleens were isolated from M3R$^{+/+}$ and M3R$^{-/-}$ mice. The spleens from each group of mice (each $n = 3$) were pooled and homogenized. The red blood cells were removed from the splenocytes by treatment with 0.16 M NH_4Cl solution. After washing with phosphate buffered saline (PBS), the cells were resuspended in PBS.

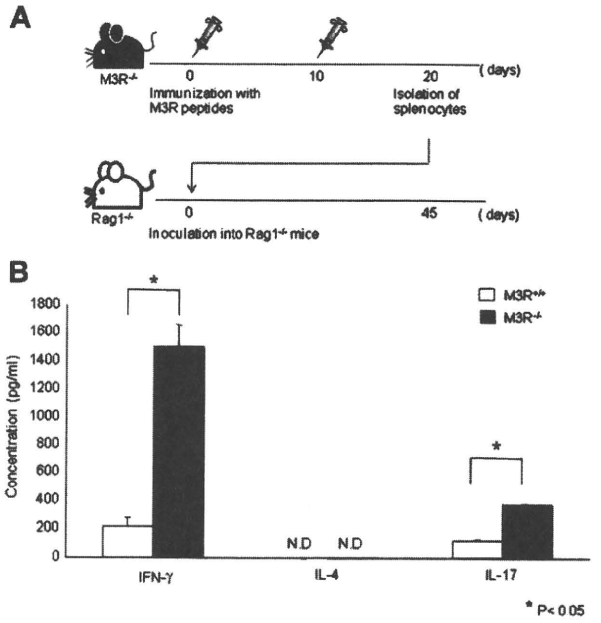

Fig. 1. Cytokine profile of M3R reactive T cells in M3R$^{-/-}$ mice immunized with M3R peptides. (A) M3R$^{-/-}$ and M3R$^{+/+}$ mice were immunized with a murine M3R peptide mixture on days 0 and 10. On the day of immunization, 500 ng of pertussis toxin was injected intraperitoneally. Ten days after booster immunization, the spleens were isolated and transferred into Rag1$^{-/-}$ mice. At day 45 after the transfer, the salivary glands of Rag1$^{-/-}$ recipient mice were analyzed histologically. (B) M3R$^{-/-}$ and M3R$^{+/+}$ mice were immunized followed by a booster dose of M3R peptides mixture emulsified in IFA plus M. tuberculosis. Ten days later, the spleens were isolated from M3R$^{+/+}$ and M3R$^{-/-}$ mice and stimulated with the M3R peptides mixture. The concentrations of IFN-γ, IL-4 and IL-17 in the culture supernatants were measured by enzyme-linked immunosorbent assay. N.D.: not detected. Values are mean ± SD of three independent experiments ($n = 3$ mice per experiment). *$P < 0.05$ (Mann–Whitney U test).

$CD3^{+}$ T cells were sorted from splenocytes of M3R$^{+/+}$ and M3R$^{-/-}$ by using the Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The high purity of $CD3^{+}$ T cells (>93%) was confirmed by FACSCalibur (BD PharMingen, San Diego, CA). Cells were resuspended in PBS and 1×10^7 cells were inoculated intravenously into Rag1$^{-/-}$ mice (Fig. 1A).

2.6. Measurement of salivary volume

Mice were first anesthetized with intraperitoneal injection of pentobarbital (1.0 mg/kg), then injected subcutaneously with pilocarpine (25 mg/kg). This was followed 15 min later by collection of saliva from the oral cavity using a 200-μl micropipette. The volume of the sample was measured and expressed relative to body weight. Changes in saliva volume were calculated relative to the volume measured at baseline, using the formula [dayN saliva volume (ml)/weight (g)]/[day0 saliva volume (ml)/weight (g)].

2.7. Analysis of antibody levels by enzyme-linked immunosorbent assay

The peptide solution containing all types of extracellular peptides (each 5 μg/ml) was mixed with 0.1 M Na_2CO_3 buffer, pH 9.6, absorbed onto a Nunc-Immunoplate (Nalge Nunc Int, Rochester, NY) at 4 °C overnight, then blocked with 1% bovine serum

albumin (Wako Pure Chemical Industries, Osaka, Japan) in PBS for 1 h at room temperature. Serum at 1:50 dilution in blocking buffer was incubated for 2 h at room temperature. The plates were then washed five times with 0.05% Tween 20 in PBS, and HRP-conjugated anti-mouse IgG antibody diluted 1:1000 in blocking buffer was added for 1 h at room temperature. After washing, 100 μl of TMB solution (Kirkegaard & Perry Laboratories, Inc, Washington, DC) was added as a substrate and then the plates were incubated for 15 min at room temperature in the dark. After adding 50 μl of 1 M phosphate buffer to stop the enzymatic reaction, the optical density was measured at 450 nm by plate spectrophotometry (Bio-Rad Laboratories, Hercules, CA).

2.8. Histological score

The inflammatory lesions were graded histologically (histological sore) using the method proposed by Greenspan et al. [14] as follow: focus score was described as the number of a focus composed of >50 mononuclear cells per 4 mm^2 of tissue. Histological evaluation of the salivary glands was performed in a blind manner, and at least one tissue section from each salivary gland was examined.

2.9. Immunohistochemical analysis

Sections of the salivary glands were thawed, dried, and then fixed with acetone for 10 min. They were incubated with biotin-conjugated anti-mouse CD4, CD8, I-A^b, CD11c (Biolegend, San Diego, CA), rat anti-mouse Thy1 (Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-mouse B220, CD86, F4/80 (Biolegend) or HRP-conjugated rabbit anti-mouse IgG antibodies (DAKO, Japan) for 2 h. To detect primary antibodies, horseradish peroxidase (HRP)-conjugated streptavidin or HRP-conjugated anti-rat IgG antibodies were added for 30 min. HRP activity was detected using 3,3-diaminobenzidine (DAB; Nichirei, Tokyo) as a substrate. The stained sections were counterstained with Mayes's hematoxylin for 30 s, and mounted with aqueous medium. Control slides were incubated with a dilution buffer containing isotype-matched antibodies instead of the primary antibodies.

Double immunofluorescence staining was performed as follow: Sections of the saliva glands were thawed, dried, and then fixed with 4% paraformaldehyde PBS (Wako) for 10 min. The sections were incubated with rat anti-mouse IFN-γ (Biolegend) and rat anti-mouse IL-17 antibodies (Santa Cruz Biotechnology) for 30 min. To detect primary antibodies, Alexa Fluor 546 goat anti-rat IgG antibody was added for 30 min. The nuclear DNA was stained with DAPI (Sigma–Aldrich).

2.10. Terminal deoxynucleotidyl transferase-mediated nick and labeling staining

Apoptotic cells were detected by *in situ* apoptosis detection kit (Takara Biomedicals, Tokyo). Briefly, frozen sections were fixed with 4% paraformaldehyde for 30 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase/methanol. After washing in PBS, the sections were treated for 5 min on ice with the permeabilisation buffer and then incubated with TdT enzyme reaction for 90 min at 37 °C. An HRP-conjugated anti-FITC antibody was added for 30 min at 37 °C, and the HRP activity was detected using DAB as a substrate. Nuclei were counterstained with hematoxylin.

2.11. Statistical analysis

Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Mann–Whitney *U* test. *P* values less than 0.05 were considered significant.

3. Results

3.1. High production of IL-17 and IFN-γ by splenocytes of $M3R^{-/-}$ mice

To compare the immune response to M3R between in $M3R^{-/-}$ and $M3R^{+/+}$ mice, both groups of mice were immunized with the M3R peptide mixture and their splenocytes were cultured with M3R *in vitro* at 20 days after first immunization (Fig. 1A). As shown in Fig. 1B, the production of IL-17 and IFN-γ by splenocytes of immunized $M3R^{-/-}$ mice far exceeded that of $M3R^{+/+}$ mice. On the other hand, IL-4 production was below the detection level in both groups. These results suggest a higher immune response to M3R in $M3R^{-/-}$ than $M3R^{+/+}$ mice.

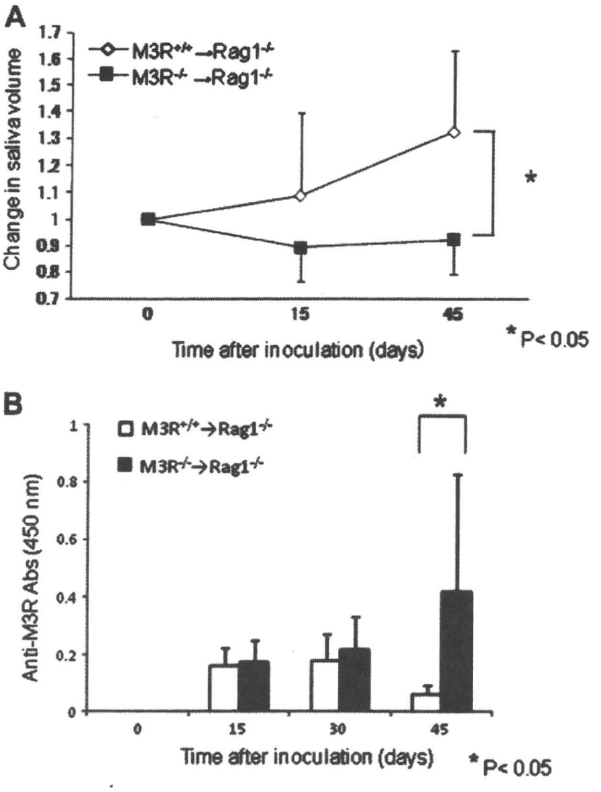

Fig. 2. Reduced salivary secretion and increased levels of anti-M3R antibodies in $M3R^{-/-}$→$Rag1^{-/-}$ mice. $Rag1^{-/-}$ mice inoculated with splenocytes from immunized $M3R^{-/-}$ and $M3R^{+/+}$ mice by intravenous injection into the tail vein. (A) Saliva was collected from $Rag1^{-/-}$ mice at 0, 15, 45 days after the adoptive transfer. The saliva samples were collected from each mouse 15 min after stimulation with pilocarpine. The volume of each saliva sample was measured and adjusted for body weight, and calculated relative to the volume measured at baseline, using the formula [dayN saliva volume (μl)/weight (g)]/[day0 saliva volume (μl)/weight (g)]. (B) Titer of anti-M3R antibodies in sera of $Rag1^{-/-}$ mice obtained at days 0, 15, 30, and 45 after adoptive transfer, determined by ELISA. *$P < 0.05$ versus saliva volume and M3R antibodies derived from $Rag1^{-/-}$ mice inoculated with splenocytes of immunized $M3R^{+/+}$ mice at the same time point, by Mann–Whitney *U* test. Values are mean ± SD of two independent experiments ($n = 6$ mice per experiment).

3.2. Development of sialoadenitis in Rag1$^{-/-}$ mice inoculated with splenocytes from immunized M3R$^{-/-}$ mice

To expose M3R reactive cells to the antigen, we isolated splenocytes from the immunized M3R$^{-/-}$ and M3R$^{+/+}$ mice and inoculated them into M3R-positive Rag1$^{-/-}$ mice (Fig. 1A). First, we examined saliva volume at days 0, 15, and 45 to determine the effect of inoculation of splenocytes of immunized M3R$^{-/-}$ or M3R$^{+/+}$ mice in Rag1$^{-/-}$ mice. The saliva volume decreased gradually from day 15–45 in Rag1$^{-/-}$ mice inoculated with splenocytes of M3R$^{-/-}$ mice compared with those inoculated with splenocytes of M3R$^{+/+}$ mice (Fig. 2A). At day 45 after the cell transfer, serum anti-M3R antibodies were significantly higher in M3R$^{-/-}$→Rag1$^{-/-}$ mice than M3R$^{+/+}$→Rag1$^{-/-}$ mice (Fig. 2B).

To examine the influence of transferred splenocyte from M3R$^{-/-}$ or M3R$^{+/+}$ immunized with the M3R peptides on salivary glands of Rag1$^{-/-}$ mice, M3R$^{-/-}$→Rag1$^{-/-}$ mice and M3R$^{+/+}$→Rag1$^{-/-}$ mice were analyzed histologically. Histological examination of mice at day 45 after inoculation showed marked mononuclear infiltration in the salivary glands and lacrimal glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice but not in M3R$^{+/+}$→Rag1$^{-/-}$ mice (Fig. 3A). However, no inflammatory lesions were observed in the intestine or liver (Fig. 3C). Quantitative analysis of the inflammatory lesions of the salivary glands indicated a higher histological sore for M3R$^{-/-}$→Rag1$^{-/-}$ mice than M3R$^{+/+}$→Rag1$^{-/-}$ mice (Fig. 3B). These results suggested that autoimmune response against M3R-induced infiltration of mononuclear cells in the salivary glands and salivary dysfunction.

To characterize the infiltrating cells in the inflammatory lesions of salivary glands, the frozen sections were analyzed immunohistochemically. The majority of infiltrating cells in M3R$^{-/-}$→Rag1$^{-/-}$ mice were Thy1^{+} CD4^{+} T cells, with only few CD8^{+} T cells. Other cells such as B220^{+}, CD11c^{+} cells and macrophage formed a minor population and were localized around T cells (Fig. 4A). Furthermore, deposition of IgGs was sometimes noted in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice (Fig. 4A). MHC class II and CD86 were expressed in ductal epithelial cells of the inflammatory lesions of salivary glands from M3R$^{-/-}$→Rag1$^{-/-}$ mice, suggesting that activated epithelial cells acted as APC.

We also examined cytokine production in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ by immunofluorescence staining. The salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice, but not those of M3R$^{+/+}$→Rag1$^{-/-}$ mice, showed differential expression of IFN-γ and IL-17 (Fig. 4B). A few apoptotic cells were noted in the lesions of salivary glands isolated from M3R$^{-/-}$→Rag1$^{-/-}$ mice on TUNEL staining (Fig. 4D), which might explain the salivary destruction and dysfunction.

3.3. M3R reactive T cells and development of sialoadenitis

The above findings indicated that the infiltrated cells in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice were mainly T cells. To confirm that M3R reactive T cells play a role in the development of sialoadenitis in M3R$^{-/-}$→Rag1$^{-/-}$ mice, we transferred purified splenic CD3^{+} T cells or CD3^{+} T cells-free splenocytes (CD3^{-} cells) from M3R$^{-/-}$ mice immunized with M3R peptides mix into Rag1$^{-/-}$ mice (M3R$^{-/-}$ CD3+

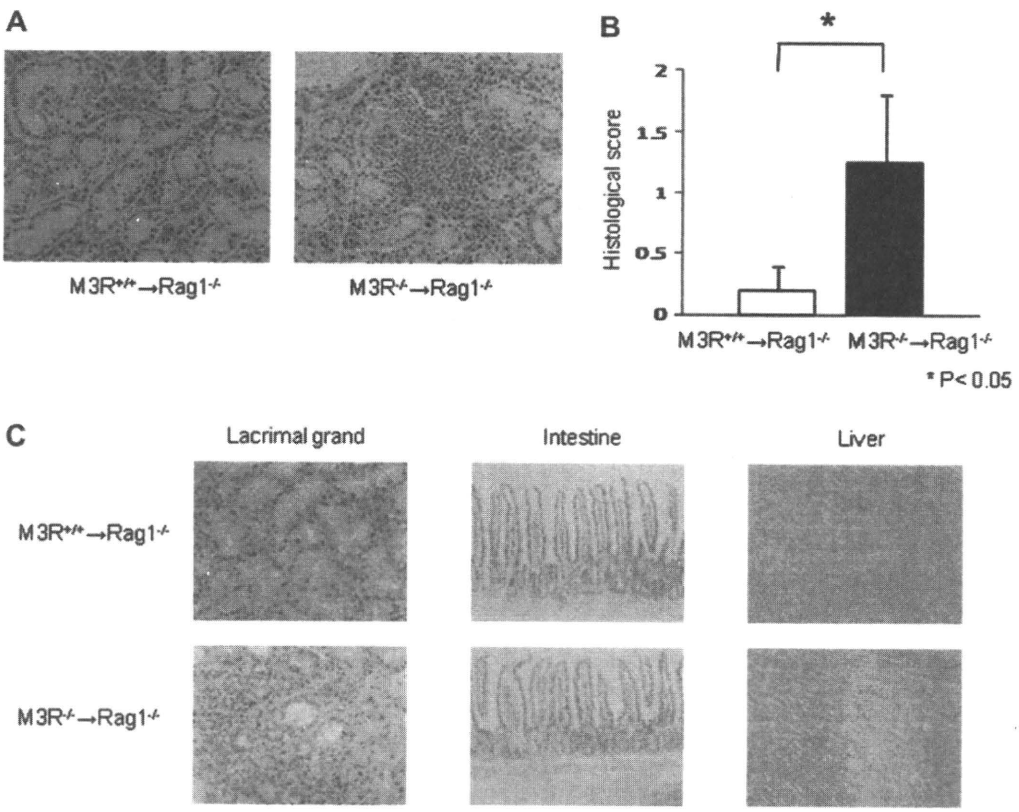

Fig. 3. Infiltration of salivary glands in M3R$^{-/-}$→Rag1$^{-/-}$ mice. Salivary glands isolated from Rag1$^{-/-}$ mice at day 45 after inoculation of splenocytes from immunized M3R$^{-/-}$ and M3R$^{+/+}$ mice. (A) Salivary glands were prepared into 4-μm thick sections, and each section was stained with Mayer's hematoxylin and eosin (H&E). Representative images of five to seven mice in each group. (B) Mean grade (histological score) of inflammatory lesions in salivary glands of M3R$^{+/+}$→Rag1$^{-/-}$ and M3R$^{-/-}$→Rag1$^{-/-}$ mice. Values are mean ± SD of five mice. (C) H&E-stained sections of the lacrimal glands, intestine and liver isolated from M3R$^{+/+}$→Rag1$^{-/-}$ mice and M3R$^{-/-}$→Rag1$^{-/-}$ mice. (Original magnification 100×).

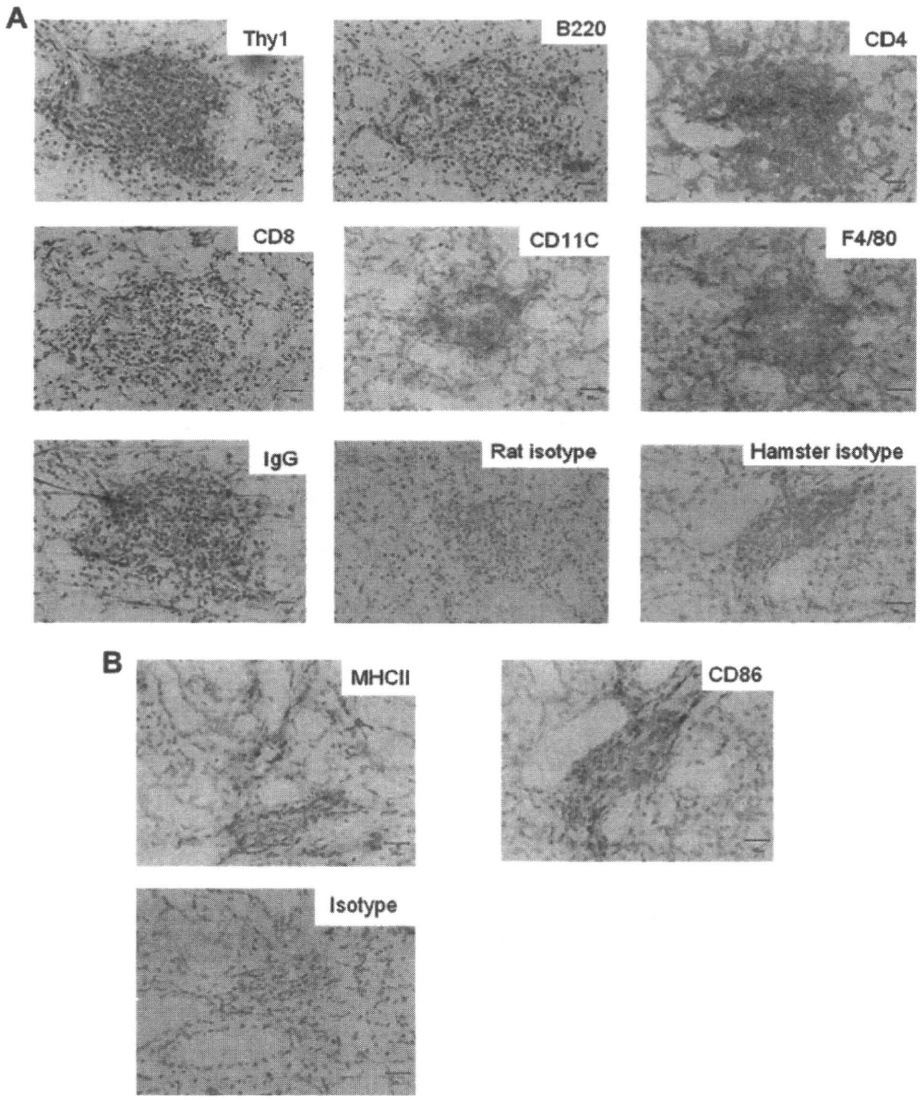

Fig. 4. Histological analysis of salivary glands isolated from M3R$^{-/-}$→Rag1$^{-/-}$ mice. (A) Inflammatory lesions in salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice stained with anti-Thy1, B220, CD4, CD8, CD11c, F4/80 as macrophage specific marker, IgG antibodies and control antibodies. Representative images of five to seven mice. (B) Expression of MHC class II and CD86 detected by immunohistochemistry using anti-I-A^{b}, CD86 and control antibodies. Representative images of three to five mice. (C) Immunofluorescence analysis of IFN-γ and IL-17 in salivary glands of M3R$^{+/+}$→Rag1$^{-/-}$ and M3R$^{-/-}$→Rag1$^{-/-}$ mice. The stained sections were counterstained with H&E, and mounted with aqueous medium. Stained sections were observed at 100× original magnification. Representative images of three to five mice from each group. (D) Apoptotic cells in sections of salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice detected by the TUNEL method. Note the presence of a few apoptotic cells in the salivary glands of M3R$^{+/+}$→Rag1$^{-/-}$ mice. Representative images of three to five mice from each group. Bars = 30 μm.

cells→Rag1$^{-/-}$ mice or M3R$^{-/-}$CD3^{-} cells→Rag1$^{-/-}$ mice). The purity of CD3^{+} T cells was 93.6% and CD3^{-} T cell included only 1.6% of CD3^{+} T cells (Fig. 5A). Histological analysis showed marked cell infiltration in the salivary glands of M3R$^{-/-}$ CD3+ cells→Rag1$^{-/-}$ mice, but not in M3R$^{-/-}$CD3^{-} cells→Rag1$^{-/-}$ mice (Fig. 5A). The majority of infiltrating cells in the salivary glands of M3R$^{-/-}$ CD3+ cells→Rag1$^{-/-}$ mice were Thy1+ CD4+ T cells, compared with only a few CD8^{+} T cells (Fig. 5B). These findings suggest that M3R reactive T cells play a crucial role in the pathogenesis of sialoadenitis in the M3R$^{-/-}$→Rag1$^{-/-}$ mice.

4. Discussion

Our previous results (6) demonstrated that 14% of patients with SS harbor autoantibodies against M3R in their sera. In another study, we also reported that peripheral lymphocytes of 4 out of 9 patients with SS included M3R-reactive T cells (12). These results suggested that the underlying pathomechanism of sialoadenitis in patients with SS could perhaps involve the immune response to M3R. The present study is an extension to our previous investigation and was designed to clarify the role of the M3R reactive immune response in the development of SS. For this purpose, M3R$^{-/-}$ mice were immunized with M3R peptides, their splenocytes were transferred into Rag1$^{-/-}$ mice, and the development of SS-like sialoadenitis was examined. Our results showed the development of mild to severe sialoadenitis in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice, indicating the involvement of the M3R reactive immune response in the development of sialoadenitis. The infiltrating cells in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice were mainly CD4^{+} T cells with a few surrounding B cells. The histological findings resemble those of infiltration of lymphocyte subset in the labial salivary glands of

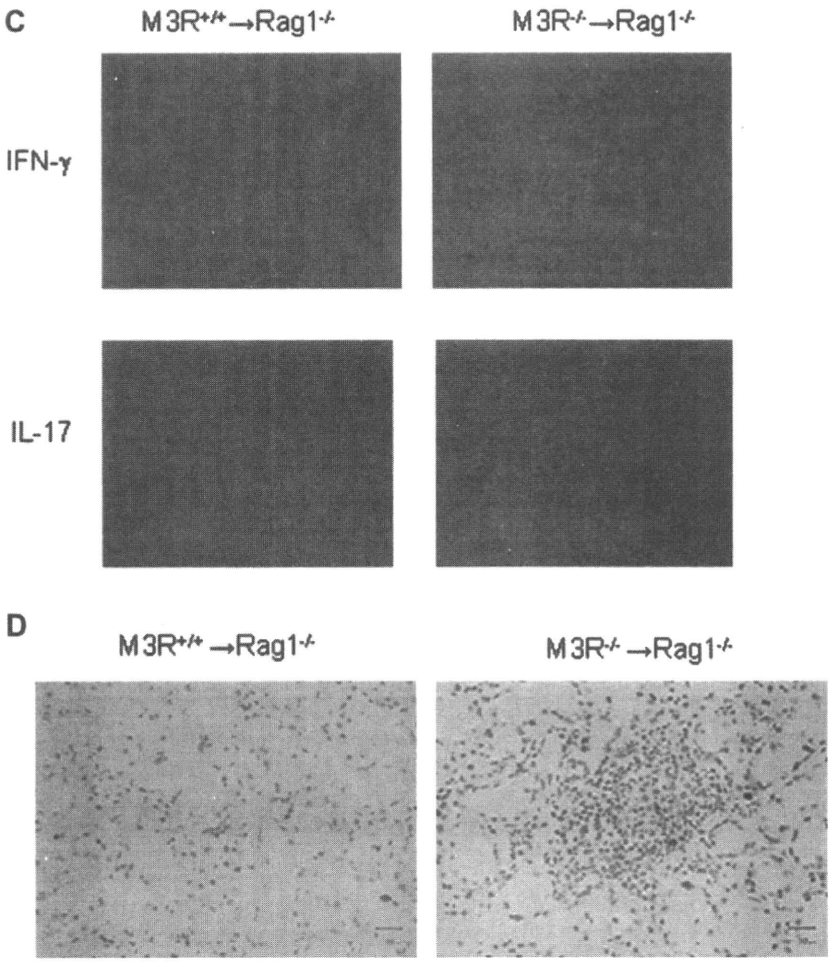

Fig. 4. (*continued*).

patients with SS [15]. Moreover, the $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice had high levels of anti-M3R antibodies and low saliva volume, similar to patients with SS. Previous studies analyzed the levels of anti-M3R antibodies in patients with SS by three methods including peptide-based ELISA, flow cytometry assay and western blot analysis [9,16,17]. Previously, we established peptide-based ELISA for detection of anti-M3R antibodies in SS patients [10,12] and thus applied this method in our mouse model in this study. The results suggest that the M3R molecule acts as an autoantigen to induce an autoimmune reaction in some patients with SS as well as experimental M3R-induced sialoadenitis.

What is the mechanism of M3R-induced sialoadenitis in our mouse model? In $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice, anti-M3R autoantibodies were detected in the serum. IFN-γ– and IL-17-producing cells were also detected in the salivary glands of $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Moreover, the adoptive $CD3^+$ T cell transfer experiments showed that $CD3^+$ T cells are essential for the development of sialoadenitis in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Thus, M3R reactive T cells are important in the development of antigen-induced sialoadenitis in our mouse model. Although we have no direct evidence in support of a pathogenic role for IFN-γ-producing Th1 cells or IL-17-producing Th17 cells, the latter type of cells might be important in the pathogenesis of M3R-induced sialoadenitis, because none of the IFN-γ dominant T-bet transgenic mice immunized with M3R peptides developed sialoadenitis (data not shown). In this regard, recent studies reported the expression of IL-17 in the salivary glands of patients with SS [18,19]. Thus, further studies using $IFN\text{-}\gamma^{-/-}$ mice or $IL\text{-}17^{-/-}$ mice are necessary.

Why does the combination of $M3R^{-/-}$ mice immunized M3R peptides and $Rag1^{-/-}$ mice enhance the generation of antigen-driven sialoadenitis? It is possible that immunization using a mixture of M3R peptides that includes the N region, and the 1st, 2nd, and 3rd extracellular domains of the M3R molecule, could have induced the immune response against exogenous antigens such as M3R in $M3R^{-/-}$ mice. In fact, M3R reactive T cells and anti-M3R antibodies appeared in $M3R^{-/-}$ mice immunized with M3R peptides. It is possible that these M3R reactive lymphocytes proliferate in $Rag1^{-/-}$ mice and attack the M3R molecule expressed in their own salivary glands, resulting in sialoadenitis.

Why is tolerance to M3R autoantigens broken down in patients with SS? While there is no evidence for the expansion of M3R reactive T cells and B cells in the salivary glands of patients with SS, it is possible that overexpression of M3R autoantigen on APC present in the salivary glands plays a role in the induction of M3R lymphocytes in the peripheral blood with subsequent infiltration of these cells into the tissues [20,21]. The high expression of M3R in the salivary glands may be triggered by previous bacterial or viral infections in the local tissues [22–24].

Based on the results of the present study, we propose that the pathogenesis of sialoadenitis in patients with SS might involve

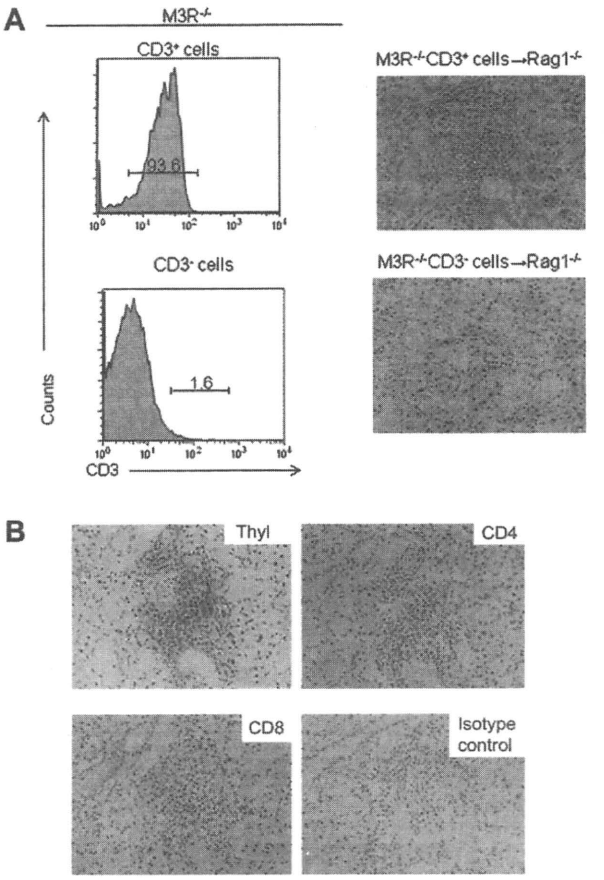

Fig. 5. Role of M3R reactive T cells in the development of sialoadenitis. $CD3^+$ or $CD3^-$ splenocytes of $M3R^{+/+}$ and $M3R^{-/-}$ mice immunized with M3R peptide mix obtained on day 20 after immunization were inoculated into $Rag1^{-/-}$ mice. (A) Salivary glands isolated from $Rag1^{-/-}$ mice at day 45 after transfer of $CD3^+$ or $CD3^-$ cells. Salivary glands were prepared into 4-μm thick sections, and each section was stained with H&E. Representative images of five to seven mice. (B) Sections of salivary glands of $M3R^{-/-}$ $CD3^+$ cells→$Rag1^{-/-}$ mice stained with anti-CD4, CD8, Thy1 and isotype control. Representative images of three to five mice from each group. Magnification 40×.

induction of M3R reactive T cells and B cells in the salivary glands. Importantly, the altered peptide ligands (APL) for M3R binding to the HLA-DR molecule are known to suppress the M3R reactive T cells *in vitro* [25,26]. Therefore, the antigen-induced sialoadenitis in patients with SS could be regulated by APL of M3R. Targeting this antigen-specific modification of autoimmunity in SS could be a potentially useful therapeutic strategy.

In conclusion, we established a new model mouse of sialoadenitis in SS using M3R immunized $M3R^{-/-}$→$Rag1^{-/-}$ mice. Experiments using the new model suggest that the M3R reactive immune response, especially M3R reactive T cells, play a crucial role in the pathogenesis of SS. Targeting this antigen-specific modification of autoimmunity could be a potentially suitable therapeutic strategy in autoimmune diseases such as SS.

References

[1] Fox RI, Stern M. Sjögren's syndrome: mechanisms of pathogenesis involve interaction of immune and neurosecretory systems. Scand J Rheumatol Suppl 2002;116:3–13.
[2] Adamson 3rd TC, Fox RI, Frisman DM, Howell FV. Immunohistologic analysis of lymphoid infiltrates in primary Sjögren's syndrome using monoclonal antibodies. J Immunol 1983;130:203–8.
[3] Sumida T, Namekawa T, Maeda T, Nishioka K. New T-cell epitope of Ro/SS-A 52 kD protein in labial salivary glands from patients with Sjögren's syndrome. Lancet 1996;348:1667.
[4] Matsumoto I, Maeda T, Takemoto Y, Hashimoto Y, Kimura F, Iwamoto I, et al. Alpha-amylase functions as a salivary gland-specific self T cell epitope in patients with Sjögren's syndrome. Int J Mol Med 1996;3:485–90.
[5] Sumida T. T cells and autoantigens in Sjögren's syndrome. Mod Rheumatol 2000;10:193–8.
[6] Naito Y, Matsumoto I, Wakamatsu E, Goto D, Sugiyama T, Matsumura R, et al. Muscarinic acetylcholine receptor autoantibodies in patient with Sjögren's syndrome. Ann Rheum Dis 2005;64:510–1.
[7] Dawson LJ, Stanbury J, Venn N, Hasdimir B, Rogers SN, Smith PM. Anti-muscarinic antibodies in primary Sjögren's syndrome reversibly inhibit the mechanism of fluid secretion by human submandibular salivary acinar cells. Arthritis Rheum 2006;54:1165–73.
[8] Matsui M, Motomura D, Karasawa H, Fujikawa T, Jiang J, Komiya Y, et al. Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M3 subtype. Proc Natl Acad Sci USA 2000;97:9579–84.
[9] Bacman S, Berra A, Sterin-Borda L, Borda E. Muscarinic acetylcholine receptor antibodies as a new marker of dry eye Sjögren's syndrome. Invest Ophthalmol Vis Sc 2001;742:321–7.
[10] Nakamura Y, Wakamatsu E, Matsumoto I, Tomiita M, Kohno Y, Mori M, et al. High prevalence of autoantibodies to muscarinic-3 acetylcholine receptor in patients with juvenile-onset Sjögren syndrome. Ann Rheum Dis 2001;67:136–7.
[11] Robinson CP, Brayer J, Yamachika S, Esch TR, Peck AB, Stewart CA, et al. Transfer of human serum IgG to nonobese diabetic $Ig\mu^{null}$ mice reveals a role for autoantibodies in the loss of secretory function of exocrine tissues in Sjögren's syndrome. Proc Natl Acad Sci USA 1998;95:7538–43.
[12] Naito Y, Matsumoto I, Wakamatsu E, Goto D, Ito S, Tsutsumi A, et al. Altered peptide ligand regulate muscarinic acetylcholine receptor reactive T cells of patients with Sjögren's syndrome. Ann Rheum Dis 2006;65:269–71.
[13] Amagai M, Tsunoda K, Suzuki H, Nishifuji K, Koyasu S, Nishikawa T. Use of autoantigen-knockout mice in developing an active autoimmune disease model for pemphigus. J Clin Invest 2000;105:625–31.
[14] Greenspan JS, Daniels TE, Talal N, Sylvester RA. The histopathology of Sjögren's syndrome in labial salivary gland biopsies. Oral Surg Oral Med Oral Pathol 1974;37:217–29.
[15] Skopouli FN, Fox PC, Galanopoulou V, Atkinson JC, Jaffe ES, Moutsopoulos HM. T cell subpopulations in the labial minor salivary gland histopathologic lesion of Sjögren's syndrome. J Rheumatol 1991;18:210–4.
[16] Gao J, Cha S, Jonsson R, Opalko J, Peck AB. Detection of anti-type 3 muscarinic acetylcholine receptor autoantibodies in the sera of Sjögren's syndrome patients by use of a transfected cell line assay. Arthritis Rheum 2004;50:2615–21.
[17] Sumida T, Tsuboi H, Iizuka M, Nakamura Y, Matsumoto I. Functional role of M3 muscarinic acetylcholine receptor (M3R) reactive T cells and anti-M3R autoantibodies in patients with Sjögren's syndrome. Autoimmun Rev 2010;9:615–7.
[18] Sakai A, Sugawara Y, Kuroishi T, Sasano T, Sugawara S. Identification of IL-18 and Th17 cells in salivary glands of patients with Sjögren's syndrome, and amplification of IL-17-mediated secretion of inflammatory cytokines from salivary gland cells by IL-18. J Immunol 2008;181:2898–906.
[19] Nguyen CQ, Hu MH, Li Y, Stewart C, Peck AB. Salivary gland tissue expression of interleukin-23 and interleukin-17 in Sjögren's syndrome. Arthritis Rheum 2008;58:734–43.
[20] Beroukas D, Goodfellow R, Hiscock J, Jonsson R, Gordon TP, Waterman SA. Up-regulation of M3-muscarinic receptors in labial salivary gland acini in primary Sjögren's syndrome. Lab Invest 2002;82:203–10.
[21] Wang F, Jackson MW, Maughan V, Cavill D, Smith AJ, Waterman SA, et al. Passive transfer of Sjogren's syndrome IgG produces the pathophysiology of overactive bladder. Arthritis Rheum 2004;50:3637–45.
[22] Fox RI, Luppi M, Pisa P, Kang HI. Potential role of Epstein-Barr virus in Sjögren's syndrome and rheumatoid arthritis. J Rheumatol 1992;19:18–24.
[23] Font J, Tassies D, Garcia-Carrasco M, Ramos-Casals M, Cervera R, Reverter JC, et al. Hepatitis G virus infection in primary Sjögren's syndrome: analysis in a series of 100 patients. Ann Rheum Dis 1998;57:42–4.
[24] Kamimura T, Sato H, Iwamoto M, Nara H, Torikoe K, Masuyama J, et al. Sjögren's syndrome associated with chronic hepatitis C, severe thrombocytopenia, hypertrophic cardiomyopathy, and diabetes mellitus. Intern Med 2005;44:657–61.
[25] Ohnishi Y, Tsutsumi A, Matsumoto I, Goto D, Ito S, Kuwana M, et al. Altered peptide ligands control type II collagen-reactive T cells from rheumatoid arthritis patients. Mod Rheumatol 2006;16:226–8.
[26] Iwanami K, Matsumoto I, Yoshiga Y, Inoue A, Kondo Y, Yamamoto K, et al. Altered peptide ligands inhibit arthritis induced by glucose-6-phosphate isomerase peptide. Arthritis Res Ther 2009;11:R167.

Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a multisystem, autoimmune disease that predominantly affects women. Previous findings that duplicated Toll-like receptor 7 (*Tlr7*) promotes lupus-like disease in male BXSB mice prompted us to evaluate *TLR7* in human SLE. By using a candidate gene approach, we identified and replicated association of a *TLR7* 3′UTR SNP, rs3853839 (G/C), with SLE in 9,274 Eastern Asians ($P_{combined} = 6.5 \times 10^{-10}$), with a stronger effect in male than female subjects [odds ratio, male vs. female = 2.33 (95% CI = 1.64–3.30) vs. 1.24 (95% CI = 1.14–1.34); $P = 4.1 \times 10^{-4}$]. G-allele carriers had increased *TLR7* transcripts and more pronounced IFN signature than C-allele carriers; heterozygotes had 2.7-fold higher transcripts of G-allele than C-allele. These data established a functional polymorphism in type I IFN pathway gene *TLR7* predisposing to SLE, especially in Chinese and Japanese male subjects.

functional polymorphism | disease susceptibility | autoimmunity | type I interferon

Systemic lupus erythematosus [SLE; Online Mendelian Inheritance in Man (OMIM) no. 152700] is a multisystem, autoimmune disease with strong genetic and environmental components (1). SLE predominantly affects women, with a female-to-male ratio of approximately 9:1. Male patients with SLE, although rare, tend to have more severe disease and poorer outcome (2), suggesting potential sex dimorphism in the disease development. Although the sex effect has often been attributed to sex hormones, the fact that XXY male subjects have approximately a 14-fold higher risk of developing SLE than 46 XY men indicates that X-linked genes may be risk factors for human SLE (3).

Located at Xp22.2, Toll-like receptor 7 (*TLR7*; OMIM no. 300365) and its functionally related gene *TLR8* (OMIM no. 300366) encode proteins that play critical roles in pathogen recognition and activation of innate immunity (4). They recognize endogenous RNA-containing autoantigens and induce the expression of type I IFN, a pivotal cytokine in the pathogenesis of SLE (5). In lupus-prone BXSB mice, the translocation of a segmental duplication of X chromosome to Y chromosome creates the Y-linked autoimmune accelerator (Yaa) locus, which was associated with autoreactive B cell responses to RNA-related antigens and exacerbation of glomerulonephritis in male mice (6). Although translocated X chromosome segment in Yaa may contain as many as 16 genes, the major gene for causation of the autoimmune phenotypes was identified to be *TLR7* (7), making it a potential susceptibility gene for SLE. By using a candidate gene approach, we report herein that a functional polymorphism in 3′UTR of *TLR7* is associated with SLE in Chinese and Japanese populations, with a stronger effect in male than female subjects.

Results

Discovery and Replication of the Association of a *TLR7* 3′UTR SNP with SLE in Eastern Asian Population. We genotyped 27 SNPs from the *TLR7–TLR8* region (12 in *TLR7* and 15 in *TLR8*) in 1,434 SLE cases and 1,591 control subjects of Eastern Asian ancestry using the Beadstation Infinium II platform (Illumina). Eleven SNPs in *TLR7* and 12 SNPs in *TLR8* that showed a minor allele frequency greater than 0.01 were included in association analysis (Fig. 1*B* and Table S1). We observed evidence of association with SLE in 2 *TLR7* SNPs (rs5935436 and rs3853839) and 2 *TLR8* SNPs (rs3764880 and rs4830805; Fig. 1*B*). Only rs5935436 and rs3853839 in *TLR7* remained significant after Bonferroni correction ($P = 0.041$ and $P = 0.016$, respectively). The strongest association signal was detected at rs3853839 among Chinese subjects [cases vs. controls, 563 vs. 522; $P = 6.3 \times 10^{-6}$; odds ratio (OR) = 1.67 (95% CI = 1.33–2.08)], but not in Korean subjects [$P = 0.32$; OR = 0.92 (95% CI = 0.79–1.08); Fig. 1*B*], suggesting potential genetic heterogeneity of SLE between the two populations.

We next performed a haplotype-based association test using Haploview 4.03 software. The "GAACAC" haplotype formed by rs2897827, rs5935436, rs2302267, rs179019, rs5743740, and rs179016 had a frequency of 3.1% in SLE cases and 4.8% in con-

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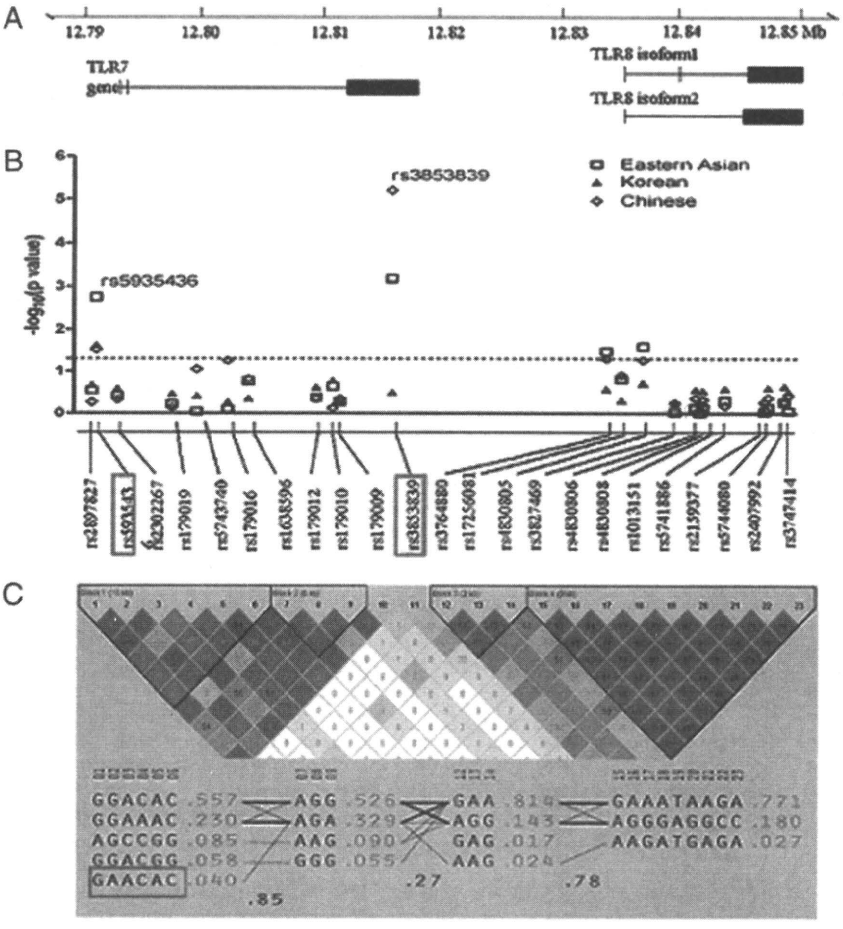

Fig. 1. A functional SNP rs3853839 in 3′UTR of *TLR7* is associated with SLE in a Chinese population. (*A*) *TLR7* and *TLR8* gene structure. (*B*) Eleven *TLR7* SNPs and 12 *TLR8* SNPs were genotyped in 1,434 SLE cases and 1,591 healthy controls of Eastern Asian descent. The two SNPs (rs5935436, rs3853839) that showed significant association after Bonferroni correction are highlighted. (*C*) Two haplotype blocks were constructed based on the strength of LD in each gene region. The R^2 values of each SNP pair are depicted. The protective haplotype GAACAC is highlighted.

trols ($P = 0.0017$; Fig. 1*C*). Given that only this *TLR7* protective haplotype carries the minor allele of rs5935436, we genotyped rs5935436 in replication studies to represent the SLE-associated haplotype. Rs3853839 was in low linkage disequilibrium (LD) with other SNPs in the region. To minimize missing any other common polymorphisms, we sequenced 5′ promoter region (2 kb upstream) as well as three exons (including 3′UTR) of *TLR7* in 48 Chinese female patients with SLE, which revealed no additional polymorphism and raised the possibility that rs3853839 might be causal.

To verify the association of rs3853839 and rs5935436 with SLE, we then conducted a replication study in two independent case-control Chinese and Japanese panels. Whereas rs5935436 was not replicated in these studies ($P = 0.97$ and $P = 0.25$, respectively), rs3853839 showed a consistent association with SLE in both replication panels [Chinese, 2,340 vs. 2,436; $P = 9.0 \times 10^{-4}$; OR = 1.21 (95% CI = 1.08–1.35); and Japanese, 560 vs. 913; $P = 0.007$; OR = 1.28 (95% CI = 1.07–1.53); Table 1]. A combined analysis of discovery and replication panels showed compelling evidence supporting that G allele of *TLR7* SNP rs3853839 conferred risk for SLE [$P_{combined} = 6.5 \times 10^{-10}$, $OR_{combined} = 1.27$ (95% CI = 1.17–1.36)]. Furthermore, G allele showed an additive effect in female subjects, as the OR for female homozygous GG versus CC was 1.45 (95% CI = 1.17–1.79) and OR for heterozygous GC versus CC was 1.15 (95% CI = 0.93–1.43), which reinforced a role of rs3853839 in developing SLE. Stratification by clinical subsets including age of disease onset, presence of mucocutaneous manifestations, lupus nephritis, dsDNA, or anti-RNA binding protein antibodies (presence of one or more autoantibodies to Ro/SSA, La/SSB, RNP, and Sm) (8) revealed a weak association of G allele with the presence of anti-RNA binding protein [$P = 0.049$, OR = 1.16 (95% CI = 1.00–1.35); missing data, 45%], suggesting that *TLR7* binding of RNA containing immune complexes might play a role in the initiation and perpetuation of autoimmunity.

Significant Male Effect of rs3853839 in the Risk of Developing SLE. Of interest, we observed a stronger association of rs3853839 with male SLE. In the discovery panel, G allele showed higher OR in male patients compared with female patients [OR = 1.79 (95% CI = 1.03–3.13) vs. 1.22 (95% CI = 1.06–1.39); Table 1], especially in Chinese subjects [OR = 5.56 (95% CI = 1.85–16.7) vs. 1.54 (95% CI = 1.22–1.96)]. This notable male effect was verified in both replication panels: Chinese male patients with SLE had a significantly higher frequency of G allele than controls [92% vs. 81%; OR = 2.73 (95% CI = 1.57–4.74)]; in contrast, female cases only showed a modest increase of G allele versus controls [83% vs. 80%; OR = 1.19 (95% CI = 1.06–1.35)]. A similar finding was also observed in the Japanese subject replication panel. In the combined analysis, G allele occurred in 89% of male cases ($n = 358$), but only 77% of male controls ($n = 1,550$),

Table 1. Association of rs3853839 with SLE in Eastern Asian populations

Ethnicity and panel	Case/control	G risk allele frequency		*P* value	OR (95% CI)
		Case	Control		
Eastern Asian*:					
Discovery					
Male	126/229	0.83	0.73	0.038	1.79 (1.03–3.13)
Female	1,308/1,362	0.79	0.76	3.00E-03	1.22 (1.06–1.39)
All	1,434/1,591	0.80	0.76	6.70E-04	1.24 (1.1–1.41)
Chinese: Replication 1					
Male	196/931	0.92	0.81	2.31E-04	2.73 (1.57–4.74)
Female	2,144/1,505	0.83	0.80	4.24E-03	1.19 (1.06–1.34)
All	2,340/2,436	0.83	0.80	9.02E-04	1.21 (1.08–1.35)
Japanese: Replication 2					
Male	36/390	0.89	0.69	0.014	3.51 (1.22–10.2)
Female	524/523	0.75	0.71	0.037	1.23 (1.01–1.49)
All	560/913	0.75	0.70	7.00E-03	1.28 (1.07–1.53)
Eastern Asian*: Combined					
Male	358/1,550	0.89	0.77	1.33E-06	2.33 (1.64–3.30)
Female	3,976/3,390	0.80	0.77	1.19E-07	1.24 (1.14–1.34)
All	4,334/4,940	0.81	0.77	6.50E-10	1.27 (1.17–1.36)

*Includes subjects of Chinese, Korean, or Japanese descent.

showing a strong association with SLE ($P = 1.33 \times 10^{-6}$) and significantly higher OR in male versus female subjects [2.33 (95% CI = 1.64–3.30) vs. 1.24 (95% CI = 1.14–1.34); $P = 4.1 \times 10^{-4}$]. The significant male sex effect of rs3853839 in SLE was confirmed by a higher G allele frequency in male cases than female cases ($P = 1.1 \times 10^{-4}$; OR = 1.90 (95% CI = 1.36–2.64)] and by a higher OR in male subjects than the OR calculated by using only female homozygotes [GG vs. CC, OR = 1.45 (95% CI = 1.17–1.79); $P = 0.02$].

***TLR7* SNP rs385839 Confers Elevated Expression of *TLR7* in Vivo.** The consistent association of rs3853839 with SLE led us to investigate the potential functional consequences of the G risk allele. We first examined whether the 3′UTR polymorphism affected the expression level of *TLR7* transcripts. We isolated peripheral blood mononuclear cells (PBMCs) from selected Chinese individuals carrying various genotypes and measured the mRNA expression levels of *TLR7* by using real-time PCR. As shown in Fig. 2*A*, male controls carrying G allele had significantly higher *TLR7* transcripts than those carrying C allele ($P = 0.02$). Similarly, female cases and controls homozygous for G allele showed notably higher *TLR7* mRNA expression than those carrying homozygous C allele ($P = 7.4 \times 10^{-6}$ and $P = 0.03$, respectively). No sex differences in *TLR7* mRNA expression levels were observed between normal controls carrying the same allelic genotype of rs3853839 ($P = 0.07$ or $P = 0.55$ for GG females vs. G males or CC females vs. C males, respectively; Fig. 2*A*). The sex comparison was not replicated in patients with SLE because of insufficient sample size of blood collected from male patients with SLE.

To exclude potential bias between individuals, we next assessed the allelic specific transcriptional expression of *TLR7* in Chinese female G/C heterozygotes by pyrosequencing. We extracted genomic DNA (gDNA) and cDNA from PBMCs of heterozygous SLE patients and controls and determined the exact ratios of G/C in gDNA and cDNA for each individual. As shown in Fig. 2*B*, the mean G/C ratio in gDNA of all individuals was 1.05, which was consistent with the theoretical value. However, SLE and control female heterozygotes showed a 2.7-fold higher G/C ratio in *TLR7* transcripts than that in gDNA ($P = 2 \times 10^{-4}$ and $P = 2 \times 10^{-3}$, respectively; $P_{overall} = 1.2 \times 10^{-6}$), meaning that there was significantly higher level of G allele–containing *TLR7* transcripts than C allele–containing transcripts in PBMCs, even though there was same amount of gDNA that contains either allele.

To investigate whether the disparity observed between transcript and gDNA was a result of imbalanced X chromosome inactivation (XCI) in female subjects, we analyzed the XCI pattern in these individuals by measuring the degree of methylated CAG alleles in androgen receptor (*AR*) gene as previously described (9). Fig. 2*C* showed no significant skewed XCI in either SLE or controls ($P = 0.8$), suggesting that skewed XCI or somatic selection might not account for the current finding. Taken together, these data suggested rs3853839 polymorphism could play a functional role in the regulation of *TLR7* transcript levels in vivo.

To investigate whether allelic differences of rs3853839 corresponded to TLR7 protein levels, we isolated proteins from PBMCs of healthy Chinese male subjects carrying the G or C allele for Western blot analysis. Fig. S1 showed that five of the six male G allele carriers had higher expression levels of TLR7 protein than the two C allele carriers after normalization of the amount of protein loading using β-actin. Although the result was not statistically significant ($P = 0.21$, probably because of the very small sample size), the finding of higher TLR7 protein expression of the G risk allele carriers was consistent with what we observed at mRNA levels of *TLR7*.

We next investigated that whether the functional SNP rs3853839 might influence the IFN reaction downstream of *TLR7*. A prominent feature of patients with SLE is an increased expression of type I IFN–regulated genes (i.e., IFN signature) in PBMCs compared with unaffected controls (10, 11). We measured expression levels of four type I IFN–regulated genes, including lymphocyte antigen 6 complex, locus E (*LY6E*), myxovirus resistance 1 (*MX1*), IFN-inducible protein with tetratricopeptide repeats 1 (*IFIT1*), and *IFIT3* by real-time PCR in PBMCs from clinically inactive female SLE patients carrying various rs3853839 genotypes and calculated an IFN score for each individual. As shown in Fig. 2*D*, female SLE patients carrying GG genotype showed a more pronounced IFN signature in PBMCs compared with those carrying GC and CC genotypes, indicating rs3853839 might modulate expression levels of *TLR7*, affecting IFN response in vivo.

Discussion

There is compelling evidence supporting the contribution of *TLR7* to the development of autoimmunity. Transgenic mice with a twofold overexpression of *TLR7* would have increased production of RNA-related autoantibodies and develop spontaneous