can alter the efficacy of biologic agents in particular populations. These factors are important to consider in the identification of predictive disease markers or development of personalized treatment regimens.

DMARD therapy in Japan

Although the focus of this article is on the use of biologic agents in Japanese patients with RA, nonbiologic DMARDs are often administered together with biologic agents, and biologic agents can be indicated for patients with RA who have failed to respond to nonbiologic DMARD therapy. These nonbiologic DMARDs will, therefore, also be briefly discussed here.

Methotrexate

At present, methotrexate is the drug of choice for RA therapy in Japan, as in many other countries. Methotrexate was approved in 1999 in Japan, but only as a second-line agent for patients with RA who failed to respond to other DMARDs. Moreover, the approved maximum dosage of methotrexate in Japan is 8 mg per week, whereas 15–25 mg per week is the recommended dosage of methotrexate in the USA and many European countries. The approved dosage of methotrexate in Japan was based on a multicenter, randomized, double-blind clinical trial that compared three different once-weekly doses of methotrexate (2 mg, 6 mg and 9 mg) administered for 12 weeks to patients with DMARD-resistant RA. The trial demonstrated that the efficacy of the 6 mg weekly dose was superior to that of the 2 mg weekly dose and comparable to that of 9 mg per week, whereas levels of liver enzymes (aspartate transaminase and/or alanine transaminase) were elevated by 4%, 15%, and 22% (>2.5 times the upper limit of normal) in patients who received the 2 mg per week, 6 mg per week and 9 mg per week doses, respectively. These results suggested that a weekly dose of methotrexate <9 mg was preferable.

Methotrexate at an average dose of 7 mg per week was considered to be effective in approximately half of methotrexate-treated patients with RA,[8] although remission, as determined by the lack of tender or swollen joints and normal serum C-reactive protein levels (0.76–28.5 nmol/l), was only achieved in 11% of patients. Owing to the current requirements for achieving strict treatment targets, such as clinical remission or low scores for disease activity,[9] and the results of a meta-analysis that favored rapid escalation to high doses of methotrexate,[10] the Japan College of Rheumatology has, however, recommended that methotrexate should be approved at a dose of 16 mg per week or higher.

Bucillamine

10 years ago, bucillamine, a D-penicillamine analogue that was only available in Japan and South Korea, was the most popular DMARD used to treat RA in Japan despite its modest efficacy.[11] The combination of methotrexate and bucillamine is clinically more effective (as confirmed with radiographical findings) than methotrexate monotherapy[12] and this combination therapy is currently used in Japan.

Key points

- The effectiveness and safety of biologic agents in Japanese patients with rheumatoid arthritis (RA) could be influenced by the unique genetic, environmental and medical backgrounds of these individuals
- Japanese patients with RA with highly active disease and rapid progression of structural joint damage have favorable clinical responses (including retardation of joint destruction) when treated with biologic agents
- Post-marketing surveillance data for all patients with RA treated with biologic agents in Japan demonstrated a low incidence of adverse reactions (~5%)
- In Japanese patients, important adverse reactions to biologic agents include pneumonia, tuberculosis, *Pneumocystis jirovecii* pneumonia and interstitial pneumonitis
- Identification of predictive markers could facilitate personalized therapy with biologic agents and optimize the outcomes of patients with RA

Leflunomide

Leflunomide was approved for the treatment of patients with RA in Japan in September 2003.[13] However, because of an unexpectedly high prevalence of interstitial lung disease among leflunomide-treated patients in Japan (1.20%, versus an estimated ~0.02% in Western countries)[14] and a high mortality rate (9 of 22 deaths were judged by the study committee members to be caused by leflunomide-induced lung injury),[15] only 6,994 Japanese patients with RA had received leflunomide as of November 2009.[16]

Tacrolimus

Tacrolimus was approved as a treatment for RA in Japan in April 2005. A positive response to tacrolimus therapy (according to European League Against Rheumatism criteria, that is the Disease Activity Score using 28 joint counts [DAS28]) was achieved in 20% of patients with RA in a single-center cohort study in Japan.[17] This drug has been used, either alone or in combination with methotrexate, in >10,000 Japanese patients with RA.[18]

Biologic agents used in Japan

Several multicenter, randomized, double-blinded clinical trials of biologic agents have been conducted in Japan and have exhibited excellent rates of remission and ACR 20 responses (a 20% improvement in disease activity according to American College of Rheumatology [ACR] criteria) in patients with RA, as shown in Figures 1 and 2.[4,6,19–23] We believe the clinical trials in Japan are of high quality, that the appropriate patients were selected for inclusion and that clinical assessments by the attending rheumatologists were reproducible; notably, the response rates in placebo groups were low (<10% for monotherapy trials and ~20% for trials in which methotrexate was added to biologic therapy). One of the caveats of the conclusions drawn from these studies is that the dose of biologic agents needed for clinical efficacy is unclear, as detailed below.

Infliximab

Abe *et al.*[4] evaluated the efficacy of 3 mg/kg and 10 mg/kg doses of infliximab in combination with low-dose methotrexate for the treatment of Japanese patients with

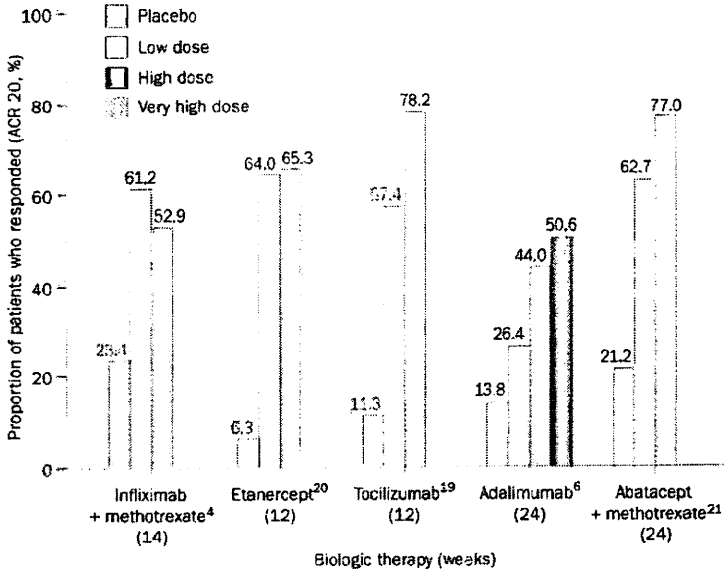

Figure 1 | Japanese clinical trials of biologic therapies for RA. Clinical trials in Japan of biologic agents (including infliximab, etanercept, adalimumab and abatacept) reported good disease response rates (with or without concomitant methotrexate) in Japanese patients with RA. Most trials assigned roughly equal numbers of patients to receive placebo, low-dose biologic agents or high-dose biologic agents, as follows (placebo versus low dose versus high dose): placebo* (47 patients) versus infliximab, 3 mg/kg (49 patients), versus 10 mg/kg (51 patients) at weeks 0, 2 and 6 (in combination with a minimum of 6 mg per week methotrexate);[4] placebo (48 patients) versus etanercept, 10 mg twice weekly (50 patients) versus 25 mg twice weekly (49 patients);[20] placebo (53 patients) versus tocilizumab, 4 mg/kg (54 patients) every 4 weeks versus 8 mg/kg every 4 weeks (55 patients);[19] placebo‡ (66 patients) versus abatacept (in combination with maximum of 8 mg per week methotrexate), 2 mg/kg every 4 weeks (67 patients) versus 10 mg/kg every 4 weeks (61 patients) after 0, 2 weeks induction.[21] Trials of adalimumab compared three different dose regimens: 20 mg twice weekly (87 patients), 40 mg twice weekly (91 patients) and 80 mg twice weekly (87 patients) versus placebo (87 patients).[6] Patients who received infliximab and abatacept had no previous response to methotrexate, whereas patients who received etanercept, tocilizumab and adalimumab were DMARD-resistant. *Active treatment with methotrexate only. ‡No DMARD treatment. Abbreviations: ACR 20, a 20% improvement in disease activity according to American College of Rheumatology criteria; RA, rheumatoid arthritis.

RA. In this trial, there was no upper limit to the dose of methotrexate, but the average dose of methotrexate was ~7 mg per week for all treatment arms (7.4 mg per week for the placebo group, 7.1 mg per week for the 3 mg/kg infliximab group, and 7.1 mg per week for 10 mg/kg infliximab treatment arm). ACR 20 response rates were similar in the two groups of infliximab-treated patients.[4] This result could have been related to the relatively short period of observation (14 weeks), small sample size ($n = 147$ patients in total) and the choice of measurement used to evaluate treatment efficacy (the ACR 20 response rate).

A series of investigator-initiated clinical studies, including RECONFIRM (Retrospective Clinical Study on the Notable Efficacy and Related Factors of Infliximab Therapy in a RA Management Group in Japan),[24] and the subsequent follow-up studies RECONFIRM 2[25] and RECONFIRM 2J,[26] demonstrated that a 3 mg/kg dose of infliximab administered at 0, 2, and 6 weeks, and thereafter every 8 weeks, produced excellent clinical responses as well as inhibition of progressive joint destruction in radiographic studies of patients with RA (Figure 2 and Table 1). The inhibition of structural joint damage in RECONFIRM 2J[26] was comparable to that reported in studies conducted in Western countries.[27] A slight decrease in the clinical response to 3 mg/kg of infliximab at around 30–38 weeks was, however, observed.[25] This transient secondary loss in efficacy could be related to insufficient trough levels of infliximab in serum remaining from the previous infusion, presumably because of the 8-week intervals between infusions.

RISING (Impact of Trough Serum Level on Radiographic and Clinical Response to Infliximab Plus Methotrexate in Patients with RA) compared 3 mg/kg, 6 mg/kg and 10 mg/kg doses of infliximab (both in combination with methotrexate).[22] The study used the ACR N index of improvement (or ACR-N, based on the percentage improvement analogous to the ACR 20, ACR 50 and ACR 70 criteria) to define response and included a larger sample of patients ($n = 327$ patients in total) than the study by Abe and colleagues.[4] The investigators found that 10 mg/kg dose had markedly superior efficacy compared to the 3 mg/kg dose: the DAS28 rate of remission was 45.2% (compared to 30.3% in the low-dose groups), and serum trough levels were increased in patients treated with the high dose of infliximab.[22] Infliximab treatment at both doses was also associated with reduced progression of structural joint damage.[22]

Etanercept

The JESMR study (Efficacy and Safety of Etanercept on Active RA Despite Methotrexate Therapy in Japan) compared the efficacy of continuation versus discontinuation of methotrexate at the initiation of etanercept. The findings revealed superior clinical efficacy for continuation of methotrexate therapy (~8 mg per week) at the commencement of etanercept treatment.[23,28] Striking inhibition of structural joint damage was also observed.[28]

As shown in Figure 1, patients treated with the twice-weekly, low dose of 10 mg etanercept had similar ACR 20 response to that of patients treated with the twice-weekly high dose of 25 mg etanercept (ACR 20 response was 64.0% for the low-dose group and 65.3% for the high-dose group),[20] which suggests a ceiling effect with regard to the dose response. A fixed, twice-weekly 25 mg dose of etanercept in Japanese patients with RA[20] (who on average have body weight approximately 70% that of their Western counterparts) might be speculated to exhibit a ceiling effect with regard to achieved blood levels of this agent similar to that observed in patients from Western countries. However, a pharmacokinetic study of etanercept in both Japanese and American individuals (body weight of Japanese healthy volunteers was 63 (±5) kg, while that of American healthy volunteers was 76.1 (±14) kg) showed that serum concentrations and absorption rates of etanercept were similar in both groups of patients, despite receiving the same initial dose of this

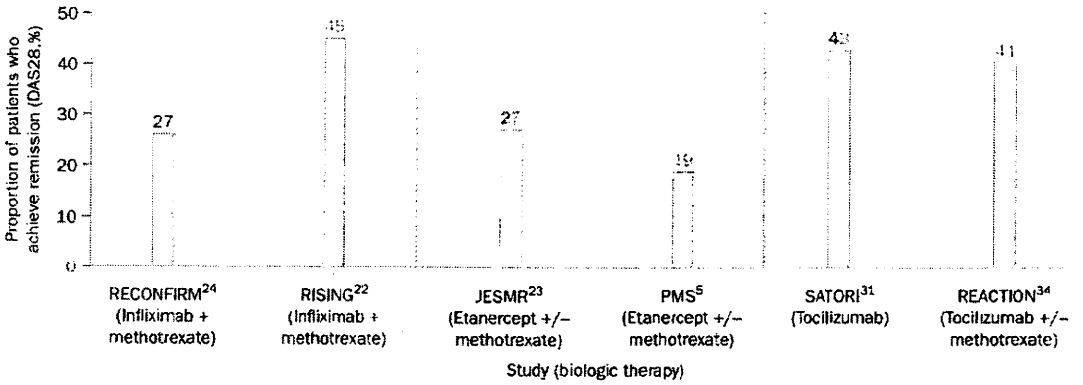

Figure 2 | Effectiveness of biologic agents in Japanese patients with RA. Disease activity was scored according to the DAS28 criteria in Japanese patients with RA after treatment with infliximab, etanercept and tociluzumab (either as a monotherapy or in combination with methotrexate). Remission was defined by the DAS28 criteria plus erythrocyte sedimentation rate <2.6 (except in the RECONFIRM study, in which DAS28 criteria <2.3 plus C-reactive protein levels were used). Abbreviations: DAS, disease activity score; JESMR, Efficacy and Safety of Etanercept on Active RA Despite Methotrexate Therapy in Japan; RA, rheumatoid arthritis; REACTION, Efficacy of Tocilizumab in RA Patients With Daily Clinical Practice in Japan; RECONFIRM, Retrospective Clinical Study on the Notable Efficacy and Related Factors of Infliximab Therapy in a RA Management Group in Japan; RISING, Impact of Trough Serum Level on Radiographic and Clinical Response to Infliximab Plus Methotrexate in Patients with RA; SATORI, Study of Active Controlled Tocilizumab Monotherapy for RA Patients with an Inadequate Response to Methotrexate.

Table 1 | Japanese studies of structural joint damage after biologic agent therapy for RA

Study characteristics	RECONFIRM 2J[26]	RISING[22]	JESMR[28]	SAMURAI[32]
Biologic therapy	Infliximab	Infliximab	Etanercept	Tocilizumab
Design	Retrospective	Double-blind, controlled	Prospective comparison	Double-blind, controlled
Patients' age per treatment group (years)	50.8	3 mg/kg: 49.9 6 mg/kg: 48.8 10 mg/kg: 50.4	Etanercept: 58.1 Etanercept + methotrexate: 56.5	DMARD: 53.1 Toculizumab: 52.9
Duration of study per treatment group (year)	7.9	3 mg/kg: 8.3 6 mg/kg: 7.2 10 mg/kg: 8.4	Etanercept: 10.6 Etanercept + methotrexate: 8.1	DMARD: 2.4 Toculizumab: 2.2
Mean estimated YP at 0 weeks per treatment group (median)	21.3 (16.2)	3 mg/kg: (5.5) 6 mg/kg: (7.6) 10 mg/kg: (6.5)	Etanercept: 17.7 Etanercept + methotrexate: 20.8	DMARD: 12.3 Toculizumab: 14.1
Mean TSS at 0 weeks per treatment group (median)	104.4 (73.5)	3 mg/kg: (28.0) 6 mg/kg: (32.2) 10 mg/kg: (38.3)	Etanercept: 114.5 Etanercept + methotrexate: 113.1	DMARD: 30.6 Toculizumab: 28.3
Mean change in TSS over 1 year per treatment group (median)	−0.03 (0)	3 mg/kg: (0) 6 mg/kg: (0.5) 10 mg/kg: (0)	Etanercept: 3.6 Etanercept + methotrexate: 0.8	DMARD: 6.2 (2.5) Toculizumab: 2.3 (0.5)
Increase in TSS per treatment group (% change from baseline)	0	3 mg/kg: (0) 6 mg/kg: (1.6) 10 mg/kg: (0)	Etanercept: 2.8 Etanercept + methotrexate: 0.7	DMARD: 20.5 Toculizumab: 8.1

Abbreviations: JESMR, Efficacy And Safety of Etanercept on Active RA Despite Methotrexate Therapy in Japan; RA, rheumatoid arthritis; RECONFIRM 2J, Retrospective Clinical Study on the Notable Efficacy and Related Factors of Infliximab Therapy in a RA Management Group in Japan: 1 Year Outcome of Joint Destruction; RISING, Impact of Trough Serum Level on Radiographic and Clinical Response to Infliximab Plus Methotrexate in Patients with RA ; SAMURAI, Study of Active Controlled Monotherapy Used for RA, an IL-6 Inhibitor; TSS, total Sharp score; YP, yearly progression (TSS/disease duration).

agent.[29] This observation argues against body weight as the sole explanation for the existence of the ceiling effect in Japanese patients. An alternative explanation for this ceiling effect might be that patients with mild RA were selected for the study, in part because of the reduced maximum doses of methotrexate in Japan. In patients with milder RA, levels of inflammation and TNF could be lower than in those with severe RA and, therefore, only low doses of TNF inhibitors are needed to neutralize these lower levels of inflammation and TNF.

Adalimumab

In an adalimumab monotherapy trial,[6] a higher incidence of development of antibodies to adalimumab was observed after 24 weeks of treatment in Japanese patients than was detected in a similar study in Western patients (40.2%, 44.0%, and 26.4% of Japanese patients treated with 20 mg, 40 mg, and 80 mg adalimumab every other week, respectively[6] versus 12% of patients treated with adalimumab in the Western study[30]). This high incidence of antibodies against adalimumab might

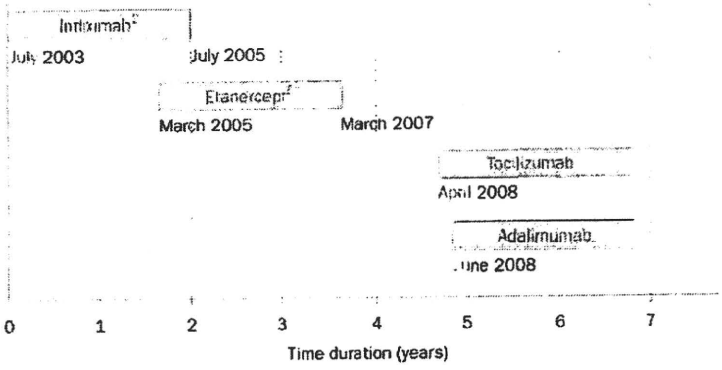

Figure 3 | Collection of post-marketing surveillance data on biologic therapies for RA in Japan. Several strategies have been initiated in Japan to monitor the efficacy and safety of biologic agents in patients with RA. Patients are centrally registered and their baseline demographic data are noted. Clinical disease characteristics and drug safety and efficacy are recorded during and after treatment with biologic agents. These data have been collected since 2003 by pharmaceutical companies, the Japan College of Rheumatology and attending physicians at their respective institutions. Post-marketing surveillance for infliximab (5,000 patients) and etanercept (13,842 patients, 7,091 of whom were included in interim reports) has already been completed. Post-marketing surveillance for tocilizumab and adalimumab is ongoing and expected to be completed by the end of 2010, whereas that for abatacept is yet to be initiated. Abbreviation: RA, rheumatoid arthritis.

have contributed to the low response rates (in patients treated with 20 mg and 40 mg adalimumab every other week, ACR 20 response was 28.7% and 44%, respectively in the Japanese trial,[6] whereas the ACR 20 was 35.8% and 46.0%, respectively in the Western study[30]) to adalimumab therapy in Japanese individuals,[6] and implies that different genetic backgrounds affect the production of antibodies that are specific for biologic agents. In future studies, Japanese patients treated with adalimumab in combination with methotrexate might need to be evaluated for the presence of anti-adalimumab antibodies to see whether this combination therapy could reduce the occurrence of these antibodies and result in similar efficacy to those in Western countries.

Tocilizumab

Tocilizumab, a humanized monoclonal antibody against the interleukin (IL)-6 receptor, was first approved in 2008 for the treatment of RA in Japan, and was subsequently approved for use in Europe in 2009 and USA in 2010. Excellent efficacy of tocilizumab monotherapy has been demonstrated in phase II clinical trials with 12 weeks of treatment[19] and with 24 weeks of treatment in the SATORI (Study of Active Controlled Toculizumab Monotherapy for RA Patients with an Inadequate Response to Methotrexate) trial.[31] In the SAMURAI (Study of Active Controlled Monotherapy Used for RA, an IL-6 Inhibitor) trial, both the total Sharp score and the erosion and joint narrowing scores were markedly lower in the tocilizumab monotherapy group than the conventional DMARD therapy group.[32] This result was the first evidence that biologic agents are superior to DMARDs for inhibiting structural joint damage in Japanese patients with RA (Table 1). No information has been obtained on the efficacy of combination therapy with tociluzumab and methotrexate in Japanese clinical trials (Figures 1 and 2).

A phase II clinical trial provided the first evidence of the long-term safety and sustainability of using tocilizumab to block IL-6 receptor signaling in patients.[33] The efficacy of tocilizumab was confirmed in the REACTION (Efficacy of Tocilizumab in RA Patients with Daily Clinical Practice in Japan) study, which reported high remission rates (40.7%) at 24 weeks (Figure 2).[34] The findings from REACTION generated interest in whether tocilizumab and methotrexate combination therapy was superior to tocilizumab alone in inhibiting joint damage in Japanese patients with RA, which is currently under investigation in Japan.

Guidelines

In Japan, biologic agents are used according to guidelines proposed by the Japan College of Rheumatology.[35,36] These guidelines list the indications for biologic therapy in patients with RA: ≥6 swollen joints, ≥6 tender joints and high levels of inflammatory markers (C-reactive protein ≥190.48 nmol/l) or an erythrocyte sedimentation rate of ≥28 mm/h) despite ≥3 months of treatment with one of the DMARDs (including methotrexate, bucillamine and sulfasalazine). These guidelines were further revised to include the administration of etanercept and infliximab to patients with both moderate disease activity and progression of bony erosions.[37] Criteria for the use of tocilizumab[38] and adalimumab[39] have also been devised and used since 2008.

The safety of biologic agents in Japan

PMS programs

The primary objectives of PMS are to determine the exact frequency of drug-related adverse events (especially opportunistic infections, such as TB) and the factors that affect drug safety and efficacy. PMS data have been collected for all patients with RA who have received biologic agents in Japan since 2003. Pharmaceutical companies, the Japan College of Rheumatology, and attending physicians at their respective institutions have collected data on adverse effects and their treatment (Figure 3). All patients with RA in Japan are centrally registered and baseline demographic information, clinical characteristics and drug safety and efficacy are recorded during the 6 months after starting biologic agents. PMS for infliximab[2] and etanercept[5] in Japan has been completed. Two other PMS programs for tocilizumab and adalimumab in patients with RA were initiated in 2008 and are expected to be completed by the end of 2010 (interim data for tocilizumab is discussed below), at which time PMS data for abatacept will begin to be collected (Figure 2).

The 5,000 patients with RA who received infliximab were 3 years younger on average, consisted of fewer elderly individuals (>70 years of age) and had lower comorbidities than those patients treated with etanercept (interim report for 7,091 patients) (Table 2).[2,5] In addition, all the patients who received infliximab were

also treated with methotrexate in combination, but only 53% of patients were treated with both etanercept and methotrexate (Table 2).

Adverse drug reactions

Despite the differences in patients' characteristics, treatments, and the time frames of PMS programs, rates of adverse drug reactions (ADR) and severe ADR were comparable for etanercept and infliximab (incidence of ADR 28.0% and 30.6%; incidence of severe ADR 6.2% and 5.7% for infliximab and etanercept, respectively; Table 2).[2,5] The rates of ADR and severe ADR for these two agents were also comparable to those reported in patients treated with tacrolimus (ADR 36.0% and severe ADR 6.4%) in a PMS initiated by a pharmaceutical company that included 3,175 patients treated with tacrolimus for 24 weeks).[18] The similarity of these results indicated that biologic agents are generally well tolerated in Japanese patients with RA.

Rates of adverse events were comparable between tocilizumab-treated and methotrexate-treated groups of patient in short-term trials.[19,31,32] Concerns, however, were raised in relation to the high rates of serious infections in 143 patients exposed to tocilizumab up to 5 years in a phase II clinical trial.[33] 27.5 serious adverse events per 100 patient-years of treatment and 5.7 serious infections per 100 patient-years of treatment occurred.[33] These findings were, however, obtained in a small group of patients (only 66% of the cohort of 143 patients completed 5 years of treatment with tocilizumab)[33] and might be influenced by attrition bias. PMS data for tocilizumab should provide further information with regard to these adverse effects.

Infections such as TB, bacterial pneumonia, *Pneumocystis jirovecii* pneumonia (PCP) and interstitial pneumonitis were the most frequent and important severe ADR in Japanese patients with RA treated with biologic therapy. The risk factors associated with pneumonia or severe infections in PMS for infliximab and etanercept have been identified as advanced age, pulmonary comorbidity, advanced RA (as assessed by disease stage or functional class) and concomitant use of glucocorticoids.[2,5,37]

Tuberculosis

The overall incidence of TB was 0.3% (14 of 5,000 patients) among patients treated with infliximab enrolled in the PMS program.[2] A substantially higher rate of TB infection was observed in the initial 2,000 patients treated with this agent, which led to concerns about the potential reactivation of latent TB.[40] The initial 2,000 individuals treated with infliximab had not received prophylactic isoniazid despite having risk factors for TB, such as a positive Tuberculin skin test, radiography results that were suspicious for past TB infection, and a history of contact with patients with active TB.[2] Subsequent patients were assessed for risk factors associated with TB and those at risk of the infection received isoniazid prophylaxis, with the result that the rate of TB decreased from 0.55% in the initial 2,000 patients to 0.1% in the subsequent 3,000 individuals. As PMS information on infliximab was extensively shared among rheumatologists in Japan, the incidence of TB in patients treated with etanercept was comparable to that in the final 3,000 infliximab-treated patients (0.1%),[5] owing to the implementation of measures to reduce the risk of this infection

Table 2 | Summary of PMS data in Japan for infliximab and etanercept

Parameter	Infliximab (2008)[2]	Etanercept (2009)[5]
Characteristics of treated patients		
Total treated	5,000	7,091
Mean age (years)	55.1	58.3
>70 years old (%)	11.4	19.9
Treated with concomitant methotrexate (%)	100	52.7
Comorbidity (%)	38.4	58.2
Adverse drug reactions*		
Total (%)	28.0	30.6
Severe adverse drug reactions (%)	6.2	5.7
Pneumonia (%)	2.2	1.4
Pneumocystis jirovecii pneumonia (%)	0.4	0.2
Tuberculosis (%)	0.3	0.1
Interstitial pneumonitis (%)	0.5	0.6
Severe infusion reaction (%)	0.5	0

*Only the five most clinically important adverse reactions are individually mentioned. Abbreviation: PMS, post-marketing surveillance data.

Pneumocystis jirovecii pneumonia

PCP is an important opportunistic infection in patients with AIDS, but the exact incidence of this form of pneumonia in patients with RA who are treated with TNF inhibitors was not well characterized until publication of the Japanese PMS data for infliximab.

The Japan College of Rheumatology PMS committee reported that the incidence of PCP was 0.4% in the 5,000 patients enrolled in the PMS program for infliximab,[2] which is more than 10 times higher than the reported incidence of this infection in data from Western studies of this agent.[41] The median time to develop PCP was 8.5 weeks from the first infusion of infliximab; this complication tended to develop in elderly patients, those with pulmonary comorbidities such as interstitial lung diseases, and those who received high doses of glucocorticoids.[42] In addition, decreased serum albumin and IgG levels were also identified in infliximab-treated patients with RA at the onset of PCP; however, these risk factors are not specific to this infection and can be observed in other opportunistic and common infections.

Interestingly, *P. jirovecii* infection in patients with RA who were receiving immunosuppressive treatment seemed to have different clinical features and radiographic findings (in high-resolution chest CT scans) from those of patients with HIV infections.[43] Patients with RA who were treated with biologic agents tended to deteriorate rapidly (within weeks), while

patients with HIV infection progressed more slowly (within weeks to months). The disparity between these two groups of patients suggests that a unique mechanism might underlie the pathogenesis of PCP in patients with RA, possibly resulting from macrophage activation by C-type lectin domain family 7 member A (also termed dectin 1) or lactosylceramide.[44–46]

80% of infliximab-treated patients with RA who had PCP developed acute respiratory failure, although all survived with appropriate treatment. During the PMS for etanercept, PCP developed in 0.2% of patients enrolled in PMS programs for etanercept in Japan. Six of these 25 patients died.[5] This result raised a particular concern that Japanese patients treated with TNF-blocking agents might have increased risk of PCP and highlighted the need for antibiotic prophylaxis with co-trimoxazole (sulfamethoxazole combined with trimethoprim) in patients at risk of this infection. In this regard, measuring serum levels of β-D-glucans might be useful for the diagnosis and monitoring of the development of PCP in patients with RA.[47]

Interstitial lung disease

Interstitial lung disease was observed in 0.5% and 0.6% of individuals treated with infliximab and etanercept, respectively[2,5], which is consistent with the incidence of this complication in DMARD-treated patients (0.38–1.53 per 100 patient-year).[48,49] Findings from PMS data, however, show that 75% of patients who developed interstitial lung disease during etanercept treatment were not concomitantly treated with methotrexate, which implies that interstitial lung disease in etanercept-treated patients cannot be considered a confounding effect of concomitant methotrexate treatment. The incidence of interstitial lung disease was considerably increased in leflunomide-treated patients (1.2% in PMS data).[14]

Findings from studies of dermatomyositis and gefitinib-induced or leflunomide-induced lung injury suggest that Japanese patients might be particularly predisposed to develop interstitial lung disease, or at least have increased susceptibility to its exacerbation.[50–52] In addition to the influence of genetic background, environmental factors (such as the presence of airway colonization with *P. jirovecii*) could underlie the apparently elevated incidence of both PCP and interstitial lung disease in Japanese patients with RA. Patients with pre-existing lung disease, such as subclinical pulmonary inflammation elicited by either colonization with pathogens or by RA itself (owing to the immunomodulatory effects of biologic and DMARD therapy) might, therefore, subsequently develop clinical pulmonary complications.

Discontinuation of biologic agents

Clinical studies have evaluated whether biologic agents can be discontinued in patients with RA who maintain a low disease activity owing to the sustained benefit of these drugs after treatment withdrawal. A clinical study to examine the pharmacokinetics of infliximab was carried out in Japan with a small number of established DMARD-resistant patients with RA.[53] These patients received 3 mg/kg infliximab at 0, 2, and 6 weeks without concomitant methotrexate. After completion of the trial, the patients did not receive further biologic agents, but were treated with DMARDs, including methotrexate. Surprisingly, nearly half of the patients had persistently stable levels of disease activity 1 year after the trial ended, which demonstrated that infliximab could be discontinued in selected patients with RA.[53] Given the evidence that suggested patients with early RA can discontinue infliximab if they achieve stable remission or low disease activity scores,[54,55] a clinical study of infliximab discontinuation in patients with established RA was initiated. Of the 102 patients who could be evaluated, around half successfully discontinued infliximab during the 1-year follow-up. 67% of the patients who were able to discontinue infliximab had no radiographic evidence of disease progression.[56] A treatment strategy that uses tightly controlled doses of biologic agents to induce clinical remission or low disease activity, followed by discontinuation of the biologic agents once a low disease activity has been achieved, could be advantageous from both the patient's health status and economic points of view. This 'response-driven' treatment strategy is now being examined in several clinical studies and trials in Japan for several biologic agents, including infliximab, etanercept, tocilizumab and abatacept.

Predicting response to biologic agents

Medical treatment can be personalized for individual patients with RA by predicting the efficacy and safety of a given biologic agent. An enormous effort is underway to identify factors that can accurately predict responses to treatment. Several clinical variables have been associated with efficacy and safety, but these markers are often unrealistic markers to use in clinical practice. For example, the finding that the response rate to a certain biologic agent is increased in young patients with RA (<45 years of age)[57] is not applicable to the treatment of elderly individuals (>65 years of age). Clinical studies using modern technology and analyses of molecular data (including genetic polymorphisms[58,59] and comprehensive messenger RNA expression analysis)[60,61] have identified numerous candidate molecules for predicting the efficacy of biologic agents: tristetraprolin,[62] the products of interferon-related genes,[60] phosphoserine phosphatase[61] and disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5).[63] At present, however, consensus has not been reached as to which molecules offer truly accurate and generalizable predictions of the efficacy of biologic therapies for RA.[64,65] Many investigators are also extensively searching for factors that can predict ADR (including infections[66] and infusion reactions) associated with biologic agents by examining genetic polymorphisms in patients with RA.

Conclusions

Experience with biologic therapies in Japanese patients with RA has shown that these agents have good efficacy. However, several important adverse effects, such as

opportunistic infections, have an elevated incidence in Japanese patients, which could amplify the risks associated with biologic therapies for RA in Japan. Attention should be closely focused on the differences between patients with RA from different ethnic backgrounds. Treatment of RA should ultimately be individualized to each patient's needs to maximize drug efficacy and minimize the associated risks. Future research could identify factors that can predict the response to treatment with biologic agents and the risks of adverse effects associated with these drugs to improve patients' outcomes further.

Review criteria

We searched for original and review articles focusing on rheumatoid arthritis, biologic agents and Japan in MEDLINE and PubMed published between 2003 and 2010 in the English language. The search terms we used were "arthritis", "rheumatoid", "infliximab", "TNFR-Fc fusion protein", "adalimumab", "tocilizumab", "abatacept" and "Japan". We also searched the reference lists of identified articles for further papers and also included articles on websites, some of which are in Japanese.

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Author contributions
Both authors contributed equally to all aspects of this manuscript.

nature genetics

A regulatory variant in *CCR6* is associated with rheumatoid arthritis susceptibility

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Rheumatoid arthritis is a common autoimmune disease with a complex genetic etiology. Here, through a genome-wide association study of rheumatoid arthritis, we identified a polymorphism in *CCR6*, the gene encoding chemokine (C-C motif) receptor 6 (a surface marker for Th17 cells) at 6q27, that was associated with rheumatoid arthritis susceptibility and was validated in two independent replication cohorts from Japan (rs3093024, a total of 7,069 individuals with rheumatoid arthritis (cases) and 20,727 controls, overall odds ratio = 1.19, $P = 7.7 \times 10^{-19}$). We identified a triallelic dinucleotide polymorphism of *CCR6* (CCR6DNP) in strong linkage disequilibrium with rs3093024 that showed effects on gene transcription. The CCR6DNP genotype was correlated with the expression level of *CCR6* and was associated with the presence of interleukin-17 (IL-17) in the sera of subjects with rheumatoid arthritis. Moreover, CCR6DNP was associated with susceptibility to Graves' and Crohn's diseases. These results suggest that *CCR6* is critically involved in IL-17–driven autoimmunity in human diseases.

Rheumatoid arthritis manifests as inflammation of synovial tissue and joint destruction. The T-cell–driven autoimmune response and the inflammatory cytokine cascade are two important arms of rheumatoid arthritis pathogenesis, involving both environmental and genetic factors[1]. The etiological role of $CD4^+$ helper T cells is supported by evidence that the specific alleles of the *HLA-DRB1* gene encoding the major histocompatibility complex class II proteins are associated with disease risk[2]. Among the subsets of helper T cells, Th1 cells that characteristically produce interferon-γ are considered to have a prominent role in rheumatoid arthritis, as they are highly prevalent in the synovial tissues of subjects with the disease[3,4]. Moreover, a newly identified subset of helper T cells that produces IL-17, hence termed Th17 cells[5], are now thought to have an important role in rheumatoid arthritis, as has been demonstrated in several mouse arthritis models[6–8]. However, less is known about the role of Th17 cells in human rheumatoid arthritis. In fact, among the loci identified in recent genome-wide association studies (GWAS) of rheumatoid arthritis, few have been identified that may affect the axis of Th17 cells specifically, with the exception of the genetic markers near *IL21*[9–12].

To identify rheumatoid arthritis susceptibility loci in the Japanese population, we performed a GWAS by genotyping over 550,000 SNP markers. We applied stringent quality-control criteria and tested 2,303 rheumatoid arthritis cases (of whom 79.2% were positive for anti-cyclic citrullinated peptide (anti-CCP) antibodies; **Supplementary Table 1**) and 3,380 controls for 393,217 autosomal SNPs with minor allele frequency of >0.05. The results of principal-component analysis (PCA)[13] did not demonstrate substantial population stratification in our study population (**Supplementary Fig. 1** and **Supplementary Note**), as has not been previously anticipated for the Japanese population[14]. The inflation of test statistics, $\lambda_{\text{genomic control}}$ (λ_{GC})[15], was 1.097. We identified significant associations in two chromosomal regions (6p21 and 6q27) that satisfied a genome-wide significance threshold of $P < 5 \times 10^{-8}$ (**Fig. 1a** and **Supplementary Fig. 2**). The peak of association in 6p21 was observed at a SNP near *HLA-DRB1* at the major rheumatoid arthritis susceptibility locus (rs13192471, $P = 1.9 \times 10^{-58}$, OR = 1.97, 95% CI 1.82–2.14; **Supplementary Table 2**). Among the markers in 6q27, the smallest P value was observed at a SNP in the region containing *CCR6* (rs3093024, $P = 4.5 \times 10^{-10}$, OR = 1.27, 95% CI 1.18–1.37; **Fig. 1b**, **Table 1** and **Supplementary Tables 2** and **3**). The association of rs3093024 was still significant after PCA correction[13] ($P = 5.5 \times 10^{-9}$). We observed suggestive associations ($5 \times 10^{-8} \leq P < 1 \times 10^{-5}$) in five

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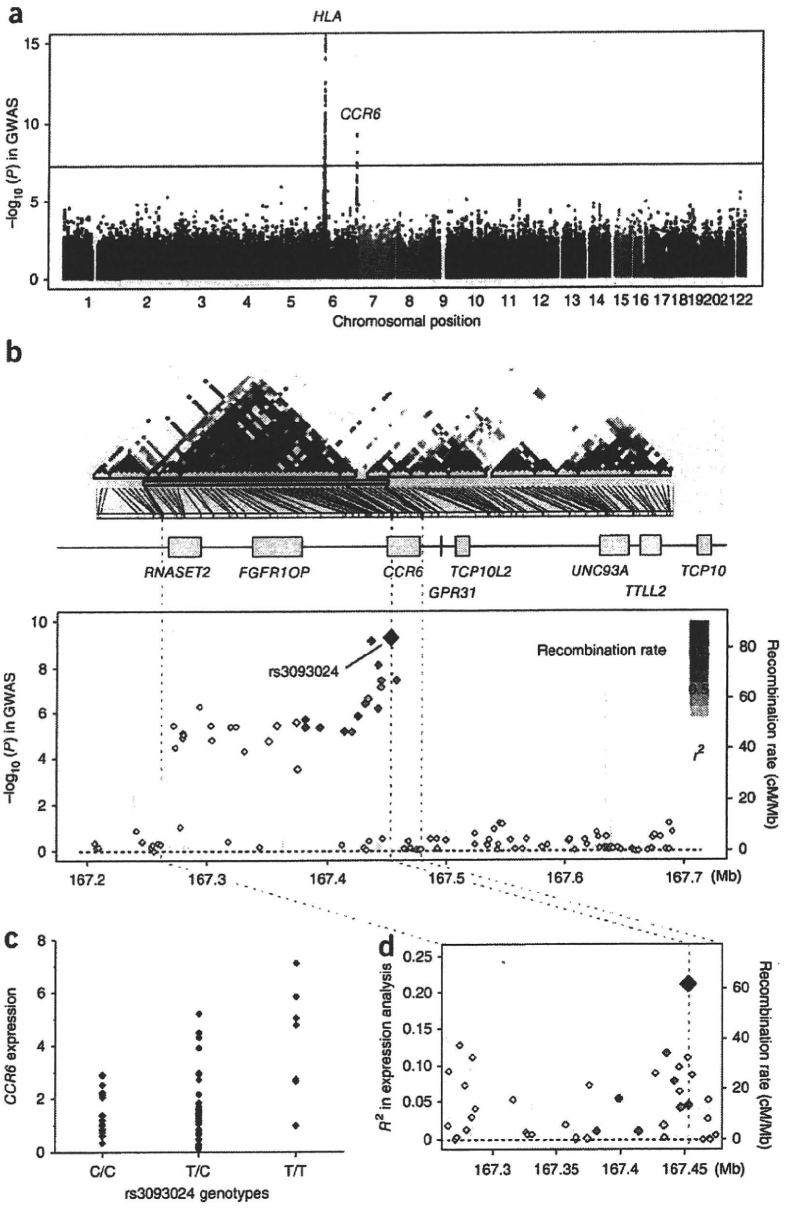

Figure 1 Results of GWAS and expression analysis around *CCR6*. (**a**) A Manhattan plot showing the $-\log_{10}$ (*P* value) of SNPs in the GWAS for 2,303 Japanese rheumatoid arthritis cases and 3,380 controls. Some SNPs in the HLA region are not included because they exceeded the upper limit of the plot. (**b**) LD map (upper), genomic structure (middle) and $-\log_{10}$ (*P* value) of SNPs in the GWAS (lower) around *CCR6*. The *D'*-based LD map (upper) is drawn based on the genotype data of rheumatoid arthritis cases and controls enrolled in the GWAS using Haploview software version 4.1. LD blocks involved in the selection of tag SNPs and the expression analysis are highlighted with a yellow bar. The diamond dots (lower) represent respective SNPs. Their densities in red represent LD indices (R^2), with rs3093024 indicated as the largest dot. (**c**) Correlation between the genotypes of rs3093024 and the transcript levels of CCR6-a (NM_031409) in EBV-transfected cell lines ($n = 57$) stimulated with PMA. (**d**) The coefficients of determination, r^2, of SNPs in the correlation analysis between the genotypes and the expression levels of CCR6-a transcripts. The correlation peak was observed at rs3093024. The red or gray dotted lines represent the correspondence of the chromosomal positions in **b** and **d** (the red line represents rs3093024). The plots of **b** and **d** were drawn by using SNAP version 2.1.

chromosomal regions, which included the region containing the previously associated *STAT4* locus[16–18] (**Supplementary Table 2**). The association results for other previously reported loci[9–12,16,19] are summarized in **Supplementary Table 4**, including replications for the *PADI4* and *TNFAIP3* loci (both $P < 0.01$).

To validate the association with *CCR6*, we performed a replication study using two independent Japanese rheumatoid arthritis case-control cohorts (cohort 1 consisted of 3,662 cases and 15,873 controls, and cohort 2 consisted of 1,106 cases and 1,486 controls). The association of rs3093024 was replicated in both sets ($P = 6.3 \times 10^{-9}$ and $P = 3.1 \times 10^{-3}$ in the two individual cohorts, respectively; combined-analysis $P = 7.7 \times 10^{-19}$, OR = 1.19, 95% CI 1.15–1.24; **Table 1**). A concurrent study[20] also identified an association at the *CCR6* region with rheumatoid arthritis in European populations by performing a meta-analysis of GWAS from a collection of 5,539 autoantibody-positive rheumatoid arthritis cases and 20,169 controls, in which the strongest signal was observed at a SNP in *CCR6* (rs3093023, $P = 3.3 \times 10^{-7}$, OR = 1.13). The landmark SNP in the Japanese population

Table 1 Association analysis of rs3093024 in *CCR6* with rheumatoid arthritis

		Number of subjects		Allele T frequency			
Population	Study set	Cases	Controls	Cases	Controls	OR (95%CI)	*P* value
Japanese	GWAS	2,301	3,368	0.52	0.46	1.27 (1.18–1.37)	4.9×10^{-10}
	Replication study 1[a]	3,662	15,873	0.50	0.46	1.16 (1.11–1.22)	6.3×10^{-9}
	Replication study 2[a]	1,106	1,486	0.51	0.47	1.18 (1.06–1.32)	0.0031
	Combined analysis[b]	7,069	20,727	0.51	0.46	1.19 (1.15–1.24)	7.7×10^{-19}
European	GWAS meta-analysis[c]	5,539	20,169	0.47	0.43	1.13 (1.08–1.18)	3.6×10^{-7}

[a]Cochran-Armitage trend test. [b]Meta-analysis of GWAS and replication studies 1 and 2 (Mantel-Haenszel method). [c]Additive model logistic regression (Wald test) in six GWAS collections and meta-analysis across collections by inverse variance-weighted *z*-scores. Details of the European GWAS meta-analysis are described elsewhere[20]. GWAS, genome-wide association study; OR: odds ratio.

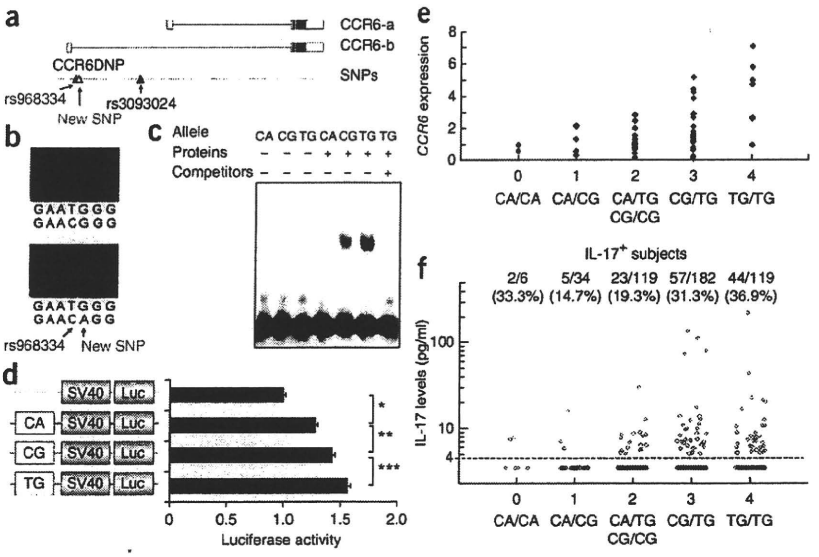

Figure 2 CCR6DNP genotype is correlated with the expression level of *CCR6* and IL-17 status of rheumatoid arthritis cases. (**a**) Genomic position of disease-associated polymorphisms in *CCR6* (CCR6-a and CCR6-b correspond to transcripts NM_031409 and NM_004367 in GenBank, respectively). (**b**) A newly identified SNP in the 3′ flanking sequence of rs968334. (**c**) Binding of nuclear factors from PSC cells to the 31-bp sequences around each allele of CCR6DNP was evaluated by EMSA. Unlabeled probes in 400-fold excess as compared to the labeled probes were used for the competition experiment. The densities of bands were quantified and normalized to that of the TG allele, and significant allelic differences were observed (the mean intensities from four independent experiments were 0.26, 0.76 and 1 for CA, CG and TG, respectively; $P < 0.0001$ by Student's *t*-test). (**d**) Enhanced activity of the 31-bp sequence region around CCR6DNP as evaluated by luciferase assay. SV40, promoter sequence of SV40; luc, luciferase). Data represent mean ± s.e.m. Representative data from three experiments performed in octuplicate are shown. $*P = 1.3 \times 10^{-7}$, $**P = 7.4 \times 10^{-5}$ and $***P = 0.0024$ by Student's *t*-test. (**e**) Correlation between CCR6DNP genotype and expression of *CCR6* as measured by quantitative TaqMan PCR of PSC cells ($n = 57$). (**f**) Correlation between CCR6DNP genotype and IL-17 status in the sera of rheumatoid arthritis cases. The ratios of cases that showed detectable levels of IL-17 (>4 pg/ml) are shown above (IL-17 active cases) and are significantly associated with the CCR6DNP genotype ($P = 1.2 \times 10^{-3}$ by trend test).

(rs3093024) was in almost absolute linkage disequilibrium (LD) with rs3093023 in the European population ($r^2 > 0.99$ and $D' > 0.99$) and showed a similar level of association ($P = 3.6 \times 10^{-7}$, OR = 1.13). Together, these results provide strong evidence for association of the *CCR6* locus with risk of rheumatoid arthritis. This locus encompasses several genes, three of them in the region showing strong LD with rs3093024 (**Fig. 1b**) and six located within 0.5 Mb of either side of this LD region. Although the disease-associated polymorphisms may affect the function of these flanking genes, there is no bibliographical evidence so far showing their functional relevance in rheumatoid arthritis pathogenesis other than that previously shown for *CCR6*.

Recent studies have shown that CCR6 is a specific marker for Th17 cells, distinguishing them from other helper T-cells[8,21], although CCR6 is also expressed in IL-17–producing γδ T cells[22] as well as in subsets of B cells and dendritic cells[23]. Considering the roles of these cells in the pathogenesis of rheumatoid arthritis, *CCR6* is a strong candidate gene in the locus. In a search for causal variants, we first sequenced the *CCR6* coding region in 24 rheumatoid arthritis cases but found no variation that alters its amino acid sequence. We then evaluated whether the *CCR6* polymorphisms could affect gene expression. Epstein-Barr virus (EBV)-transfected cell lines established from Japanese individuals (Pharma SNP Consortium (PSC) cells, $n = 57$) were examined for the expression levels of two major *CCR6* transcripts (NM_031409 and NM_004367 in GenBank, termed here CCR6-a and CCR6-b, respectively). Correlation analysis between the SNP genotype and gene expression level showed a significant correlation between rs3093024 and CCR6-a, not present in resting cells but rather seen in cells stimulated with phorbol myristate acetate (PMA) ($R^2 = 0.21$, $P = 3.2 \times 10^{-4}$ **Fig. 1c**). The expression levels increased with the number of risk (T) alleles. A similar trend was observed for CCR6-b, but this trend was not statistically significant at a level of $\alpha = 0.05$. These observations suggested the presence of regulatory variants in this region that could affect expression levels, especially those of CCR6-a.

To search for regulatory variants, we expanded the region for analysis to approximately 210 kb, covering the entire *CCR6* gene and the surrounding LD blocks containing disease-associated SNPs with $P < 10^{-3}$ (**Fig. 1b**). In addition to the SNPs registered in the Phase II HapMap database for Japanese samples[24], we identified 19 newly discovered SNPs by resequencing the 12-kb region that comprises the 5′ flanking region of *CCR6* in 24 rheumatoid arthritis cases (**Supplementary Fig. 3a**). Of these 312 SNPs, 40 tag SNPs were selected for further correlation analysis with gene expression using a threshold of $r^2 > 0.8$. Correlation analysis revealed a peak of correlation at rs3093024 with the expression level of CCR6-a in cells stimulated with PMA (**Fig. 1d**).

This concordance of peaks in rs3093024 that correlated disease susceptibility with *CCR6* expression made this SNP, or variants in LD with it, a strong candidate as a causal variant. We thus examined all identified SNPs in strong LD ($r^2 > 0.8$) with rs3093024 for their potential to affect gene expression (**Supplementary Fig. 3a**). Allelic differences in binding of nuclear proteins were first evaluated for the sequences surrounding each SNP by electrophoretic mobility shift assays (EMSAs) using nuclear extracts from PSC cells. Of the six SNPs examined, only rs968334 (located 6.7 kb upstream of rs3093024 and with $r^2 = 0.89$) showed a substantial difference in binding of nuclear proteins between the two alleles (**Supplementary Fig. 3b**). Because the nucleotide immediately 3′ of rs968334 was also found to be polymorphic through resequencing this region (**Fig. 2a,b** and **Supplementary Fig. 3a**), we analyzed the haplotype frequency of these two SNPs in 376 rheumatoid arthritis cases by direct sequencing and haplotype phasing. These two SNPs comprised three haplotypes (CA, CG and TG). We termed this 2-base-pair polymorphism *CCR6* dinucleotide polymorphism (CCR6DNP) and thereafter analyzed it as a single, tri-allelic variant. When the binding of nuclear proteins was examined for these three alleles, higher binding was seen in the order of CA < CG < TG (**Fig. 2c**). Although no significant shift was detected in supershift assays using antibodies for nuclear proteins predicted by *in silico* procedures, including YY1 and LYF1 (data not

Table 2 Association analysis of CCR6DNP and autoimmune diseases

		Genotype count						Allele frequency			
		CA/CA	CG/CA	CG/CG	TG/CA	TG/CG	TG/TG	CA	CG	TG	
Disease	Number of subjects	0	1	2	2	3	4	0	1	2	*P* value[a]
Rheumatoid arthritis	2,297	29	161	398	208	897	604	0.09	0.40	0.50	2.4×10^{-9}
Graves' disease	1,783	25	133	334	150	715	426	0.09	0.43	0.48	2.4×10^{-5}
Crohn's disease	483	5	40	81	52	195	110	0.11	0.41	0.48	0.020
Controls	1,808	28	189	379	168	683	361	0.11	0.45	0.44	–

[a]*P* values to determine trends for genotype using the χ^2 test.

shown), this differential binding of unknown nuclear proteins should affect the transcriptional activity of each allele. We next performed reporter assays using oligonucleotides of these three alleles cloned into a plasmid vector containing the viral SV40 promoter. In parallel with the results from the gel shift assays, different enhancing activities were detected in the order of CA < CG < TG (**Fig. 2d**). Taken together, these results suggest that CCR6DNP is a strong candidate for a disease-causing variation that affects gene expression.

To analyze the effect of CCR6DNP on disease susceptibility and on phenotypes within individuals, we established a genotyping assay for CCR6DNP (**Supplementary Fig. 4**). We scored the alleles as 0, 1 and 2 for CA, CG and TG, respectively, according to the enhanced transcription observed in the *in vitro* assays. The diplotypes of individuals were in turn scored as 0, 1, 2, 3 and 4 by summing each individual's allele scores. When the expression of *CCR6* in PSC cells ($n = 57$) was regressed with this diplotype score for CCR6DNP, a significant correlation was observed ($R^2 = 0.23$, $P = 1.7 \times 10^{-4}$; **Fig. 2e**), supporting the regulatory effect of CCR6DNP. When the distribution of the diplotype scores for CCR6DNP was compared between 2,303 rheumatoid arthritis cases and 1,820 controls, a significant association was also observed ($P = 2.4 \times 10^{-9}$ by trend test; **Table 2** and **Supplementary Table 1**). Compared with the CA allele, the ORs of the other alleles demonstrated an identical trend order (CA, 1.00; CG, 1.14; and TG, 1.25) with those indicated in the *in vitro* assays, which supports the hypothesis that the triallelic variant is causal. Conditional regression analysis of the newly identified SNP with rs968334 did not indicate a significant independent association at a level of $\alpha = 0.05$, presumably because of the strong LD between the SNPs ($D' = 1$) or because of inadequate statistical power due to the low minor-allele frequency of the newly identified SNP (minor allele frequency = 0.10). We also genotyped individuals with Graves' disease ($n = 1,783$) and Crohn's disease ($n = 483$); we found significant association of CCR6DNP with both Graves' disease ($P = 2.4 \times 10^{-5}$) and Crohn's disease ($P = 0.020$; **Table 2**).

To seek further association between the CCR6DNP genotype and phenotypes within individuals, we analyzed the serum level of IL-17 in rheumatoid arthritis cases. Among 451 cases examined, 28.5% showed detectable levels of IL-17 (here termed 'IL-17 active cases'). There was a significant association between the CCR6DNP genotype and the proportion of IL-17 active cases ($P = 1.2 \times 10^{-3}$ by trend test; **Fig. 2f**). This association was still significant after adjustment for age and sex using a logistic regression model ($P = 1.4 \times 10^{-3}$). No significant correlation was observed between CCR6DNP and the titer of IL-17 at $\alpha = 0.05$. We also analyzed the association between the CCR6DNP genotype and autoantibody positivity (anti-CCP antibodies and rheumatoid factors) in the same rheumatoid arthritis cases, but did not observe any significant association (**Supplementary Fig. 5**). These results suggest that CCR6DNP has a more direct link to the activation of Th17 cells and their secretion of IL-17 than in autoantibody response in rheumatoid arthritis.

The role of Th17 cells in autoimmune arthritis has been well demonstrated in SKG mice, an animal model for rheumatoid arthritis which results from a mutation in *Zap70* (ref. 8). In this model, $Ccr6^+$ Th17 cells are recruited into the inflamed joints by Ccl20 (a known ligand for Ccr6) that is produced by synoviocytes and Th17 cells themselves. The arthritogenic role of Th17 cells in SKG mice is supported by the observation that the administration of blocking anti-Ccr6 antibodies substantially inhibits arthritis[8]. In humans, the majority of circulating Th17 cells express CCR6 (ref. 21), and its ligand, CCL20, is also detected in inflamed synovial tissues[25]. Preferential expression of *CCR6* in synovial tissues from rheumatoid arthritis cases compared to those from osteoarthritis cases has been previously described[25] and was confirmed in our samples (**Supplementary Fig. 6**). Our observation that CCR6DNP is associated with rheumatoid arthritis susceptibility, as well as our detection of enhanced expression of *CCR6* and the status of IL-17 in the serum of rheumatoid arthritis cases, implies that the same mechanism found in the mouse model might also underlie the pathogenesis of human rheumatoid arthritis.

The CCR6-CCL20–mediated migration of Th17 cells to inflamed tissues may also be important in other autoimmune diseases, as is implicated by the association of Graves' disease and Crohn's disease susceptibility to CCR6DNP observed in the present study. In Crohn's disease, a genetic marker in *FGFR1OP* (adjacent to *CCR6*) was also implicated in a meta-analysis of GWAS[26], supporting the association of *CCR6* to disease. As several other genes implicated in Th17 function, such as *IL23R* and *STAT3*, have been associated with Crohn's disease susceptibility, Th17 cells are considered to play a substantial role in Crohn's disease[27,28]. In contrast, the lack of confirmatory evidence for an association of *IL23R* and *STAT3* in rheumatoid arthritis[9–12] implies that this pathway might be less important in rheumatoid arthritis as compared to Crohn's disease. Therefore, it is possible that the *CCR6* polymorphism could be involved in rheumatoid arthritis pathogenesis by influencing *CCR6*-expressing cell types other than Th17 cells. As enhanced expression of *CCR6* may differentially contribute to pathogenesis between different autoimmune diseases, studies of other autoimmune diseases are needed to clarify the etiological role of the *CCR6* polymorphism in autoimmunity.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession numbers. RefSeq, NM_031409 and NM_004367.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Y.K., Y.O. and K. Yamamoto. designed the study and drafted the manuscript. Y.O., A.T., T.T. and R.Y. analyzed the GWAS data. N.H. and M.K. performed the genotyping for the GWAS. Y.K. performed expression analysis of *CCR6* and functional analysis of *CCR6* polymorphisms. Y.K. and K.M. established the genotyping method for CCR6DNP. K.I., S.M. and H.Y. analyzed data for the first replication cohort of rheumatoid arthritis. C.T., K.O., T.M., R.Y. and F.M. analyzed the data for the second replication cohort of rheumatoid arthritis. Y.K. analyzed the data for the Graves' disease cohort. K. Yamazaki analyzed the data for the Crohn's disease cohort. T.F. and S.I. analyzed the data for the fourth control cohort. T.I. and K.I. analyzed *CCR6* expression in the synovial tissues. Y.K. and A.S. analyzed the sera of subjects with rheumatoid arthritis. M.K., N.K. and Y.N. contributed to overall GWAS study design.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Samples enrolled in the study. We enrolled 7,090 rheumatoid arthritis cases, 1,783 Graves' disease cases, 483 Crohn's disease cases and 21,712 unaffected controls through several medical institutes in Japan under the support of the BioBank Japan Project[29], the University of Tokyo, Institute of Rheumatology Rheumatoid Arthritis (IORRA), Kyoto University and the GARNET consortium. All subjects were of Japanese origin and provided written informed consent. Details of the samples are summarized in **Supplementary Table 1** and **Supplementary Note**. This research project was approved by the ethical committees of the BioBank Japan Project, RIKEN, the University of Tokyo, Tokyo Women's Medical University and Kyoto University.

Genotyping and quality control. In the GWAS, 2,322 rheumatoid arthritis cases and 3,473 controls were genotyped using the Illumina Human610-Quad and Illumina Human 550v3 Genotyping BeadChips (Illumina), respectively. After the exclusion of samples with low call rates (<98%), SNPs with low call rates (<99%), non-autosomal SNPs and those SNPs not shared among cases and controls were also excluded.

We excluded closely related samples using identity by descent estimated by PLINK[30] version 1.06. For sample pairs in a first degree of kinship, we excluded the individual who was a control, or in cases where that had not uniquely been determined, the individual had lower call rate than the other. We next excluded samples who were outliers in terms of ancestries. We performed PCA for the genotype data of the samples along with European (CEU), African (YRI) and East-Asian (Japanese and Han Chinese, denoted JPT + CHB) individuals obtained from Phase II HapMap database (release 22)[24] using EIGENSTRAT[13] version 2.0. A PCA plot clearly separated the samples into three clusters, as previously indicated[14] (**Supplementary Fig. 1**). The concordance of the cluster between JPT + CHB individuals and our samples suggested that our samples had homogeneous ancestries. We visually excluded the samples who were estimated to be outliers.

We excluded SNPs with minor allele frequency less than 0.05 in either rheumatoid arthritis cases or controls, or with Hardy-Weinberg equilibrium P value[31] $>10^{-6}$ in controls. Cluster plots of SNPs were checked by visual inspection, and SNPs with ambiguous calls were excluded. Finally, 393,217 SNPs for 2,303 rheumatoid arthritis cases and 3,380 controls were obtained. The genotyping methods for the replication studies are described in **Supplementary Table 1**.

Association analysis. The associations of the SNPs were tested with the Cochran-Armitage trend test. Combined analysis was performed with the Mantel-Haenszel method. Correction for potential population stratification was performed by the genomic control method[15] and by using the results of PCA[13] (see **Supplementary Note** for details).

Replication study for previously reported rheumatoid arthritis susceptibility loci. We evaluated the associations in previously reported rheumatoid arthritis susceptibility loci[9–12,16–19,32–39] using the samples in our GWAS (2,303 rheumatoid arthritis cases and 3,380 controls) and the controls in the first replication study (15,873 controls). We imputed the SNPs that were not genotyped using MACH 1.0 (see URLs), with Phase II HapMap JPT + CHB individuals as references[24]. All the imputed SNPs demonstrated *Rsq* values more than 0.95.

Genotyping method for CCR6DNP. The triallelic dinucleotide polymorphism in *CCR6* (CCR6DNP) was genotyped using two sets of TaqMan MGB probes (**Supplementary Table 5**). Approximately 4 ng DNA was amplified in a 5 μl reaction mixture comprising 200 nM each of probe and 900 nM each of primer in TaqMan genotyping master mix (Applied Biosystems). After denaturation at 95 °C for 10 min, 50 cycles of denaturation at 95 °C for 15 s and annealing and extension at 58 °C for 1 min were performed. The results for the reactions of two probe sets were analyzed together and the genotype of each sample was determined by an algorithm as shown in **Supplementary Figure 4**. The genotypes of 376 individuals completely matched (100%) those obtained by direct sequencing.

DNA resequencing. Unknown genetic variants were revealed by directly sequencing of the DNA of 24 individuals affected with rheumatoid arthritis. DNA fragments were amplified for sequencing with appropriate primers and were purified using a MultiScreen PCR filter plate (Millipore). The amplified DNAs were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and signals were detected using an Applied Biosystems ABI 3700 DNA Analyzer.

Quantification of gene expression. EBV-transformed lymphoblastoid cell lines (n = 57) were established by the Pharma SNP Consortium (PSC, Tokyo, Japan) using peripheral blood lymphocytes of Japanese healthy individuals. Cells were incubated for 2 h in medium alone (RPMI 1640 medium containing 10% FBS albumin, 1% penicillin and 1% streptomycin) or with 100 ng/ml PMA. Conditions for cell stimulation were optimized before the experiment as previously described[40]. Cells were then harvested and total RNA was isolated using an RNeasy Mini Kit (Qiagen) with DNase treatment. Total RNA from synovial tissues was isolated from rheumatoid arthritis cases (n = 74) and osteoarthritis cases (n = 8) who underwent total knee replacement surgery. Total RNA (1 μg) was reverse transcribed using TaqMan Gold RT-PCR reagents with random hexamers (Applied Biosystems). Real-time quantitative PCR was performed in triplicate using an ABI PRISM 7900 and TaqMan gene expression assays (Applied Biosystems). Specific probes for each transcript of *CCR6* were used: Hs00171121_m1 was used for CCR6-a (NM_031409) and a custom-made probe set was used for CCR6-b (NM_004367); both the custom probe and Hs00171121_m1 were comprised of a minor groove binder probe (**Supplementary Table 5**). Possible contamination of DNA was excluded by performing PCR with total RNA without the reverse transcription step. The data were normalized to *GAPDH* levels. *GUS* and 18S rRNA levels were also evaluated for internal control, and similar results were obtained among the three probes.

Correlation analysis between genotype and gene expression. DNA was extracted from PSC cells and genotyped for the tag SNPs using TaqMan genotyping assays (Applied Biosystems). The levels of each transcript of *CCR6* were regressed with the genotype in a linear model, assuming an additive effect of the allele. The correlation coefficients R^2 were used for fine mapping of the correlation between SNP genotypes and *CCR6* expression in this region. The significance of regression was tested by an F test.

Electrophoretic mobility shift assay (EMSA). EMSA and preparation of nuclear extract from PSC cells were performed as previously described[41]. Following stimulation with 50 ng/ml PMA for 2 h, cells were collected and lysed in Buffer A (20 mM HEPES at pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA at pH 8.0, 1 mM DTT, 0.1% NP-40 detergent and a protease inhibitor cocktail). The cells were then incubated on ice for 10 min, centrifuged, and the pellets were resuspended in Buffer B (which contains Buffer A with 500 mM NaCl). Following incubation on ice for 30 min and centrifugation, the supernatant fraction containing nuclear proteins was collected. Oligonucleotides (31 base pairs) were designed that corresponded to genomic sequences surrounding the SNPs of interest. Single-stranded oligonucleotide probes were labeled using a Biotin 3′ End DNA Labeling Kit (Pierce Biotechnology), and sense and antisense oligonucleotides were then annealed. DNA-protein interactions were detected by using a LightShift Chemiluminescent EMSA kit (Pierce Biotechnology) according to the manufacturer's instructions. The DNA-protein complexes were separated on a nondenaturing 5% polyacrylamide gel in 1× TBE (Tris-borate-EDTA) running buffer for 70 min at 150 V. The gel was transferred to a nitrocellulose membrane, and signals were detected using a LAS-3000 lumino image analyzer (Fujifilm).

***In silico* prediction of transcription factor binding site.** DNA sequences around each allele of the CCR6DNP were evaluated for the potential of transcriptional factor binding using Match software version 1.0 (see URLs). This software uses a library of mononucleotide weight matrices from TRANSFAC 6.0 (see URLs).

Luciferase assay. Oligonucleotides (31 base pairs) were generated using the allelic sequences of nucleotides surrounding CCR6DNP (**Supplementary Table 6**). A single copy of the oligonucleotide was cloned into the pGL3-Promoter Vector (Promega) at the MulI and BglII restriction enzyme binding sites. Each construct was then transformed into *Escherichia coli* DH5α (Toyobo). These plasmids were purified using a HiSpeed Plasmid Midi Kit (Qiagen)

after confirmation of the sequence. HEK293A cells (Invitrogen) were grown in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% FBS and antibiotics. Cells ($n = 2 \times 10^5$) were transfected with 0.8 μg constructs and 0.2 μg pRL-TK vector (an internal control for transfection efficiency) using the TransFast Transfection Reagent (Promega). After 20 h, 100 ng/ml PMA was added to the medium. Following an additional incubation for 4 h, cells were collected and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 Ultra Sensitive Tube Luminometer (Berthold Technologies). Each experiment was independently repeated three times and octuplicate samples were assayed each time.

Measurement of autoantibodies and IL-17. Anti-citrullinated peptide antibodies were measured using a MESACUP CCP test (Medical & Biological Laboratories) according to the manufacturer's instructions. Rheumatoid factors were measured by ELISA as previously described[42]. Human IL-17 was measured by ELISA, with a detection limit of 4 pg/ml (eBioscience).

URLs. MACH, http://www.sph.umich.edu/csg/abecasis/mach/; Match software, http://www.gene-regulation.com/; TRANSFAC, http://www.biobase-international.com/.

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Brief Definitive Report

Complement drives Th17 cell differentiation and triggers autoimmune arthritis

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Activation of serum complement triggers Th17 cell–dependent spontaneous autoimmune disease in an animal model. In genetically autoimmune-prone SKG mice, administration of mannan or β-glucan, both of which activate serum complement, evoked Th17 cell–mediated chronic autoimmune arthritis. C5a, a chief component of complement activation produced via all three complement pathways (i.e., lectin, classical, and alternative), stimulated tissue-resident macrophages, but not dendritic cells, to produce inflammatory cytokines including IL-6, in synergy with Toll-like receptor signaling or, notably, granulocyte/macrophage colony-stimulating factor (GM-CSF). GM-CSF secreted by activated T cells indeed enhanced in vitro IL-6 production by C5a-stimulated macrophages. In vivo, C5a receptor (C5aR) deficiency in SKG mice inhibited the differentiation/expansion of Th17 cells after mannan or β-glucan treatment, and consequently suppressed the development of arthritis. Transfer of SKG T cells induced Th17 cell differentiation/expansion and produced arthritis in C5aR-sufficient recombination activating gene (RAG)$^{-/-}$ mice but not in C5aR-deficient RAG$^{-/-}$ recipients. In vivo macrophage depletion also inhibited disease development in SKG mice. Collectively, the data suggest that complement activation by exogenous or endogenous stimulation can initiate Th17 cell differentiation and expansion in certain autoimmune diseases and presumably in microbial infections. Blockade of C5aR may thus be beneficial for controlling Th17-mediated inflammation and autoimmune disease.

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Abbreviations used: CL, clodronate liposome; IC, immune complex; MASP, mannose-binding lectin-associated serine protease; RA, rheumatoid arthritis; SPF, specific pathogen–free; TLR, Toll-like receptor; TNP, trinitrophenyl.

There is recent evidence that IL-17–secreting CD4$^+$ T cells (Th17 cells) play a key role in autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (Harrington et al., 2005; Veldhoen et al., 2006; Korn et al., 2009). It remains unclear, however, how pathogenic self-reactive Th17 cells are generated from naive T cells, and are activated by external or internal stimuli in autoimmune disease.

SKG mice, a mutant of the gene encoding ZAP-70 on the BALB/c background, spontaneously develop CD4$^+$ T cell–mediated autoimmune arthritis clinically and immunologically resembling human RA (Sakaguchi et al., 2003). The mutation alters the sensitivity of developing T cells to positive and negative selection in the thymus, leading to thymic production of potentially arthritogenic autoimmune T cells (Sakaguchi et al., 2003; Hirota et al., 2007). The SKG arthritis is critically dependent on Th17 cells, as deficiency of either IL-17 or IL-6 completely inhibits the disease (Hirota et al., 2007). Importantly, they spontaneously develop severe arthritis in a microbially conventional environment but not under a specific pathogen–free (SPF) condition, suggesting that environmental stimuli such as microbial infection may

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expand or trigger the differentiation of arthritogenic Th17 cells (Yoshitomi et al., 2005). Indeed, injection of zymosan, a crude extract of yeast cell wall containing β-glucans or purified β-glucans, such as laminarin, activates innate immunity via Toll-like receptor (TLR) and Dectin-1, and drives preferential differentiation and expansion of Th17 cells, thereby triggering arthritis in SKG mice under a SPF condition (Yoshitomi et al., 2005; LeibundGut-Landmann et al., 2007). Because zymosan is also an activator of the alternative pathway of complement (Mullaly and Kubes, 2007) and β-glucan structure can be recognized by ficolin-L, an initiator of the lectin pathway (Garlatti et al., 2007), it is also likely that complement activation may contribute to triggering Th17-mediated autoimmune disease.

In this report, we show that complement activation via all three pathways (i.e., the lectin, classical, and alternative pathways) and the resulting generation of the common product C5a potently promote the differentiation/expansion of self-reactive T cells to Th17 cells that mediate autoimmune arthritis in SKG mice. The results indicate that exogenous or endogenous stimuli that activate complement can be a triggering cause of Th17-mediated autoimmune disease and that C5a is a key molecular target in controlling Th17-mediated autoimmunity as well as microbial immunity.

RESULTS AND DISCUSSION

Mannan triggers autoimmune arthritis by expanding Th17 cells

We first tested whether mannan, a prototypic activator of the lectin pathway of complement activation, was able to trigger arthritis in SKG mice (Fig. 1, A–E; Fujita, 2002). A single i.p. injection of 20 mg mannan triggered self-sustained chronic arthritis within 2 wk in all of the treated SKG mice but not in BALB/c mice. A small amount (200 μg) also elicited joint swelling, but only in a few small joints and in 50% of SKG mice. IL-17$^{-/-}$ SKG mice were completely resistant to arthritis induction by mannan (Fig. 1 F). The ratio of IL-17^{+} cells among CD4^{+} T cells was increased significantly (approximately fourfold) in regional (e.g., popliteal) lymph nodes of mannan-treated SKG mice with arthritis (e.g., in ankles) compared with control PBS-treated SKG mice without arthritis; the ratio also increased significantly, although to a much lesser degree, in mannan-treated BALB/c mice (Fig. 1, G and H). Thus, mannan can enhance the development of arthritogenic Th17 cells and evoke arthritis in SKG mice.

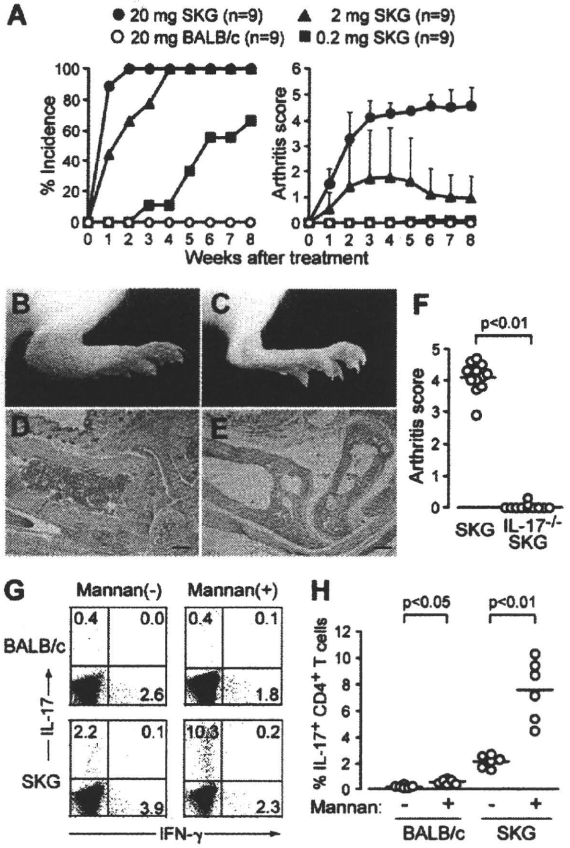

Figure 1. Mannan triggers autoimmune arthritis by expanding Th17 cells. (A) Joint score of 8∼12-wk-old SKG or BALB/c mice that received a single i.p. injection of mannan at the indicated doses. A total of two independent experiments are shown. Error bars are means ± SD of scores. (B–E) A representative joint swelling and histology of an SKG (B and D) and a BALB/c mouse (C and E) 8 wk after mannan treatment (hematoxylin and eosin staining). Bars, 200 μm. (F) Joint scores of IL-17$^{+/+}$ or IL-17$^{-/-}$ SKG mice 8 wk after mannan treatment (n = 12). Horizontal bars are the means of each group. (G) Intracellular staining of IL-17 and IFN-γ in CD4^{+} T cells in the popliteal lymph nodes from SKG or BALB/c mice 8 wk after mannan treatment (numbers indicate percentages). One representative staining out of six independent experiments is shown. (H) Percentages of IL-17^{+} cells in CD4^{+} T cells in each SKG or BALB/c group (n = 6), as shown in G. Horizontal bars are the means of each group.

C5aR is essential for driving Th17 cell differentiation and triggering arthritis

C5a, a key common product of all three complement activation pathways, is the most potent complement-derived mediator of inflammation; increases the production of IL-6, TNF, and IL-1 from TLR-stimulated macrophages; and suppresses IL-12 production (Guo and Ward, 2005; Hawlisch et al., 2005; Zhang et al., 2007). To examine possible effects of mannan treatment on C5a production via the lectin pathway, and consequently Th17 cell differentiation and expansion in SKG mice, we prepared SKG mice deficient in C5aR (CD88; Hawlisch et al., 2005). The incidence and severity of arthritis was significantly suppressed in mannan-treated C5aR$^{-/-}$ SKG mice (Fig. 2 A). The measurement of serum C3a and C5a revealed that the treatment strongly triggered complement activation for the first 3 d, with persisting low level activation over 28 d in mannan-elicited arthritic SKG mice (Fig. S1). Mannan-treated C5aR$^{+/+}$ SKG mice, when examined 8 wk (Fig. 2, B–E) or 2 wk (Fig. S1) after treatment, showed a marked hypertrophy of the regional lymph nodes (Fig. 2 B), which contained a much higher ratio and absolute number of IL-17^{+} CD4^{+} T cells compared with similarly

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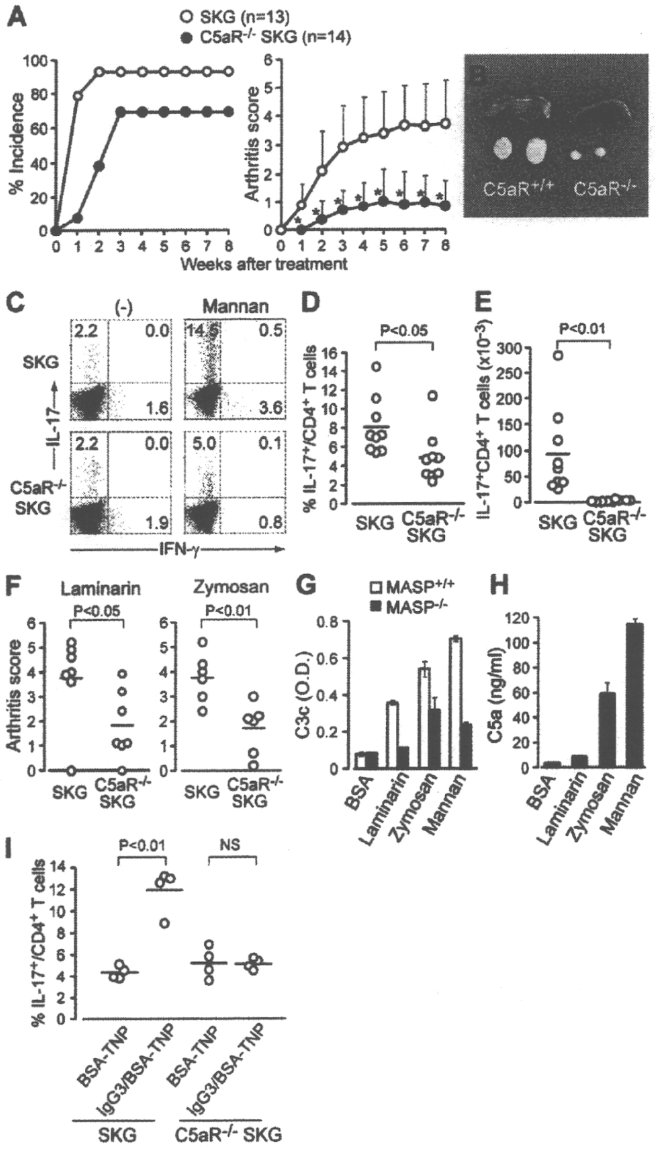

Figure 2. C5a drives Th17 cell differentiation and triggers arthritis. (A) Joint score of C5aR$^{+/+}$ or C5aR$^{-/-}$ SKG mice treated by 20 mg mannan. A total of two independent experiments are shown. Error bars are means ± SD of scores. *, $P < 0.05$. (B) Spleen and popliteal lymph nodes of C5aR$^{+/+}$ or C5aR$^{-/-}$ SKG mice 8 wk after mannan treatment. (C) Intracellular staining of IL-17 and IFN-γ in CD4$^+$ T cells in the popliteal lymph nodes of C5aR$^{+/+}$ or C5aR $^{-/-}$ SKG mice, as shown in B (numbers indicate percentages). One representative staining out of nine independent experiments is shown. (D and E) Percentages (D) and absolute numbers (E) of IL-17$^+$ cells in CD4$^+$ T cells in each group ($n = 9$), as shown in C. (F) Joint scores of C5aR$^{+/+}$ or C5aR$^{-/-}$ SKG mice 8 wk after treatment by laminarin ($n = 7$) or zymosan ($n = 6$). (G) C3 deposition assay with laminarin-, zymosan-, or mannan-coated wells incubated with 2% sera from MASP-intact or -null C57BL/6 sera. (H) C5a ELISA for the supernatant of the C3 deposition assay with BALB/c sera, as shown in G. Error bars are means ± SD. (I) C5aR$^{+/+}$ and C5aR$^{-/-}$ SKG mice were i.p. injected with TNP-BSA alone or IgG3 anti-TNP/TNP-BSA IC. The percentage of IL-17$^+$ cells in the peritoneal CD4$^+$ T cells was assessed on day 7 ($n = 4$). Horizontal bars are the means.

treated C5aR$^{-/-}$ SKG mice (Fig. 2, C–E) or BALB/c mice (not depicted).

Similar to mannan treatment, arthritis elicitation by laminarin or zymosan was significantly suppressed in C5aR$^{-/-}$ SKG mice (Fig. 2 F). The C3 deposition assay, in which the amount of activated C3c was quantified by ELISA, showed that laminarin and zymosan activated complement (Fig. 2 G). The C3c deposition was reduced by the use of mannose-binding lectin-associated serine protease (MASP)–null serum, indicating the involvement of the lectin pathway (Fujita, 2002; Iwaki et al., 2006; Takahashi et al., 2008). C5a was indeed detected in the supernatant sera of the C3 deposition assay after complement activation (Fig. 2 H). These results collectively indicate that laminarin, zymosan, and mannan in particular can commonly activate complement to produce C5a, which critically contributes to evoking autoimmune arthritis in SKG mice.

Although the lectin and alternative pathways are stimulated by microbial products such as mannan or zymosan, the classical pathway can be activated by antigen–antibody immune complexes (ICs; Guo and Ward, 2005). ICs not only activate complement but also deliver signal through FcγR on APCs (Sylvestre et al., 1996). As such, we asked whether an IgG3-IC, which is known to activate the classical and alternative complement pathways without the involvement of FcγR, would expand Th17 cells in a C5a/C5aR-dependent manner (Fig. 2 I; Díaz de Ståhl et al., 2003). C5aR$^{+/+}$ and C5aR$^{-/-}$ SKG mice were i.p. injected with trinitrophenyl (TNP) hapten–conjugated BSA alone or with ICs formed of TNP-BSA and a TNP-specific IgG3 mAb. Compared with the injection of BSA-TNP alone, IL-17$^+$ cells were markedly increased in the peritoneal cavity of C5aR$^{+/+}$ SKG but not C5aR$^{-/-}$ SKG mice after i.p. injection of the ICs, although this single-dose injection failed to elicit arthritis in the former. Collectively, activation of all three complement activation pathways facilitates Th17 cell differentiation and expansion via C5a/C5aR.

C5a acts on tissue-resident macrophages to drive Th17 cell differentiation

C5aR was highly expressed on neutrophils (as CD11b^{high}Gr-1^{high}F4/80$^-$ cells) and monocytes/macrophages (as CD11b^{high}F4/80$^+$Gr-1$^{low/-}$ cells) in the peritoneal cavity or the spleen of nontreated SKG mice (Fig. 3 A), and in arthritic joints of mannan-treated SKG mice (Fig. 3 B). To determine the cell types that received C5aR signaling and drove Th17 cell differentiation, we cultured naive BALB/c CD4$^+$ T cells with various types of APCs and stimulated them with anti-CD3 in the presence or absence of C5a and/or TGF-β. Notably,

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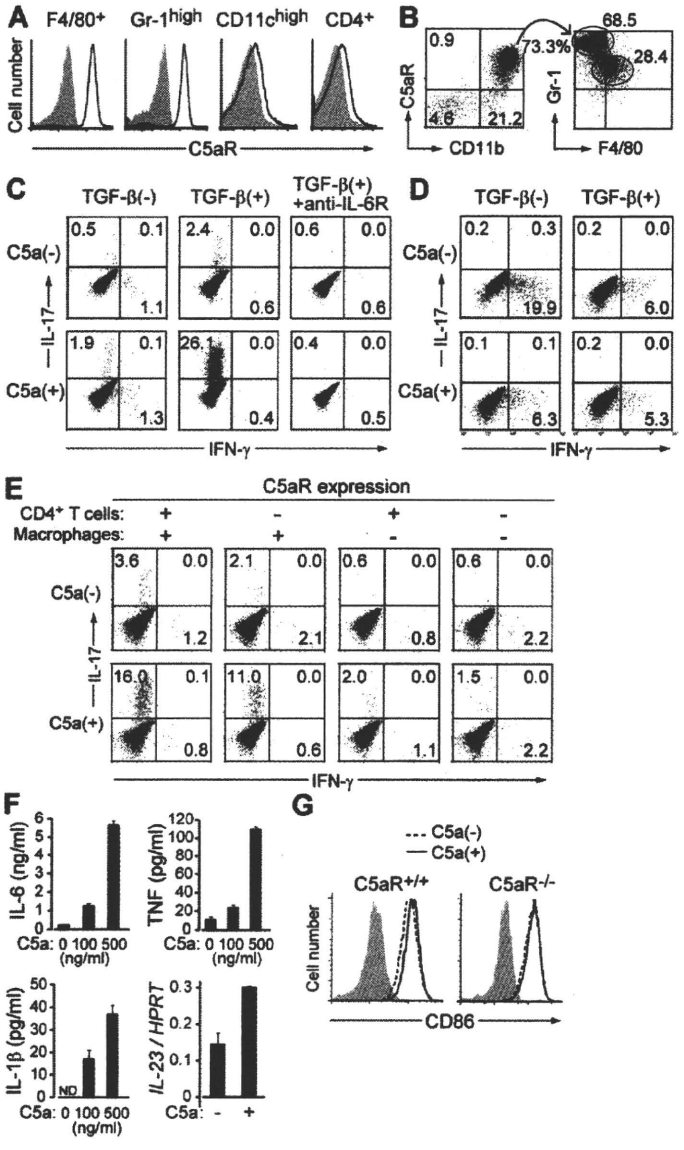

Figure 3. C5a acts on tissue-resident macrophages to facilitate Th17 cell differentiation. (A) F4/80$^+$ cells in the peritoneal cavity, and Gr-1^{high}, CD11c^{high}, and CD4$^+$ cells in the spleen of nontreated SKG mice were stained for C5aR (continuous line) or isotype control (shading). (B) Cells infiltrating in the arthritic joint of mannan-treated SKG mice were stained for C5aR, CD11b, Gr-1, and F4/80. C5aR$^+$ CD11b$^+$ cells are shown for the expression of Gr-1 and F4/80. (C) BALB/c CD4$^+$ T cells were stimulated with anti-CD3 and cultured with resident peritoneal macrophages in the presence or absence of TGF-β, recombinant C5a, or anti–IL-6R, and stained for intracellular cytokines on day 3. (D) Intracellular cytokine staining of BALB/c CD4$^+$ T cells cultured with splenic DCs in the presence or absence of TGF-β and C5a. (E) Intracellular cytokine staining of CD4$^+$ T cells after criss-cross co-culture with C5aR$^{+/+}$ or C5aR$^{-/-}$ CD4$^+$ T cells and C5aR$^{+/+}$ or C5aR$^{-/-}$ macrophages in the presence of TGF-β. (F) ELISA assessment (triplicates) for IL-6, TNF, and IL-1β produced by macrophages, or quantitative RT-PCR for their *IL-23* mRNA expression, when macrophages were stimulated overnight by C5a at the indicated doses. Error bars are means ± SD. (G) CD86 expression on C5aR$^{+/+}$ or C5aR$^{-/-}$ macrophages cultured with or without C5a for 4 h. Shading indicates isotype control. Results in A–G represent three independent experiments. Numbers in B–E indicate percentages.

naive CD4$^+$ T cells co-cultured with resident peritoneal macrophages (as CD11b^{high}F4/80$^+$Gr-1$^-$ cells) in the presence of TGF-β spontaneously differentiated into IL-17$^+$ cells, and addition of recombinant C5a dramatically increased IL-17$^+$ cells but not IFN-γ$^+$ cells; anti–IL-6R completely inhibited the increase (Fig. 3 C). This Th17-promoting effect of C5a was greater on naive (CD44^{low}CD45Rb^{high}) CD4$^+$ T cells than memory (CD44^{high}CD45Rb^{low}) CD4$^+$ T cells (unpublished data). The in vitro C5a-mediated expansion of Th17 cells also occurred with thioglycollate-elicited peritoneal macrophages, but to a much lesser extent compared with resident peritoneal macrophages (Fig. S2). Importantly, these effects with macrophages were not observed with splenic DCs (Fig. 3 D) or with DCs from mannan-treated SKG mice (Fig. S2), even in the presence of TGF-β. Without TGF-β, CD4$^+$ T cells cultured with DCs differentiated primarily into IFN-γ$^+$ cells. Addition of C5a slightly decreased the percentage of IFN-γ$^+$ cells but did not evoke Th17 cell differentiation (Fig. 3 D).

To determine whether the induction of Th17 cells by C5a in CD4$^+$ T cell/macrophage co-culture depended on a direct effect of C5a on CD4$^+$ T cells or macrophages, we performed criss-cross co-cultures with C5aR$^{+/+}$ or C5aR$^{-/-}$ CD4$^+$ T cells and C5aR$^{+/+}$ or C5aR$^{-/-}$ macrophages (Fig. 3 E). Th17 cell differentiation was inhibited when macrophages, but not CD4$^+$ T cells, lacked C5aR, suggesting that cytokines produced by C5a-stimulated macrophages were responsible for inducing Th17 cell differentiation. Indeed, C5a elicited a dose-dependent production of large amounts of IL-6 and, to a lesser degree, TNF and IL-1β from resident peritoneal macrophages (Fig. 3 F). Production of IL-23, which is essential for the survival and expansion of Th17 cells, was up-regulated at the mRNA level in C5a-treated macrophages, although the cytokine was below the detection limit (30 pg/ml) of ELISA (Fig. 3 F). C5a also up-regulated co-stimulatory molecules such as CD86 on C5aR$^{+/+}$ macrophages but not on C5aR$^{-/-}$ macrophages (Fig. 3 G; Strainic et al., 2008).

C5a drives Th17 cell differentiation in synergy with GM-CSF or TLR signaling

Next we asked whether the in vitro robust expansion of Th17 cells was mediated by C5a alone or by a synergy of C5a and LPS, because the recombinant C5a we used contained a trace amount of contaminated LPS, which could synergistically

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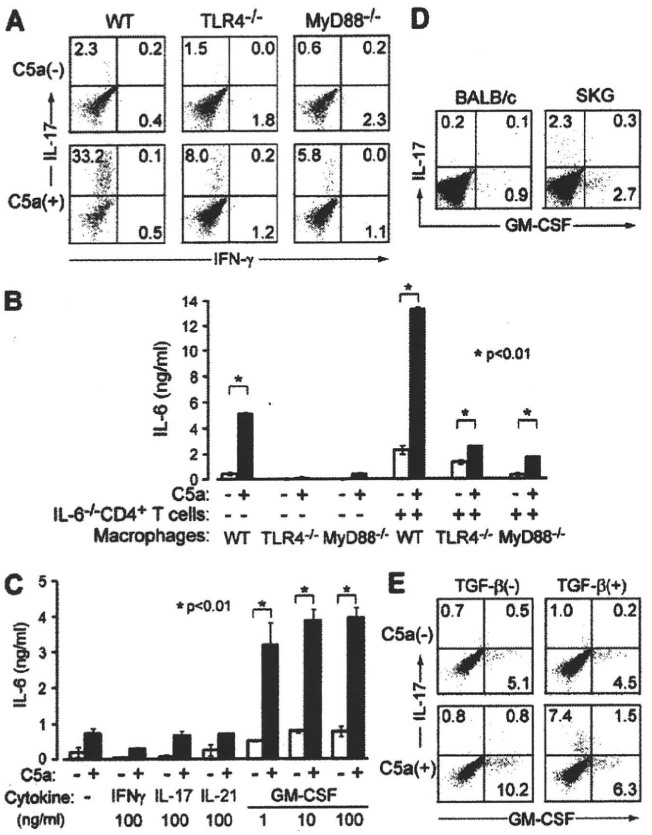

Figure 4. C5a drives Th17 cell differentiation in synergy with TLR or GM-CSF. (A) C57BL/6 CD4+ T cells were cultured with TLR4$^{-/-}$, MyD88$^{-/-}$, or wild-type C57BL/6 macrophages in the presence of TGF-β with or without C5a. (B) Macrophages alone from these mice or co-cultured with anti-CD3–stimulated CD4+ T cells from IL-6$^{-/-}$ mice were stimulated with C5a overnight. IL-6 in the supernatant was determined by ELISA. (C) MyD88$^{-/-}$ macrophages were stimulated by C5a overnight in the presence or absence of cytokines at the indicated doses. IL-6 in the supernatant was determined by ELISA (triplicates). Error bars are means ± SD. (D) Freshly isolated BABL/c or SKG splenic CD4+ T cells were stained for intracellular IL-17 and GM-CSF. (E) BALB/c CD4+ T cells were cultured with macrophages in the presence or absence of C5a and/or TGF-β, stimulated with anti-CD3, and stained for intracellular cytokines on day 3. Results in A–E represent three independent experiments. Numbers in A, D, and E indicate percentages.

act to drive Th17 cell differentiation (see Materials and methods; Fang et al., 2009). To dissect TLR-dependent and -independent effects, we used macrophages from TLR4$^{-/-}$ or MyD88$^{-/-}$ mice. Although a deficiency of TLR4$^{-/-}$ or MyD88$^{-/-}$ in macrophages substantially reduced the C5a-mediated expansion of Th17 cells, a significant proportion of Th17 cells still developed (Fig. 4 A). Further, C5a derived from LPS-free human plasma expanded Th17 cells (unpublished data; Köhl, 1997). Notably, when TLR4$^{-/-}$ or MyD88$^{-/-}$ macrophages alone were stimulated by C5a, IL-6 production was nearly abolished (Fig. 4 B). However, in co-culture with anti-CD3–stimulated IL-6$^{-/-}$ CD4+ T cells, C5a significantly enhanced IL-6 production by TLR4$^{-/-}$ or MyD88$^{-/-}$ macrophages, although much less potently compared with wild types (Fig. 4 B).

To analyze how T cells contributed to the C5a-induced IL-6 production by macrophages, we assessed the effect of costimulatory molecules and T cell–derived cytokines that could alter macrophage function (Grabstein et al., 1986). IL-6 production was partially inhibited by blockade of CD40L (Fig. S3; Hirota et al., 2007). When TLR4$^{-/-}$ or MyD88$^{-/-}$ macrophages alone were stimulated with C5a in the presence or absence of various cytokines (e.g., IL-17, IL-21, IFN-γ, and GM-CSF), only GM-CSF significantly enhanced IL-6 production even at a low concentration (e.g., 1 ng/ml; Fig. 4 C and Fig. S3; Sonderegger et al., 2008). Although freshly isolated BALB/c naive CD4+ T cells contained only a small number of GM-CSF–secreting cells (Fig. 4 D), their co-culture with resident macrophages under anti-CD3 stimulation markedly increased the proportion of GM-CSF+ T cells, and C5a further increased the proportion (Fig. 4 E). GM-CSF+ T cells were distinct from Th17 cells, whose expansion in the presence of C5a and TGF-β accompanied a slight decrease in GM-CSF+ T cells (Fig. 4 E). In vivo, splenic CD4+ T cells in SKG mice contained sizable proportions of Th17 cells and GM-CSF+ CD4+ T cells; one half of the latter also secreted IFN-γ (Fig. 4 D and not depicted).

Collectively, the results in Fig. 3 and Fig. 4 indicate that C5a acts on macrophages in the joint and other tissues to provoke their production of IL-1, IL-6, TNF, and IL-23, and together with tissue TGF-β, promotes the differentiation and expansion of self-reactive T cells into Th17 cells. C5a stimulates macrophages to produce IL-6 in at least two ways: via synergy with TLR signaling and via T cell–macrophage interaction. In the latter, GM-CSF produced by activated T cells enhances IL-6 production by C5a-stimulated macrophages, indicating a novel pathway of promoting Th17 cell differentiation and expansion.

Macrophages are required for in vivo Th17 expansion and induction of arthritis

To determine the role of macrophages in vivo, we treated SKG mice with clodronate liposome (CL), which specifically depletes monocytes and macrophages (Solomon et al., 2005). The treatment indeed efficiently depleted C5aR+ monocytes/macrophages without affecting neutrophils or DCs in SKG mice (Fig. 5 A). CL administration before mannan injection markedly attenuated the development of arthritis (Fig. 5 B) and reduced the expansion of Th17 cells (Fig. 5 C).

C5aR signaling promotes spontaneous differentiation of CD4+ T cells to Th17 cells via homeostatic proliferation

Similar to innate immune stimulation by microbial products, aseptic stimulation of SKG self-reactive T cells (e.g., via homeostatic proliferation in a lymphopenic environment) evokes