Table 1. Demographic and baseline clinical characteristics of the patients*

	Tofacitinib 5 mg twice daily (n = 321)	Tofacitinib 10 mg twice daily (n = 316)	Placebo to tofacitinib 5 mg twice daily (n = 81)	Placebo to tofacitinib 10 mg twice daily (n = 79)
Female, no. (%)	269 (83.8)	273 (86.4)	65 (80.2)	72 (91.1)
White, no. (%)	152 (47.4)	144 (45.6)	36 (44.4)	36 (45.6)
Age, mean ± SD years	53.7 ± 11.6	52.0 ± 11.4	53.2 ± 11.5	52.1 ± 11.8
Disease duration, mean (range) years	8.9 (0.3–43.0)	9.0 (0.3–42.0)	8.8 (0.6–30.8)	9.5 (0.4–43.5)
Tender joints (0–68), mean	24.1	23.0	23.3	22.6
Swollen joints (0–66), mean	14.1	14.4	14.0	14.5
Total SHS (0–448)				
Mean	31.1	37.3	35.0	30.1
Median	13.0	13.0	16.0	14.0
Average annual radiographic progression rate, units per year	5	5.5	—†	—†
Erosion score (0–280), mean	13.8	17.7	14.5	14.3
Patients with erosion score ≥3, %	60.1	65.4	—‡	—‡
JSN score (0–168), mean	17.3	19.6	20.5	15.8
HAQ DI score (0–3), mean	1.41	1.39	1.40	1.23
Four-variable DAS28-ESR (0–9.4), mean	6.34	6.25	6.25	6.29
Three-variable DAS28-CRP (0–9.4), mean	5.22	5.20	5.14	5.18
ESR, mean mm/hour	50.1	50.5	47.8	54.4
CRP, mean mg/liter	15.5	17.0	12.2	15.3
RF positive, %	75.2	77.6	79.7	75.3
Anti-CCP positive, %	85.9	84.4	84.0	82.3
Prior MTX, %	100	99.7§	100	100
Prior DMARDs other than MTX, %	60.1	60.8	76.5	58.2
Prior TNF inhibitors, %	19.3	15.8	9.9	8.9
Prior non–TNF inhibitor biologic agents, %	5.3	4.7	3.7	2.5

* In some cases the number of patients sampled was less than the total number of patients in each group. SHS = modified Sharp/van der Heijde score; JSN = joint space narrowing; HAQ DI = Health Assessment Questionnaire disability index; DAS28-ESR = Disease Activity Score in 28 joints using the erythrocyte sedimentation rate; DAS28-CRP = DAS28 using the C-reactive protein level; RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; MTX = methotrexate; DMARDs = disease-modifying antirheumatic drugs; TNF = tumor necrosis factor.
† The mean value in the 2 placebo-treated groups combined was 4.8 units per year.
‡ The mean value in the 2 placebo-treated groups combined was 68.3%.
§ One patient was randomized but died before receiving medication.

with no progression) were analyzed using normal approximation to the binomial.

The HAQ DI score was expressed as change from baseline. The analysis was performed using a mixed-effects repeated-measures model that included the fixed effects of treatment, visit, treatment-by-visit interaction, and baseline; patients were a random effect. Secondary end points that were binary variables were analyzed by NRI; last observation carried forward analysis was performed to support robustness of results. Continuous end points followed the analysis described for HAQ DI score; values were set to "missing" for months 3–6 for patients who advanced at month 3. Supplementary efficacy analyses were performed to verify the robustness of the primary results (see Supplementary Appendix 3, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract). Safety data were summarized.

To control the Type I error rate in the primary analyses, coprimary efficacy end points were assessed sequentially using a step-down approach in the following order: ACR20 response rates, mean change in total SHS, mean change in HAQ DI score, and rates of DAS28-ESR <2.6. For each end point, and for each dose group, the comparison with placebo was conducted using a significance (alpha) level set at 0.05 (2 sided) or equivalently 0.025 (1 sided); *P* values were significant based on the step-down procedure (see Supplementary Figure 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract). For key secondary end points, *P* values are presented with no adjustment for multiple comparisons, with their nominal values. For all analyses up to and including month 6, placebo sequences are pooled as 1 group, while for any analysis post–month 6, each placebo sequence is presented separately.

RESULTS

Patient disposition and demographics. Overall, 797 patients were randomized and treated (tofacitinib at 5 mg twice daily, n = 321; tofacitinib at 10 mg twice daily, n = 316; placebo to tofacitinib at 5 mg twice daily, n = 81; placebo to tofacitinib at 10 mg twice daily, n = 79). At month 3, 42 (51.9% of the placebo to 5 mg tofacitinib group) and 37 (46.8% of the placebo to 10 mg

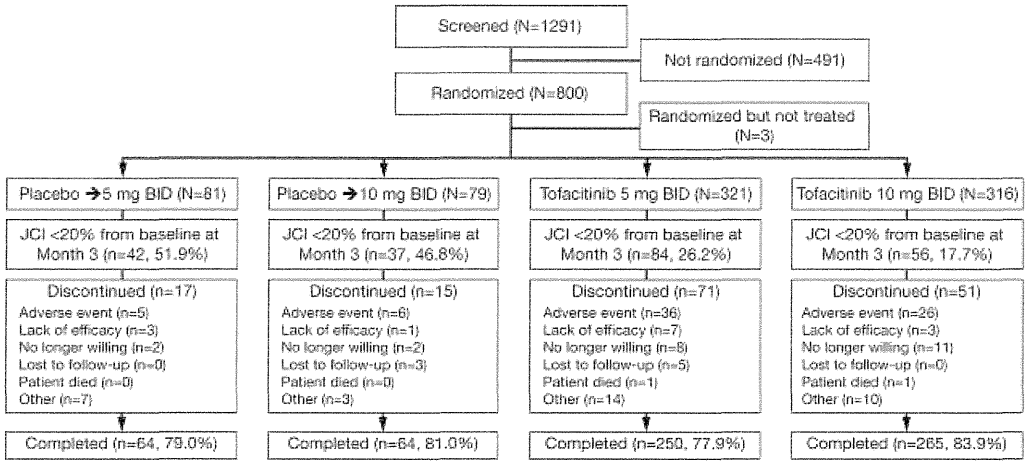

Figure 1. Disposition of the study patients. Patients in the treatment arms were randomized to receive tofacitinib starting at month 0 at either 5 mg twice daily (BID) or 10 mg twice daily. Placebo-treated patients were randomized to receive placebo during months 0–6 and then tofacitinib at either 5 mg twice daily or 10 mg twice daily during months 6–12. All placebo-treated patients who had not achieved 20% improvement in swollen and tender joint counts after 3 months were advanced in a blinded manner to receive tofacitinib at 5 or 10 mg twice daily. Completed patients were those still receiving study treatment at the date of cutoff (April 1, 2011). JCI = (swollen and tender) joint count improvement.

tofacitinib group) nonresponder placebo-treated patients advanced to tofacitinib; 84 patients (26.2%) and 56 patients (17.7%) randomized to tofacitinib 5 mg and 10 mg twice daily, respectively, were also nonresponders at month 3. Baseline demographics (Table 1) and rates of discontinuation of study treatment (Figure 1) were similar across groups. The mean age of the patients was 53 years, the mean duration of RA was 9.0 years, 53.8% of the patients were nonwhite, and 85.2% were female. At baseline, the mean total SHS ranged from 30.1 to 37.3, and average annual radiographic progression rates were similar across groups (Table 1). The proportions of patients with an erosion score ≥3 at baseline were 68.3%, 60.1%, and 65.4% in the placebo-treated, 5 mg tofacitinib–treated, and 10 mg tofacitinib–treated groups, respectively.

Efficacy. *Coprimary efficacy end points.* The ACR20 response rates at month 6 for patients receiving tofacitinib 5 mg and 10 mg twice daily were 51.5% and 61.8%, respectively, versus 25.3% for patients receiving placebo ($P < 0.0001$ for both comparisons). The least squares mean (LSM) changes in total SHS at month 6 were 0.12 and 0.06 for patients receiving tofacitinib 5 mg and 10 mg twice daily, respectively, versus 0.47 for patients receiving placebo ($P = 0.0792$ [not significant] and $P \leq 0.05$, respectively). Since tofacitinib at 5 mg twice daily failed to be statistically significant for radiographic progression, and due to the step-down procedure applied to primary efficacy end points, significance was not declared for the HAQ DI score or DAS28-ESR <2.6 for tofacitinib at 5 mg twice daily. LSM changes in the HAQ DI score at month 3 for tofacitinib at 5 mg and 10 mg twice daily were −0.40 and −0.54, respectively, versus −0.15 for placebo (5 mg twice daily, significance not declared for this coprimary end point; 10 mg twice daily, $P < 0.0001$). Rates of remission as defined by DAS28-ESR <2.6 at month 6 were 7.2% and 16.0% for tofacitinib at 5 mg and 10 mg twice daily, respectively, versus 1.6% for placebo (5 mg twice daily, significance not declared for this coprimary end point; 10 mg twice daily, $P < 0.0001$).

Signs and symptoms. Statistically significant improvements with tofacitinib were seen in ACR50 (32.4% for 5 mg twice daily, 43.7% for 10 mg twice daily, 8.4% for placebo [$P < 0.0001$ for both]) and ACR70 (14.6% for 5 mg twice daily, 22.3% for 10 mg twice daily, 1.3% for placebo [$P < 0.0001$ for both]) responses versus placebo at month 6. At month 12, ACR20, ACR50, and ACR70 response rates were 48.5%, 32.7%, and 18.8%, respectively, for tofacitinib at 5 mg twice daily and 57.0%, 41.1%, and 27.5%, respectively, for tofacitinib at 10 mg twice daily. A significant improvement in ACR20/50/70 responses for each tofacitinib dose versus placebo was seen by month 1 (first visit postbaseline). ACR response data are presented in Figures 2A and B. Changes from baseline in the ACR core set of disease activity measures (at month 6) are presented in Supplementary Table 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract. Significant effects on the rate

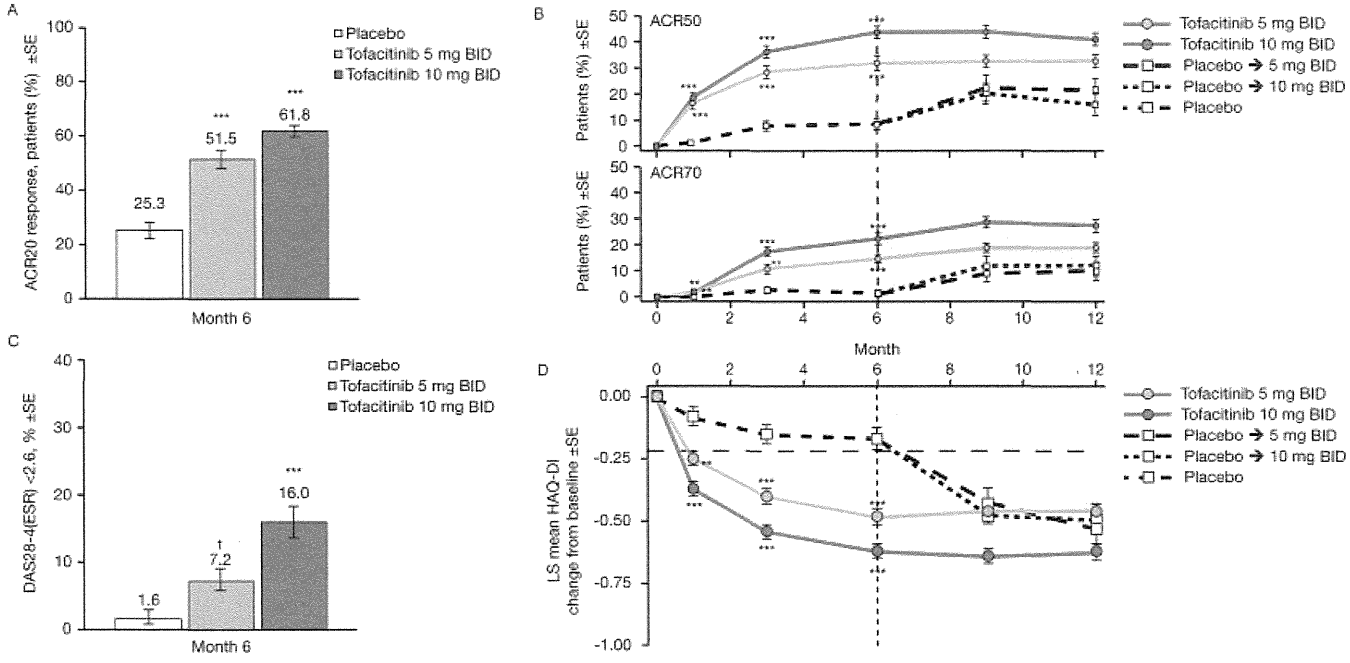

Figure 2. **A,** Response rates according to the American College of Rheumatology 20% improvement criteria (ACR20 response rates) at month 6. **B,** ACR50 and ACR70 response rates over time. **C,** Percentages of patients scoring <2.6 at month 6 on the 4-variable Disease Activity Score in 28 joints using the erythrocyte sedimentation rate (DAS28-ESR). **D,** Least squares (LS) mean changes over time in Health Assessment Questionnaire disability index (HAQ DI) scores. The dashed horizontal line represents the minimal clinically important difference of –0.22 for the HAQ DI score. Values are the mean ± SEM. *P* values are presented for analyses up to and including month 6 (the time at which all the patients in the placebo group were switched to tofacitinib), where placebo sequences are pooled as 1 group. *P* values over time are from secondary analyses where there is no adjustment for multiple comparisons; at month 3, *P* values shown are not subject to the step-down approach for the coprimary efficacy end points. ** = $P < 0.01$; *** = $P < 0.001$ versus placebo. † = significance not declared. BID = twice daily. See Figure 1 for description of groups.

of ACR20 response with tofacitinib as compared with placebo were seen in all geographic regions at month 6 ($P = 0.024$ and $P = 0.0002$ for the comparison of 5 mg and 10 mg twice daily versus placebo, respectively, in the US; $P = 0.0145$ and $P = 0.0113$, respectively, in South America; $P = 0.0045$ and $P = 0.0021$, respectively, in Europe; $P = 0.0005$ and $P < 0.0001$, respectively, in the rest of the world).

Rates of DAS28-ESR <2.6 reached 10.6% and 15.2% in the groups receiving tofacitinib at 5 mg and 10 mg twice daily, respectively, by month 12. By month 6, low disease activity (DAS28-ESR ≤3.2) was achieved by 14.3% and 28.4% of patients receiving tofacitinib at 5 mg and 10 mg twice daily, respectively, versus 3.1% of patients receiving placebo ($P < 0.0001$ for both comparisons). At month 12, the rates of DAS28-ESR ≤3.2 for patients receiving tofacitinib at 5 mg and 10 mg twice daily increased to 23.4% and 30.7%, respectively. At month 6, LSM changes from baseline in DAS28-ESR were significant for tofacitinib at both 5 mg twice daily (–2.1) and 10 mg twice daily (–2.5) versus placebo (–1.3) ($P < 0.0001$ for both comparisons); at month 12, these values were –2.3 and –2.5 for tofacitinib at 5 mg and 10 mg twice daily, respectively. Data for selected DAS28-ESR measurements are presented in Figure 2C and in Supplementary Figure 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract.

Physical function and other patient-reported outcomes. Changes from baseline in HAQ DI scores over time are presented in Figure 2D. At month 6, the LSM changes from baseline in FACIT-F for tofacitinib at 5 mg and 10 mg twice daily were 5.6 and 6.9, respectively, versus 2.1 for placebo ($P < 0.001$ and $P < 0.0001$, respectively). Significant improvements in patient's assessment of arthritis pain were also reported for tofacitinib versus placebo at month 6 (see Supplementary Table 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract).

Structural preservation. At baseline, radiographs were available for 98.7% of patients across treatment

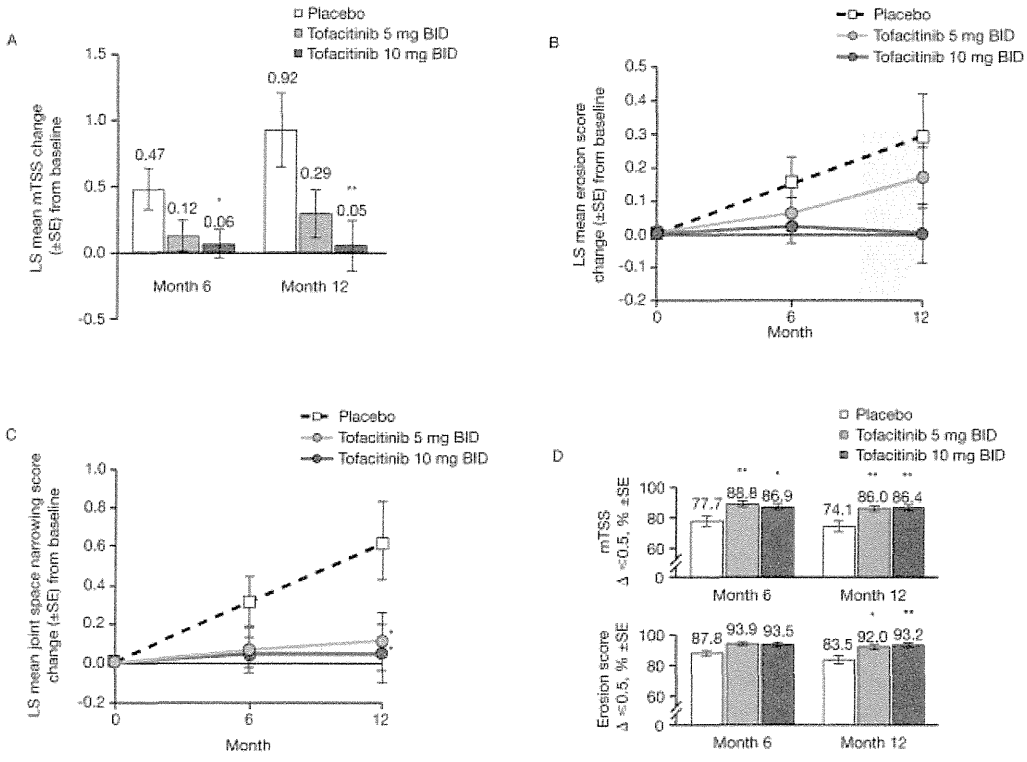

Figure 3. **A–C,** Least squares (LS) mean changes in total modified Sharp/van der Heijde score (total SHS; mTSS) at months 6 and 12 (**A**) and in erosion score (**B**) and joint space narrowing score (**C**) over time. **D,** Proportions of nonprogressors (those with changes from baseline of ≤0.5 in total SHS or erosion score) at months 6 and 12. Values are the mean ± SEM. * = $P \leq 0.05$; ** = $P < 0.01$ versus placebo. BID = twice daily.

sequences. The difference from placebo in mean changes from baseline in total SHS at month 12 was statistically significant for tofacitinib at 10 mg twice daily ($P < 0.01$) but not at 5 mg twice daily ($P = 0.0558$). Treatment with both tofacitinib doses resulted in less progression from baseline in both components of the total SHS (erosion score and JSN score) versus placebo at months 6 and 12; changes in these scores were statistically significant at month 12 for JSN, but not for erosion, for both tofacitinib-treated groups versus the placebo-treated group ($P \leq 0.05$). Mean changes from baseline in total SHS, erosion score, and JSN score are presented in Figures 3A–C.

The proportion of patients with no radiographic progression (≤0.5 unit increase from baseline in total SHS) at months 6 and 12 was similar in both tofacitinib-treated groups and significantly greater than in the placebo-treated group (both $P \leq 0.05$). At month 6, the proportion of patients with no progression in erosion score (≤0.5 unit increase from baseline) was numerically greater, but not statistically significantly different, in the tofacitinib-treated groups versus the placebo-treated group ($P > 0.05$) (Figure 3D). The proportion of patients with no progression in erosion score at month 12 was significantly greater in both tofacitinib-treated groups versus the placebo-treated group ($P \leq 0.05$) (Figure 3D).

Changes from baseline in total SHS, JSN score, and erosion score were computed for each patient, and individual values were arranged in cumulative probability plots to show the distribution of changes for the population as a whole. The plots of changes from baseline in total SHS, JSN score, and erosion score at months 6 and 12 for both tofacitinib-treated groups were very similar and were different from the plot for the placebo-treated group. Cumulative probability plots for total SHS at months 6 and 12 are presented in Figure 4.

In post hoc analyses of subsets of patients with prognostic factors predictive of greater progression of joint damage (20,21) (anti-CCP positivity, 4-variable DAS28-ESR >5.1, anti-CCP positivity and/or RF positivity with erosion score ≥3, and baseline total SHS greater than baseline median total SHS), more pronounced effects were observed for tofacitinib at 5 mg

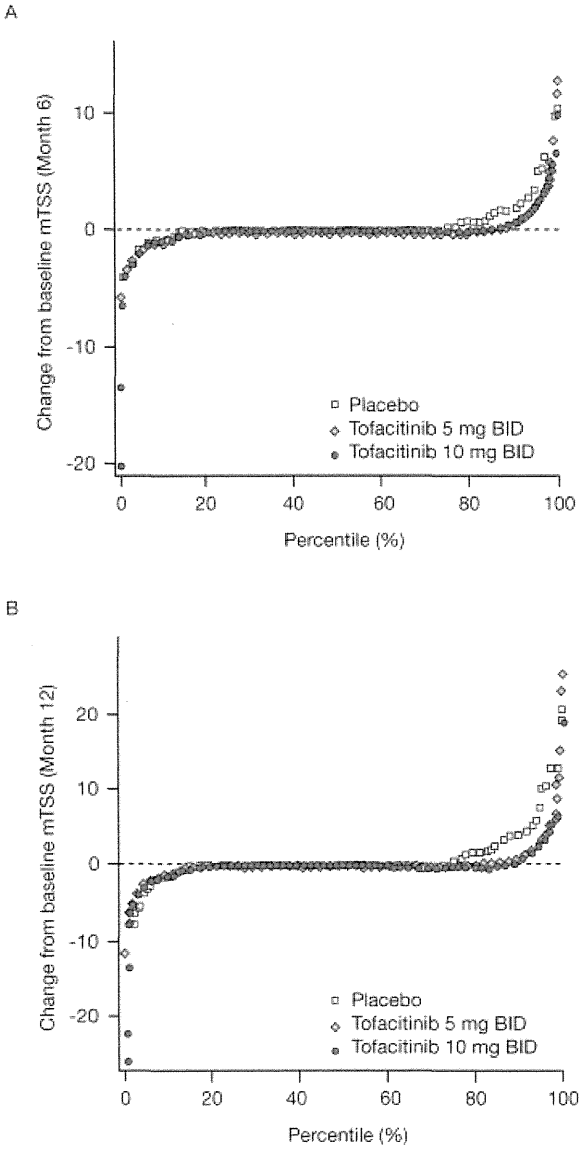

Figure 4. Cumulative probability plots showing change from baseline in total modified Sharp/van der Heijde score (total SHS; mTSS) at months 6 (**A**) and 12 (**B**). BID = twice daily.

and 10 mg twice daily, with greater differences from placebo (see Supplementary Figure 3, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract).

Supplementary analyses. Sensitivity analyses, including multiple imputation/generalized estimating equation analyses for ACR and 4-variable DAS28-ESR <2.6 response rates and a random coefficients model for total SHS, confirmed the primary analyses (see Supplementary Appendix 4, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract).

Safety and tolerability. Treatment-emergent AEs during months 0–3 were reported with similar frequency in patients treated with tofacitinib at 5 mg twice daily (157 of 321 [48.9%]), tofacitinib at 10 mg twice daily (171 of 316 [54.1%]), and placebo (73 of 160 [45.6%]). During months 3–6, treatment-emergent AEs were reported for 145 patients (45.2%) and 111 patients (35.1%) randomized to tofacitinib at 5 mg and 10 mg twice daily, respectively. In months 6–12 (when all patients randomized to placebo had advanced to active treatment), the incidence of treatment-emergent AEs was similar for tofacitinib sequences (51.7% receiving tofacitinib at 5 mg twice daily [n = 166], 55.1% receiving tofacitinib at 10 mg twice daily [n = 174]) and placebo sequences (42.0% advancing from placebo to tofacitinib at 5 mg twice daily [n = 34], 44.3% advancing from placebo to tofacitinib at 10 mg twice daily [n = 35]) (see Supplementary Table 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract).

The most frequently reported treatment-emergent AEs from months 0–12, by system organ class, were infections and infestations, gastrointestinal disorders, and abnormalities in laboratory measurements leading to investigations. Treatment-emergent AEs occurring in >2% of patients in any treatment group are summarized by Medical Dictionary for Regulatory Activities preferred terms in Supplementary Table 3, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract.

The incidence of serious AEs and discontinuations due to AEs across treatment groups was similar in each of months 0–3, 3–6, and 6–12 (see Supplementary Table 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract). Incidence rates of serious infections per 100 patient-years (95% confidence intervals [95% CIs]) through month 12 for placebo, tofacitinib at 5 mg twice daily, and tofacitinib at 10 mg twice daily were 3.68 (95% CI 0.92–14.71), 4.17 (95% CI 2.55–6.80), and 2.32 (95% CI 1.21–4.46), respectively. There were 7 opportunistic infections; doses reported were at event onset. Three were classified as serious as per the protocol (*Pneumocystis jiroveci pneumonia* [tofacitinib at 5 mg twice daily], cytomegalovirus sialadenitis [tofacitinib at 10 mg twice daily], and cytomegalovirus viremia [tofacitinib at 10 mg twice daily]) and 4 as nonserious (lymph node tuberculosis [tofacitinib at 10 mg twice daily] and esophageal candidiasis [tofacitinib at 5 mg twice daily, n = 2; tofacitinib at 10 mg twice daily, n = 1]) (see Supplemen-

tary Table 4, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract).

There were 6 deaths. Three patients receiving tofacitinib at 5 mg twice daily withdrew from the study due to AEs (acute respiratory distress syndrome and viral pneumonia, n = 1; metastatic lung cancer, n = 1; and *P jiroveci pneumonia*, n = 1) and subsequently died. One patient in the placebo-treated group withdrew due to acute renal failure before advancement to tofacitinib and then died (due to cardiac arrest and several AEs). Two patients died prior to withdrawing from the study, 1 from pneumonia (in the group receiving tofacitinib at 5 mg twice daily) and 1 from aspiration (in the group receiving tofacitinib at 10 mg twice daily). All deaths were attributed to the study treatment (including the placebo-treated patient who died) by the investigator, except for the patient who died following aspiration of a glycerine swab. Details surrounding these events are described in Supplementary Table 4, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract.

Six patients treated with tofacitinib experienced 6 nonfatal cardiovascular events that met adjudication event criteria. Three events were adjudicated as being cardiovascular: angina pectoris (in the group receiving tofacitinib at 5 mg twice daily), coronary artery disease (in the group receiving tofacitinib at 5 mg twice daily), and carotid artery stenosis (in the group receiving tofacitinib at 10 mg twice daily). Three were adjudicated as being cerebrovascular: cerebral infarction (1 in the group receiving tofacitinib at 10 mg twice daily) and lacunar infarction (2 in the group receiving tofacitinib at 10 mg twice daily [1 event occurred postrandomization but before treatment]). No patient had congestive heart failure.

Nine patients were diagnosed as having carcinomas: basal cell carcinoma (3 in the 5 mg tofacitinib–treated group, 1 in the 10 mg tofacitinib–treated group), stomach adenocarcinoma (1 in the 5 mg tofacitinib–treated group, 1 in the 10 mg tofacitinib–treated group), bone squamous cell carcinoma (1 in the 5 mg tofacitinib–treated group), breast mucinous adenocarcinoma (1 in the 10 mg tofacitinib–treated group), and non-Hodgkin's lymphoma (1 in the 10 mg tofacitinib–treated group). One patient in the 10 mg tofacitinib–treated group was diagnosed as having squamous cell carcinoma of the cervix; a biopsy sample was not available for central laboratory adjudication.

Changes in laboratory parameters observed for tofacitinib versus placebo included decreases in mean neutrophil counts, increases in mean low-density lipoprotein (LDL) cholesterol, and small increases in mean serum creatinine (Figure 5). No patient had a confirmed

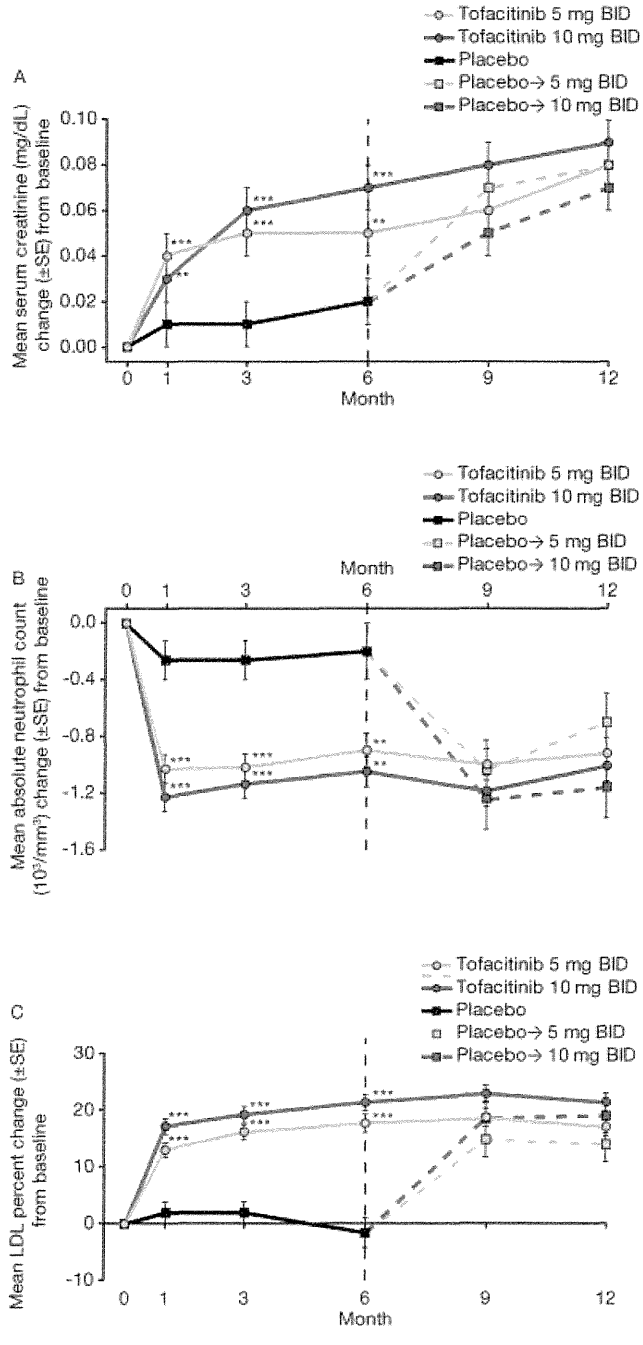

Figure 5. Mean changes from baseline in serum creatinine levels (**A**), absolute neutrophil counts (**B**), and low-density lipoprotein (LDL) cholesterol levels (**C**) over time. Values are the mean ± SEM. *P* values are presented for analyses up to and including month 6 (the time at which all the patients in the placebo group were switched to tofacitinib), where placebo sequences are pooled as 1 group. ** = $P < 0.01$; *** = $P < 0.001$ versus placebo. BID = twice daily. See Figure 1 for description of groups.

absolute neutrophil count $<0.5 \times 10^3/mm^3$ (see Supplementary Table 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract), and no patient withdrew due to leukopenia. Over the 12-month period, increases in serum creatinine >50% from baseline were observed in 5 patients: 1 in the 5 mg tofacitinib–treated group (<1.0%), 3 in the 10 mg tofacitinib–treated group (<1.0%), and 1 in the placebo to 5 mg tofacitinib–treated group after advancement to tofacitinib (1.2%); elevations were attributable to variability over time. None of these patients experienced renal failure. The patient in the placebo to 5 mg tofacitinib–treated group discontinued due to confirmed (occurring at 2 consecutive visits) elevations >50% in serum creatinine; values stabilized following discontinuation.

All mean safety laboratory values stabilized after month 3. Incidences of increases in AST and ALT ≥1× ULN at month 6 were more frequent in active treatment groups. Elevations ≥3× ULN for AST and ALT were infrequent, and were generally single occurrences that spontaneously returned to normal limits without relation to time in the study; these elevations occurred across treatment sequences (see Supplementary Table 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37816/abstract), and none were accompanied by bilirubin increases ≥2× ULN.

DISCUSSION

Tofacitinib has proven efficacious clinically in recent trials for the treatment of signs and symptoms of RA and improving physical function when given as monotherapy or in combination with MTX (6,7,22) and could provide a therapeutic alternative to augment the current therapy paradigm. The purpose of this study was to examine whether tofacitinib at 5 and 10 mg twice daily has an effect on structural progression in adult patients with active RA with an inadequate response to MTX. In addition, the study was designed to provide pivotal efficacy data concerning the reduction in signs and symptoms of RA and improvement in physical function, and to provide safety data for tofacitinib at 5 and 10 mg twice daily over 24 months.

Twelve-month data from this 24-month study provide evidence of the efficacy of inhibition of structural damage with tofacitinib. Based on published literature, the placebo was estimated to have a mean increase (deterioration) from baseline of ≥1.4 units, and the observed difference between tofacitinib and placebo would be ≥0.8 units in total SHS at month 6, whereas the observed change from baseline in mean total SHS for the placebo group at month 6 was, in fact, only 0.47 units, with both tofacitinib arms showing negligible increases (0.06 and 0.12 units) from baseline. This was approximately one-fifth of that predicted from the estimated mean annual radiographic progression at baseline of 4.8 units/year, and was significantly less than the progression of radiographic joint damage expected in DMARD-inadequate responder populations based on historical data (23–25).

These findings are also consistent with the reported trend toward decreased disease progression in RA patients over time, attributable to improved treatment (26–28), which, combined with the need to minimize duration of patient exposure to placebo treatment, makes the demonstration of a structural benefit more challenging (as seen here with the nonsignificant results with tofacitinib at 5 mg twice daily). Importantly, a substantial proportion of patients in this study also had prior treatment with tumor necrosis factor inhibitors or other biologic therapies. Despite randomization, the proportion of placebo-treated patients with prior biologic treatment was lower, which potentially disfavors the observed effect of tofacitinib as patients with prior biologic treatment usually represent a population with more severe disease.

Despite the limited degree of joint damage progression observed in the entire study population, more pronounced effects were observed for tofacitinib at 5 and 10 mg twice daily in post hoc analyses of the subset of patients with poor prognostic factors. Interestingly, these subgroups at risk for greater progression of joint damage showed maintained or increased differentiation between both doses of tofacitinib and placebo treatments.

Consistent with findings in other studies (6,7), tofacitinib at 5 and 10 mg twice daily demonstrated benefits in reducing the signs and symptoms of RA and improving physical function. Patients receiving tofacitinib also demonstrated clinically meaningful improvements in levels of fatigue and pain. Improvements were significant regardless of geographic region, consistent with previous studies (7,29). Across end points, there was no consistent pattern favoring any particular region.

Frequencies of AEs, serious AEs, and serious infections were similar across sequences. There were 6 deaths and 7 opportunistic infections (of which 3 were serious AEs) occurring during the 12-month period. Treatment with tofacitinib resulted in dose-dependent mean increases in LDL cholesterol and decreases in

mean neutrophil counts versus placebo. Elevations in serum creatinine >50% from baseline were infrequent. Potentially important increases (>3× ULN) in liver enzymes were uncommon, despite background treatment with MTX. Longer-term monitoring of patients receiving tofacitinib is ongoing in long-term extension programs from randomized studies.

Overall, the results of this 12-month analysis from a 24-month phase III study confirm findings seen previously in phase II and phase III studies in patients with active RA treated with tofacitinib and, for the first time, provide evidence of the potential to inhibit progression of structural damage.

ACKNOWLEDGMENTS

The authors would like to thank the patients who were involved in this study, the A3921044 study investigators, and the study team, including Vivianne Dillon (clinical project manager) and Allison Brailey (lead programmer). We thank Steve Gilbert, who is a statistical scientist at Rho (Chapel Hill, NC), which was a paid contractor to Pfizer Inc for statistical analysis of the data. We thank Anne Marie Reid, PhD and Gary Dever, PhD of Complete Medical Communications (Macclesfield, Cheshire, UK), who provided editorial support that was funded by Pfizer Inc.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. van der Heijde had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Van der Heijde, Tanaka, Fleischmann, Keystone, Zerbini, Cardiel, Tegzová, Wyman, Gruben, Wallenstein, Krishnaswami, Zwillich, Connell.

Acquisition of data. Van der Heijde, Tanaka, Fleischmann, Keystone, Kremer, Zerbini, Cardiel, Nash, Song, Tegzová, Wallenstein, Krishnaswami, Bradley.

Analysis and interpretation of data. Van der Heijde, Fleischmann, Keystone, Kremer, Zerbini, Cardiel, Cohen, Nash, Tegzová, Wyman, Gruben, Benda, Wallenstein, Krishnaswami, Zwillich, Bradley, Connell.

ROLE OF THE STUDY SPONSOR

The authors employed by Pfizer Inc had roles in study design, data analysis, data interpretation, writing of the manuscript, and agreement to submit the manuscript for publication. All authors, including authors employed by Pfizer Inc, approved the content of the submitted manuscript. Pfizer Inc paid a contractor for statistical analysis of the data and funded editorial support provided by a contractor.

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APPENDIX A: THE ORAL SCAN (A3921044) STUDY INVESTIGATORS

The following investigators participated in the study.

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Lupus (2013) 22, 538–540
http://lup.sagepub.com

LETTER TO THE EDITOR

Persistent memory B cell down-regulation after 6-year remission induced by rituximab therapy in patients with systemic lupus erythematosus

Sir,

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease induced by autoreactive T cell activation and autoantibody overproduction by B cells. Rituximab produces B cell depletion in patients with refractory SLE.[1–8] However, the precise mechanism of rituximab-induced long-term SLE remission remains unknown.

We investigated the phenotypic changes in lymphocytes in six patients with SLE refractory to high-dose corticosteroid and immunosuppressants including cyclophosphamide (four with lupus nephritis (WHO type I:1, type IV:3) and four with neuropsychiatric (NP)-SLE (two with both lupus nephritis and NP-SLE)). They had been in remission for 6 years since receiving rituximab. Patients' numbers (#1, 2, 3, 4, 5, 7) and their clinical background were matched with those used in our previous study.[9] Dose of rituximab was 375 mg/m^2 every 2 weeks referred to therapeutic dose of lymphoma 375 mg/m^2 weekly (4–8 weeks). Written informed consent was obtained from each patient and the study was approved by the ethics committee of our university.

Figure 1A shows a representative patient with long-term remission (Patient #4). Rituximab resulted in the disappearance of peripheral blood $CD19^+IgD^+CD27^-$ naïve B cells, $CD19^+IgD^-CD27^+$ class-switched memory B cells and $CD19^+IgD^-CD27^-$ memory B cells within 4 weeks. However, recovery of naïve B cells occurred within 3–9 months and persisted for 2–6 years, whereas memory B and plasma cells remained depleted through 0.5–6 years. The disappearance of memory B and plasma cells was observed in all six patients with long-term remission of SLE. Rituximab increased $CD19^+IgD^+CD27^-$ naive B cells (mean ± SD: 42.0 ± 18.0% to 86.5 ± 5.8% ($p < 0.05$) to 87.8 ± 5.2% ($p < 0.05$)) and reduced $CD19^+IgD^-CD27^+$ class-switched memory B cells (29.4 ± 7.2% to 6.5 ± 2.6% ($p < 0.05$) to 5.0 ± 3.5% ($p < 0.05$)) and $CD19^+IgD^-CD27^-$ memory B cells (26.9 ± 15.3% to 5.9 ± 3.0% ($p < 0.05$) to 5.7 ± 3.2% ($p < 0.05$)) at 6 years after rituximab treatment. On the other hand, $CD19^+IgD^+CD27^+$ memory B cells remained low at 6 years after treatment with rituximab (1.7 ± 0.6% to 1.0 ± 0.8% to 1.6 ± 1.4%). Little is known about the origin of $CD19^+IgD^+CD27^+$ memory B cells, which are thought to be related to splenic marginal-zone B cells since they have similar phenotypic markers with different requirements for Ig receptor mutation.[10]

During the same period, SLE remained in remission and the mean dose of corticosteroid was tapered from 35.0 to 2.9 mg/day. We reported that rituximab therapy rapidly decreased $CD19^+$ cells bearing CD80,[9] but a significant reduction from baseline levels was still noted at 2 and 6 years in CD80-expressing cells among the $CD19^+$ cells (59.9 ± 30.4% to 9.8 ± 5.4% ($p < 0.05$) to 9.6 ± 2.9% ($p < 0.05$)) (Figure 1B). Furthermore, the number of $CD4^+$ T cells (207 ± 53.0 to 340 ± 139.3 ($p < 0.05$) to 380.3 ± 209.5 ($p < 0.05$) cells/μl) and $CD45RO^-$naïve T cells (72.1 ± 36.2 to 185 ± 104.9 ($p < 0.05$) to 170 ± 108.1 ($p < 0.05$) cells/μl) increased significantly (Figure 1C) and the expression of costimulatory molecules CD40L (9.9 ± 7.8% to 2.1 ± 0.9% ($p < 0.05$) to 2.6 ± 0.7% ($p < 0.05$)) and ICOS (9.6 ± 4.9% to 2.7 ± 1.8% ($p < 0.05$) to 3.3 ± 1.6% ($p < 0.05$)) on $CD4^+$ cells remained down-regulated at 2 and 6 years in the six rituximab-treated patients (Figure 1D).

The above results indicate that rituximab induced characteristic phenotypic changes within 6 years in patients with remission: decrease in memory B cells, plasma cells, and CD80-positive B cells, increase in CD4+ naïve, and decrease in CD40L and ICOS on CD4+ T cells. Taken together, we suggest that activated T cells, in addition to activated B cells, seem to be involved in the pathogenesis of SLE and that activated B–T cell interaction may worsen the pathophysiology of SLE. B cell recovery following rituximab treatment in SLE is associated with a delay in peripheral blood memory B cell recovery that correlates with a reconstitution dominated by an expansion of naive B cells. The severely delayed maturation and/or expansion of memory B cells might, therefore, lead to the inhibition of T cell activation and

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Received 2 December 2012; accepted 12 January 2013

10.1177/0961203313477899

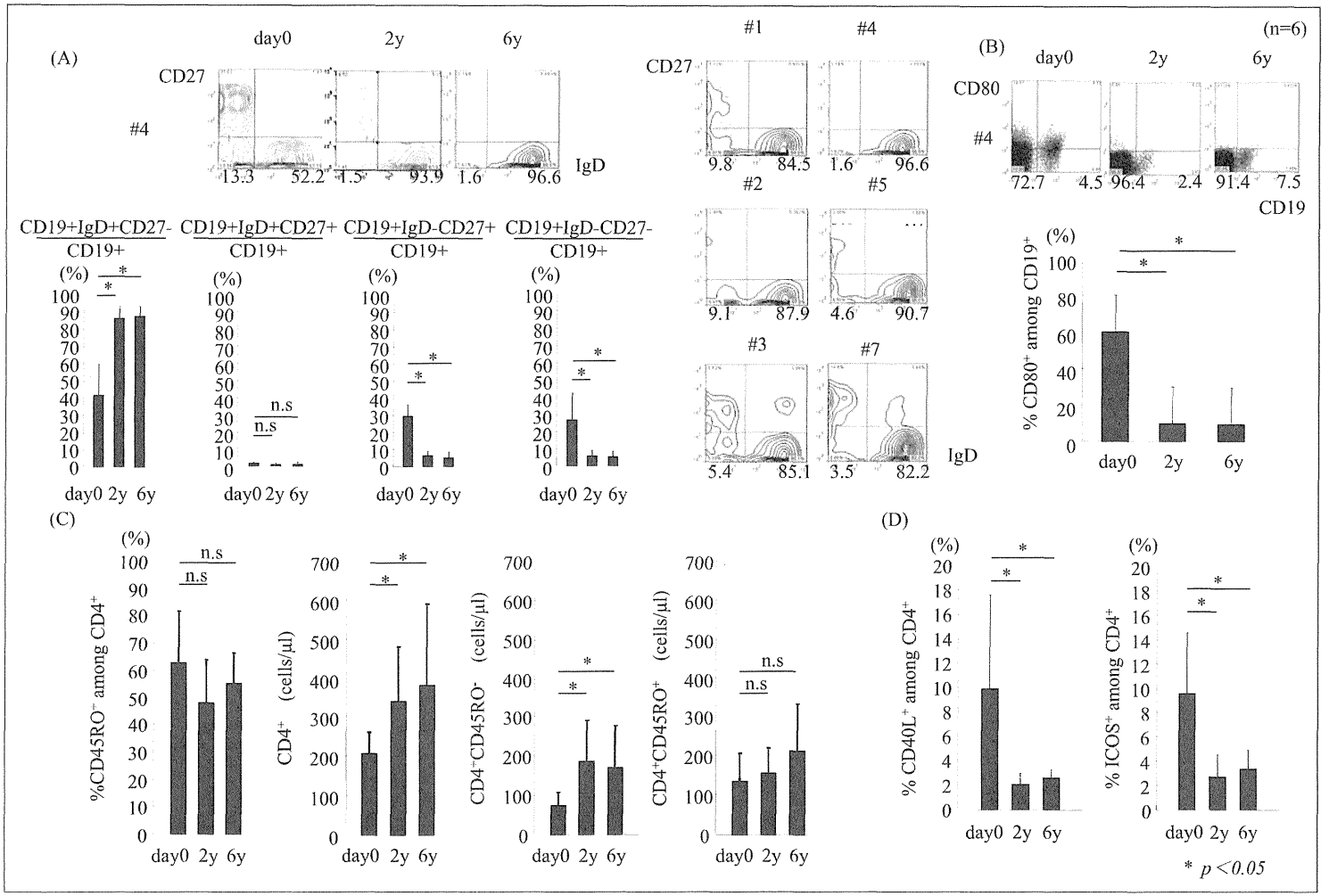

Figure 1 Changes in subsets and expression of costimulatory molecules on $CD19^+$and $CD4^+$cells in patients with SLE and prolonged remission at 6 years after treatment with rituximab therapy. (A) *Left top:* subsets on $CD19^+$cells immediately before and 2 and 6 years after rituximab treatment in Patient 4 (a representative patient following a typical course). Peripheral blood mononuclear cells were gated on $CD19^+$ cells and further separated with IgD and CD27. *Left upper quadrant:* plasma cells ($CD27^{++}$) and class-switched memory B cells ($CD27^+$). *Right upper quadrant:* IgM memory B cells. *Left lower quadrant:* double-negative memory B cells. *Right lower quadrant:* naïve B cells. *Left bottom:* changes in the percentage of each subset of $CD19^+$ cells in six patients with long-term remission. *Right:* $CD19^+$ cell subset in these six patients at 6 years. (B) *Top:* expression of costimulatory molecule CD80 on CD19-positive cells in the same patient. *Bottom:* changes in percentage of $CD80^+$ cells among $CD19^+$ cell population in the six patients with long-term remission. (C) Longitudinal changes in percentages of $CD45RO^+$ cells among CD4+ cells, absolute number of $CD4^+$ cells, absolute number of $CD4^+CD45RA^+$ cells, and absolute number of $CD4^+CD45RO^+$ cells. (D) Changes in percentages of $CD40L^+$ cells and $ICOS^+$ cells among $CD4^+$ cell population in the six patients with prolonged remission. Data are mean ± SD of six patients.

differentiation mediated by memory B cells through costimulatory molecules. Thus, the reconstitution of peripheral B cells and the possible inactivation of T cells may result in the long-term remission of the disease after the treatment with rituximab in patients with SLE.

Funding

This work was supported in part by a Research Grant-In-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the University of Occupational and Environmental Health, Japan.

Conflict of interest

Dr. Tanaka has received consulting fees, lecture fees, and/or honoraria from Mitsubishi-Tanabe Pharma, Chugai Pharma, Eisai Pharma, Pfizer, Abbott Immunology Pharma, Daiichi-Sankyo, Janssen Pharma, Astra-Zeneca, Takeda Industrial Pharma, Astellas Pharma, Asahi-kasei Pharma and GlaxoSmithKline and has received research grant support from Mitsubishi-Tanabe Pharma, Bristol-Myers Squibb, Takeda Industrial Pharma, MSD, Astellas Pharma, Eisai Pharma, Chugai Pharma, Pfizer and Daiichi-Sankyo. All other authors declare no conflict of interest.

Acknowledgments

The authors thank Ms. T. Adachi, Ms. N. Sakaguchi, and Ms. K. Noda for the excellent technical assistance.

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Diabetologia (2013) 56:1773–1780
DOI 10.1007/s00125-013-2935-8

ARTICLE

Double deficiency in IL-17 and IFN-γ signalling significantly suppresses the development of diabetes in the NOD mouse

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Received: 6 December 2012 / Accepted: 22 April 2013 / Published online: 23 May 2013

Abstract
Aims/hypothesis T helper type (Th) 17 cells have been shown to play important roles in mouse models of several autoimmune diseases that have been classified as Th1 diseases. In the NOD mouse, the relevance of Th1 and Th17 is controversial, because single-cytokine-deficient NOD mice develop diabetes similarly to wild-type NOD mice.
Methods We studied the impact of IL-17/IFN-γ receptor double deficiency in NOD mice on the development of insulitis/diabetes compared with IL-17 single-deficient mice and wild-type mice by monitoring diabetes-related phenotypes. The lymphocyte phenotypes were determined by flow cytometric analysis.
Results IL-17 single-deficient NOD mice showed delayed onset of diabetes and reduced severity of insulitis, but the cumulative incidence of longstanding diabetes in the IL-17-deficient mice was similar to that in wild-type mice. The IL-17/IFN-γ receptor double-deficient NOD mice showed an apparent decline in longstanding diabetes onset, but not in insulitis compared with that in the IL-17 single-deficient mice. We also found that double-deficient NOD mice had a severe lymphopenic phenotype and preferential increase in regulatory T cells among $CD4^+$ T cells compared with the IL-17 single-deficient mice and wild-type NOD mice. An adoptive transfer study with $CD4^+CD25^-$ T cells from young non-diabetic IL-17 single-deficient NOD mice, but not those from older mice, showed significantly delayed disease onset in immune-deficient hosts compared with the corresponding wild-type mice.
Conclusions/interpretation These results indicate that IL-17/Th17 participates in the development of insulitis and that both IL-17 and IFN-γ signalling may synergistically contribute to the development of diabetes in NOD mice.

Keywords IFN-γ · IL-17 · Lymphopenia · NOD mice · Th17 · Type 1 diabetes

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Electronic supplementary material The online version of this article (doi:10.1007/s00125-013-2935-8) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Abbreviations

CFA	Complete Freund's adjuvant
Cy	Cyanine
IAA	Insulin autoantibody
IFN-γR	IFN-γ receptor
PE	Phycoerythrin
PMA	Phorbol 12-myristate 13-acetate
SCID	Severe combined immunodeficiency
SPC	Splenocyte
TCR-Tg	T cell receptor transgenic
Teff	Effector T cell
Th	T helper type
Treg	Regulatory T cell
wt	Wild-type

Introduction

Type 1 diabetes results from the autoimmune destruction of pancreatic beta cells mediated by $CD4^+$ and $CD8^+$ T cells [1]. T helper type (Th) 1 cells are believed to play a key role in the pathogenesis of type 1 diabetes in the NOD mouse model of diabetes, because pancreatic islet-infiltrating mononuclear cells and diabetogenic T cell clones derived from NOD islets show strong expression of Th1 cytokines [2–5]. However, an unresolved issue is why the elimination of Th1 cytokines or their signalling molecules, including IFN-γ, IL-12 and IFN-γ receptor (IFN-γR), does not reduce the incidence of diabetes in NOD mice [6–10].

Th17 cells that produce IL-17 have been shown to play important roles in mouse models of several autoimmune diseases including experimental autoimmune encephalomyelitis, rheumatoid arthritis and autoimmune thyroiditis and others that had previously been thought to be Th1-dominant [11–16]. In the context of NOD mice, there are data suggesting that Th17 cells play a pathogenic role in type 1 diabetes development [17]. The administration of GAD peptide inserted into immunoglobulin molecules inhibited diabetes development, dependent on the induction of splenic IFN-γ which inhibited IL-17 production [18], and treatment with neutralising antibodies to IL-17 prevented the development of diabetes in NOD mice [19]. However, similarly to IFN-γ knockout in NOD mice, an IL-17 knockdown NOD line created by directly introducing a short hairpin RNA construct did not show altered diabetes susceptibility [20].

The differentiation of naive $CD4^+$ T cells to effector T cells (Teffs) or regulatory T cells (Tregs) is more plastic than previously thought [21]. Th17 cells have been shown to be converted into Th1 cells in a transfer model of colitis [22]. In type 1 diabetes, diabetogenic BDC2.5 $CD4^+$ T cells polarised in vitro into the Th17 cell phenotype are converted into Th1-like cells after adoptive transfer into NOD/severe combined immunodeficiency (SCID) mice, ultimately causing beta cell destruction and diabetes [23, 24]. Thus, it is possible that the conversion of Th17 cells into Th1 cells or Th1/Th17 cells coexpressing Th1 and Th17 cytokines in the pancreatic islets might counteract the disease inhibition by eliminating a single cytokine gene from the NOD mice.

To address this issue we produced NOD mice genetically deficient in both IL-17 and IFN-γR, and we evaluated insulitis/diabetes development in comparison with that in IL-17 single-deficient NOD background mice.

Methods

Mice NOD and NOD/SCID mice were purchased from Clea Japan (Tokyo, Japan). IL-17-deficient NOD mice were generated as described (originally on a 129/Sv×C57BL/6 genetic background) [25]. IFN-γR-deficient NOD mice were obtained from O. Kanagawa (Laboratory for Autoimmune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). All animal experiments described in this study were conducted with the approval of the institutional animal experimentation committee in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

Establishment of IL-17 single-deficient and IL-17/IFN-γR double-deficient NOD mice IL-17-deficient mice were backcrossed with NOD mice for eight successive generations. An analysis of the microsatellite markers of the diabetes susceptibility (*Idd1–14*) loci by PCR of the tail DNA as described [26] showed that the mice were homozygous for all of the NOD alleles (namely, $Il17^{-/-}$ NOD mice). IFN-γR-deficient NOD mice were crossed with $Il17^{-/-}$ NOD mice, and the resulting F1 hybrids, $Il17^{+/-}/Ifngr1^{+/-}$ NOD mice, were intercrossed to produce IL-17 single-deficient ($Il17^{-/-}/Ifngr1^{+/+}$), IL-17/ IFN-γR double-deficient ($Il17^{-/-}/Ifngr1^{-/-}$) and wild-type (wt; $Il17^{+/+}/Ifngr1^{+/+}$) NOD littermate mice. Only female mice were used for the present study. These mice were selected by PCR analysis of tail DNA as described [25, 26]. Tail DNA was extracted with the REDExtract-N-Amp Tissue PCR kit (Sigma, St Louis, MO, USA).

Monitoring for spontaneous diabetes Blood glucose levels were monitored using the One-touch Ultra (Johnson & Johnson, Tokyo, Japan). Mice with blood glucose levels >13.9 mmol/l in two consecutive measurements were considered diabetic.

Measurement of insulin autoantibodies Mice were bled at 8, 12 and 16 weeks of age, and serum samples were obtained and stored at −20°C until the antibody assay. The levels of insulin autoantibodies (IAAs) were evaluated by a 96-well filtration plate micro-IAA assay, as described [27]. The index value of 0.01 was selected as

the cut-off limit at the 100th percentile of 50 Balb/c and C57BL/6 mouse samples.

Histology Pancreatic sections were histologically analysed by fixing the tissue specimens in 10% formalin and staining the paraffin-embedded samples with haematoxylin and eosin. A minimum of 30 islets from each mouse were examined microscopically by two different observers for the presence of insulitis. The severity of insulitis was scored as follows: 0, no lymphocytic infiltration; 1, lymphocytic infiltration occupying <25% of the total islet cell area; 2, lymphocytic infiltration occupying 25–49% of the total islet cell area; 3, lymphocytic infiltration occupying 50–75% of the total islet cell area; 4, lymphocytic infiltration occupying >75% of the total islet cell area, or small retracted islets.

Adoptive transfer experiments Donor $CD4^+CD25^-$ T cells were purified from the spleens of 10- or 18-week-old prediabetic mice, and $CD4^+$ T cells were purified from 15- to 22-week-old newly diabetic mice, using magnetic bead cell sorting (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purified $CD4^+CD25^-$ T cells or $CD4^+$ T cells were adoptively transferred into 8- to 10-week-old NOD/SCID mice, and the recipient mice were monitored for blood glucose twice weekly after the adoptive transfer.

Flow cytometric analysis Single cell suspensions of splenocytes (SPCs) were prepared from spleens of NOD mice at 10 weeks of age. Red cells were lysed in ammonium chloride buffer. For surface staining, cells were stained for 20 min with the corresponding fluorescently labelled antibodies against surface molecules: CD3e (145-2C11), CD4 (GK1.5), CD8 (53–6.7), B220 (RA3-6B2), CD44 (IM7), CD62L (MEL-14) (all from eBioscience, San Diego, CA, USA). For the intracellular cytokine staining, the prepared SPCs were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both from Sigma) in the presence of 2 μmol/l monensin for 5 h. Thereafter, the cells were stained with allophycocyanin-cyanine (Cy)5-conjugated anti-CD4, followed by intracellular IFN-γ and IL-17 staining with phycoerythrin (PE)-Cy7-conjugated anti-IL-17 (eBio17B7) and peridinin chlorophyll protein complex-Cy5.5-conjugated anti-INF-γ (XMG1.2) antibodies (all from eBioscience). Alternatively, the cells were resuspended with PBS and stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 (PC61) (BD Biosciences, San Diego, CA, USA), followed by intracellular Foxp3 staining with PE-Cy5-conjugated anti-FoxP3 (FJK-16 s; Foxp3 staining kit; eBioscience). All cells were analysed on a FACSCanto II flow cytometry system using FACS Diva software (BD Biosciences).

Statistical analysis Group differences were analysed by Mann–Whitney *U* test or Student's *t* test, and differences between the Kaplan–Meier survival curves were estimated by the logrank test using SPSS Version 11.0 J (Chicago, IL, USA). The χ^2 test was used to compare the incidence of diabetes at each week of age. *p* values <0.05 were considered significant. The severity of the insulitis was analysed by a ridit analysis, and *t* levels higher than 1.96 or lower than −1.96 were considered significant.

Results

Diabetes and insulitis in the IL-17-deficient and IL-17/IFN-γR-deficient NOD mice In our colony, ~75% of the female and 30–40% of the male NOD mice usually develop diabetes by 48 weeks. A life-table analysis revealed that the onset of spontaneous diabetes in the $Il17^{-/-}/Ifngr1^{+/+}$ NOD mice was significantly delayed compared with the onset in wt NOD littermate mice ($p<0.05$ by the logrank test). The weekly incidence of diabetes in the $Il17^{-/-}/Ifngr1^{+/+}$ NOD mice was also significantly lower from 15 to 24 weeks of age than that in wt NOD mice ($p<0.05$ by χ^2 test). However, the cumulative incidence of diabetes at 50 weeks of age in the $Il17^{-/-}/Ifngr1^{+/+}$ NOD mice was similar to that in the wt mice (80.0% vs 85.7%, respectively) (Fig. 1a). As for the IL-17/IFN-γR double-deficient NOD mice, the onset of diabetes was significantly suppressed compared with that in the $Il17^{-/-}/Ifngr1^{+/+}$ NOD mice and wt NOD mice ($Il17^{-/-}/Ifngr1^{-/-}$ vs $Il17^{-/-}/Ifngr1^{+/+}$ and vs wt, $p=0.01$ by the logrank test). The weekly incidence of diabetes in the $Il17^{-/-}/Ifngr1^{-/-}$ NOD mice was significantly lower from 26 to 50 weeks of age compared with that in the $Il17^{-/-}/Ifngr1^{+/+}$ NOD mice ($p<0.05$ by χ^2 test) and from 16 until 50 weeks of age compared with that in wt NOD mice ($p<0.05$ by χ^2 test). The cumulative incidence of diabetes at 50 weeks of age in the $Il17^{-/-}/Ifngr1^{-/-}$ NOD mice was 43.8%, and disease suppression was maintained throughout the entire lifespan (Fig. 1a).

We next compared the severity of insulitis at 12 and 18 weeks of age in the $Il17^{-/-}/Ifngr1^{-/-}$, $Il17^{-/-}/Ifngr1^{+/+}$ and wt NOD mice. The severity of insulitis was significantly attenuated in the $Il17^{-/-}/Ifngr1^{-/-}$ and $Il17^{-/-}/Ifngr1^{+/+}$ NOD mice compared with that in the wt mice (by ridit analysis). However, there were no significant differences between the $Il17^{-/-}/Ifngr1^{-/-}$ and $Il17^{-/-}/Ifngr1^{+/+}$ NOD mice at 12 or 18 weeks of age (Fig. 1b,c).

IAA levels in the IL-17-deficient and IL-17/IFN-γR-deficient NOD mice We then determined the levels of IAAs in the $Il17^{-/-}/Ifngr1^{-/-}$, $Il17^{-/-}/Ifngr1^{+/+}$ and wt NOD mice at 8, 12 and 16 weeks of age. Despite the suppression of insulitis/diabetes development in the $Il17^{-/-}/Ifngr1^{-/-}$ and $Il17^{-/-}/Ifngr1^{+/+}$ mice, the serum levels of IAAs and

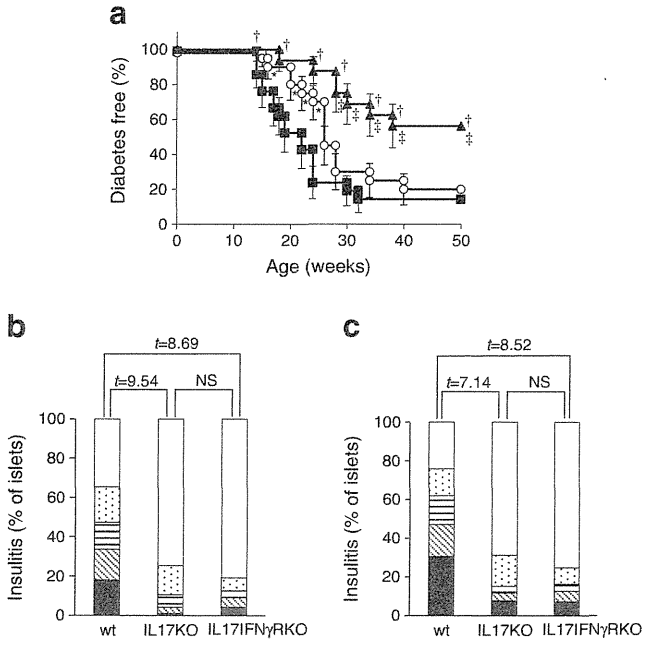

Fig. 1 Diabetes and insulitis in the IL-17-deficient and IL-17/IFN-γR-deficient NOD mice. (**a**) Incidence of diabetes in wt NOD mice (squares, $n=21$), *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ NOD mice (circles, $n=20$) and *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$ NOD mice (triangles, $n=16$). The χ^2 test was used to compare the incidence of diabetes at each week of age (*$p<0.05$, *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ vs wt; †$p<0.05$, *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$ vs wt; ‡$p<0.05$, *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ vs *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$). The severity of insulitis in 12-week-old (**b**) or 18-week-old (**c**) wt NOD mice ($n=5$), *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ NOD mice ($n=5$) and *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$ NOD mice ($n=5$). KO, knockout. The severity of insulitis was scored as described in the Methods section. Levels of insulitis: 0 (white), 1 (dotted), 2 (horizontal stripes), 3 (diagonal stripes) and 4 (black). A *t* ridit analysis was used, and *t* levels of higher than 1.96 or lower than −1.96 were considered significant

percentage of mice positive for IAAs did not significantly differ between the *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$ and wt NOD mice or between the *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ and wt mice, at all ages (data not shown).

Adoptive transfer of the CD4$^+$ *Teffs from Il17*$^{-/-}$ *NOD mice into NOD/SCID mice* Our aforementioned data suggest that IL-17 may play an important role in the development of insulitis. However, it is possible that IL-17 is associated with diabetes development only in younger NOD mice and not in older mice, since disease inhibition by IL-17 single deficiency was not maintained past 24 weeks of age. We therefore compared the diabetogenicity of CD4$^+$ Teffs from *Il17*$^{-/-}$ and wt NOD mice at different ages to adoptively transfer diabetes into NOD/SCID mice.

The adoptive transfer of purified CD4$^+$CD25$^-$ Teffs from 10-week-old non-diabetic *Il17*$^{-/-}$ mice resulted in significantly delayed disease onset compared with transfer from the corresponding wt NOD mice (Fig. 2a). No significant differences in disease development were seen in NOD/SCID mice after adoptive transfer with CD4$^+$CD25$^-$ T cells from 18-week-old non-diabetic *Il17*$^{-/-}$ or wt NOD mice (Fig. 2b), or with CD4$^+$ T cells from 15- to 22-week-old newly diabetic *Il17*$^{-/-}$ or wt NOD mice (Fig. 2c).

Flow cytometric analysis for CD4$^+$ *T cells in the IL-17-deficient and IL-17/IFN-γR-deficient NOD mice* We first counted lymphocyte numbers and found that 10-week-old *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$ NOD mice had significantly reduced numbers of lymphocytes including CD3$^+$, CD4$^+$ and CD8$^+$

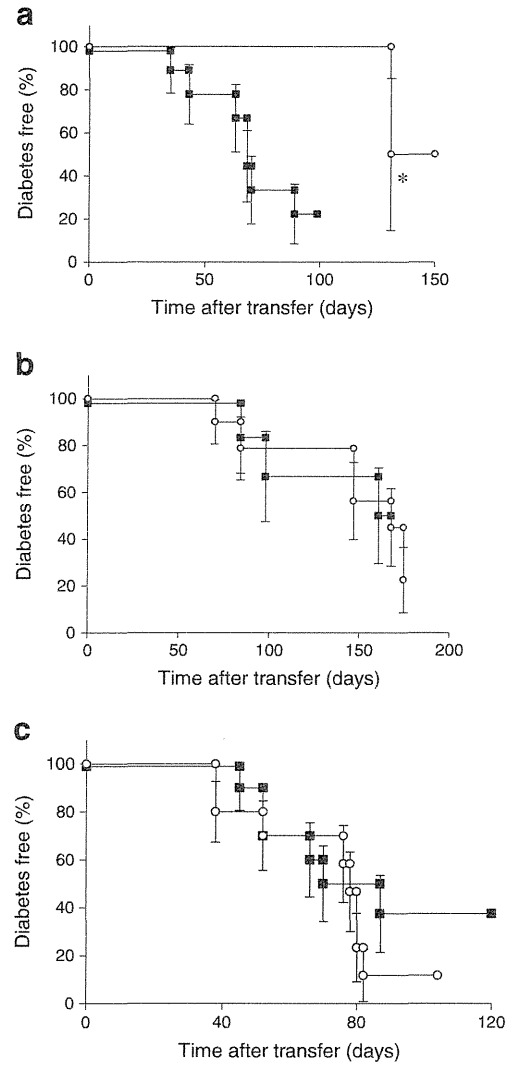

Fig. 2 Adoptive transfer of CD4$^+$ Teffs into 8- to 10-week-old NOD/SCID mice. (**a**) CD4$^+$CD25$^-$ T cells (1×10^7) from 10-week-old non-diabetic *Il17*$^{-/-}$ NOD mice (circles, $n=5$) or wt NOD mice (squares, $n=9$) were transferred into recipient NOD/SCID mice. *$p=0.03$ by the logrank test. (**b**) CD4$^+$CD25$^-$ T cells (1×10^7) from 18-week-old non-diabetic *Il17*$^{-/-}$ NOD mice (circles, $n=10$) or wt NOD mice (squares, $n=6$) were transferred into recipient NOD/SCID mice. $p=0.84$ by the logrank test. (**c**) CD4$^+$ cells (5.5×10^6) from 15- to 22-week-old freshly diabetic *Il17*$^{-/-}$ NOD mice (circles, $n=10$) or wt NOD mice (squares, $n=10$) were transferred into recipient NOD/SCID mice. $p=0.48$ by the logrank test

T cells and B cells in the spleen, and the numbers were almost half or less than half of those in the *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ or wt NOD mice. The percentages of CD4$^+$ T cells, CD8$^+$ T cells and B cells in the SPCs were not significantly different among the three groups (Table 1). As for the CD4$^+$ T cell fraction, the production of cytokines was evaluated by intracellular cytokine staining after stimulation with PMA and ionomycin for 5 h. The percentages of IFN-γ-producing cells did not significantly differ among the three groups, although the number in the *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$ mice tended to be decreased compared with the other groups (3.22±1.37% vs 4.42±0.90% in *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ mice [p=0.11] vs 4.19±0.66% in wt mice [p=0.11], respectively) (Fig. 3a–d). IL-17-producing cells among the CD4$^+$ T cells were observed only in wt mice (0.34±0.11%) as expected, and double-positive cells with IL-17 and IFN-γ were not observed in any of the groups (Fig. 3a–c,e).

We next determined the activation markers including CD44 and CD62L on CD4$^+$ T cell and Treg populations without stimulation. No significant differences were found in the level of activation markers on the CD4$^+$ T cells among the three groups (Fig. 4a–c,g). As for Tregs, the percentage of CD25$^+$Foxp3$^+$ cells to CD4$^+$ T cells was significantly higher in the *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$ mice than in the *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ mice (6.8±0.96% vs 4.7±0.54%, p<0.01) and in the wt mice (vs 5.4±0.32%, p<0.05) (Fig. 4d–f,h). The preferential increase in Tregs may be a systemic phenotype, since a higher percentage of CD25$^+$ cells to CD4$^+$ T cells was observed in mesenteric lymph nodes or pancreatic draining lymph nodes (data not shown).

Discussion

In this study, we first determined the impact of the genetic deletion of IL-17, a potent proinflammatory cytokine, in the NOD mouse to investigate whether IL-17 is involved in the pathogenesis of type 1 diabetes. Our results show that the severity of insulitis was attenuated in both the IL-17 single-deficient and IL-17/IFN-γR double-deficient NOD mice, with no significant difference between these two types of mice, indicating that IL-17 rather than IFN-γ signalling plays a key role in the build-up of the inflammatory infiltrate into islets in NOD mice, as is the case for numerous other autoimmune diseases (Fig. 1b,c) [11–16]. This result is also consistent with the finding of Martin-Orozco et al that in vitro-polarised Th17 cells derived from BDC2.5 T cell receptor transgenic (TCR-Tg) NOD mice transfer extensive insulitis, but do not produce diabetes in newborn NOD mice [23].

Regarding the development of diabetes, we found that the onset of diabetes was significantly delayed in the IL-17 single-deficient NOD mice, although they remained susceptible to longstanding diabetes, which is consistent with the report by Joseph et al [20] (Fig. 1a). In the different line of IL-17 single-deficient NOD mice (original *Il17*$^{-/-}$ NOD mice) (n=47) and the wt littermate control mice (n=44), we observed the same delayed-onset result in *Il17*$^{-/-}$ NOD mice (vs control, p<0.05 by the logrank test) (electronic supplementary material [ESM] Fig. 1).

Previous studies have demonstrated that the phenotype of delayed onset in IFN-γR-deficient NOD mice is due to the presence of 129-derived genes closely linked to the knockout gene rather than to a lack of the target gene [10, 28]. However, our mapping study with polymorphic markers on chromosome 1 distinguishing NOD from the 129 alleles showed that the maximum interval of the 129-derived genes surrounding the *Il17a* gene was less than 1 cM (ESM Fig. 2), and there are no identified insulin-dependent diabetes mellitus loci in this region, suggesting that the resistance to the development of insulitis and diabetes in the *Il17*$^{-/-}$ NOD mice is attributable to the lack of IL-17 rather than to the influence of the 129-derived genes.

The phenotype of delayed onset in the IL-17 single-deficient NOD mice indicates that IL-17 might participate in the pathogenesis of the early phase of the development of diabetes. This hypothesis was verified by our adoptive transfer study, which showed the successful adoptive transfer of diabetes by CD4$^+$CD25$^-$ Teffs from younger non-diabetic wt mice but not by those from the IL-17 single-deficient NOD

Table 1 Numbers of T cells (CD4$^+$, CD8$^+$) and B cells in the SPCs

Mice	Total cell number (×10^6)	Cell number (×10^6) (%/SPCs)			
		CD3$^+$	CD4$^+$	CD8$^+$	B220$^+$
wt (n=5)	63.4±9.7	27.0±4.8 (42.7±5.1)	18.7±3.4 (29.4±3.5)	5.8±0.9 (9.2±1.4)	16.2±1.6 (25.7±1.8)
Il17$^{-/-}$/*Ifngr1*$^{+/+}$ (n=5)	63.4±8.3	24.7±3.0 (39.2±5.2)	16.2±2.6 (25.9±4.8)	5.4±0.5 (8.6±0.9)	15.4±1.1 (24.7±3.3)
Il17$^{-/-}$/*Ifngr1*$^{-/-}$ (n=5)	26.0±12.0*	12.2±6.3** (46.1±5.6)	8.4±4.3** (32.1±4.5)	2.4±1.4** (9.0±1.4)	6.5±2.8* (25.1±2.2)

The results are shown as means±SD

*p<0.001, **p<0.005 *vs* wt or *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$

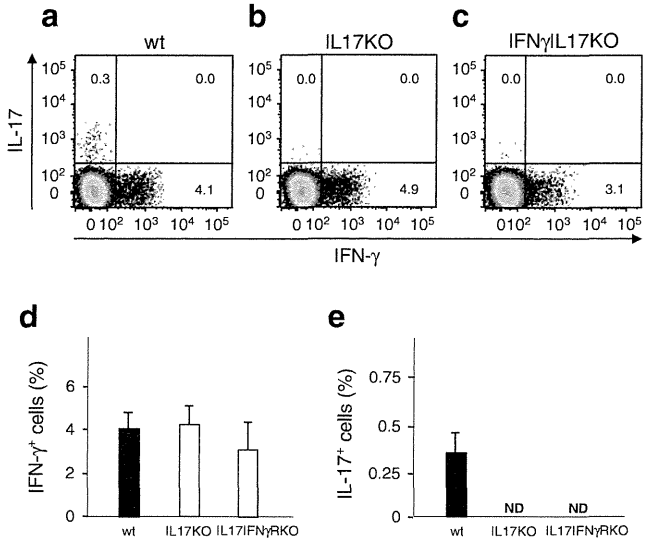

Fig. 3 IFN-γ positive or IL-17 positive cells in $CD4^+$ SPCs in the IL-17-deficient and IL-17/IFN-γR-deficient NOD mice. SPCs were prepared, stimulated with PMA and ionomycin for 5 h, stained for cell surface CD4 and intracellular IFN-γ and IL-17, and analysed with flow cytometry (**a**–**c**). Representative staining of $CD4^+$ SPCs for intracellular IFN-γ and/or IL-17. (**d**,**e**) Numeration of Th1 and Th17 cells, respectively. The data are means±SD (n=5). KO, knockout; ND, not detected

mice of the same age (Fig. 2a). However, such attenuation of diabetes development by IL-17-deficient diabetogenic T cells was no longer seen after a similar transfer of the same cells derived from older non-diabetic or diabetic mice (Fig. 2b,c). Taken together, the present results suggest that IL-17 participates in the pathogenesis of the early phase of the development of diabetes, but elimination of IL-17 could be readily dispensable in the late phase of diabetes.

In vitro-polarised Th17 cells derived from BDC2.5 TCR-Tg NOD mice do not transfer diabetes in newborn NOD mice but do transfer diabetes in immune-deficient hosts through conversion into Th1 cells or Th1/Th17 cells coexpressing Th1 and Th17 cytokines [23, 24]. We hypothesised that such a conversion of Th17 into Th1 cells or Th1/Th17 cells may have compensated for the disease inhibition by IL-17 single deficiency in NOD mice in the present study. To test our hypothesis, we also evaluated the impact of the genetic deletion of both IL-17 and IFN-γ signalling in NOD mice to determine whether such a double deficiency could clearly suppress disease. As hypothesised, IL-17/IFN-γR double deficiency significantly suppressed the longstanding incidence of diabetes compared with IL-17 single deficiency in NOD mice (Fig. 1a). These results indicate that Th1 and Th17 cytokines may synergistically contribute to the development of diabetes in NOD mice, since IFN-γR-deficient NOD mice exhibit minimal or no inhibition of disease [10, 28].

We fortuitously found that IL-17/IFN-γR double-deficient NOD mice had a severe lymphopenic phenotype. A previous study demonstrated that wt NOD mice have mild lymphopenia compared with a non-autoimmune strain, and, as a result, compensatory homeostatic expansion of T cells generates anti-islet autoimmunity resulting in the development of diabetes [29]. In contrast, NOD mice harbouring a C57BL/6-derived *Idd3* genetic interval (which encodes the *Il2* and *Il21* genes) (NOD.*Idd3* mice) are disease-resistant and not lymphopenic. It has recently been shown that naive T cells from NOD mice exhibit a greater propensity to differentiate into Th17 cells than those from NOD.*Idd3* mice, and IL-21 signalling in antigen-presenting cells plays a central role in such Th17 cell development [30].

On the other hand, several studies have demonstrated that diabetes susceptibility and protection in NOD mice correlate with lymphopenia and homeostatic expansion under a variety of experimental conditions. Thymectomy at weaning or treatment with cyclophosphamide, which causes lymphocyte apoptosis, accelerates diabetes onset in NOD mice

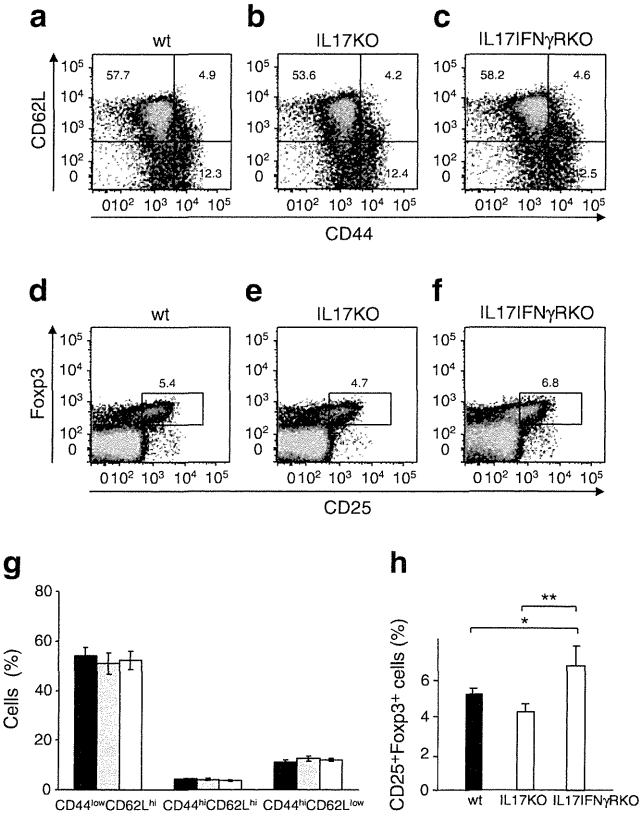

Fig. 4 Activation markers and $CD25^+Foxp3^+$cells in $CD4^+$ SPCs. Unstimulated SPCs were stained for cell surface CD4, CD62L, CD44 and CD25, and intracellular Foxp3 and analysed with flow cytometry. Representative staining of $CD4^+$ SPCs for (**a**–**c**) CD62L and CD44 and (**d**–**f**) CD25 and Foxp3. Numeration of (**g**) each $CD44^{low}CD62L^{hi}$, $CD44^{hi}CD62L^{hi}$ and $CD44^{hi}CD62L^{low}$ cells in wt NOD mice (black bar), *Il17*$^{-/-}$/*Ifngr1*$^{+/+}$ NOD mice (solid grey) and *Il17*$^{-/-}$/*Ifngr1*$^{-/-}$ NOD mice (white bar) and (**h**) $CD25^+Foxp3^+$ cells. The data are mean±SD (n=5), and group differences were analysed by Mann–Whitney U test. *p<0.05, **p<0.01. KO, knockout

[31, 32]. In contrast, immunisation with complete Freund's adjuvant (CFA) increases T cell numbers and protects NOD mice from diabetes [29, 33]. Mori et al have reported that IFN-γR deficiency abrogates cyclophosphamide-induced acceleration of diabetes and CFA-mediated protection in NOD mice [34, 35]. Thus, Th17 cell development and IFN-γ signalling may play a critical role in diabetes susceptibility mediated by lymphopenia-induced homeostatic expansion in NOD mice, although the precise kinetics and mechanisms of severe lymphopenia in these mice are still being elucidated.

We also found that double-deficient NOD mice had a preferential increase in Tregs among $CD4^+$ splenic T cells. Several recent findings highlight the plasticity within the $CD4^+$ Th cell population including Th1, Th2, Th17 and Tregs, and Th17 cells and Tregs are more plastic than Th1/Th2 cells [21]. Networks of cytokines are critical for determining $CD4^+$ T cell fates and effector cytokines [36]. For example, Bertin-Maghit et al clearly demonstrated that systemic overproduction of IL-1β in 6- to 16-week-old NOD mice impairs Treg function and promotes the Treg to Th17 conversion [37]. A pair of studies showed that Th17 cells generated ex vivo or in vitro can be converted into the Th1 phenotype by combined IFN-γ and IL-12 signalling through epigenetic processes [38, 39]. As described above, in vitro-polarised Th17 cells are readily reprogrammed into other T cell lineages on transfer into lymphopenic hosts [23, 40]. Of note, treatment with cyclophosphamide not only fails to accelerate diabetes but also confers permanent protection against diabetes by the preferential generation of Tregs in IFN-γR-deficient NOD mice [34]. It is possible that IFN-γR deficiency under lymphopenic conditions inhibits such a Th shift from Th1 to Th17 or from Tregs to another Teff lineage, resulting in a preferential increase in the Treg population and disease resistance in our double-deficient NOD mice.

Thus, we have here demonstrated that IL-17/Th17 participates in the development of insulitis and that both IL-17 and IFN-γ signalling may synergistically contribute to the Teff/Treg balance to Teffs during homeostasis expansion and the subsequent development of diabetes in NOD mice.

From the clinical point of view, the therapeutic efficacy of the inhibition of Th17 cells in some autoimmune diseases has been demonstrated [18, 19]. It has been reported that children with new-onset type 1 diabetes have an increased proportion of memory $CD4^+$ cells that have increased IL-17 secretion, suggesting that upregulation of Th17 immunity is associated with human type 1 diabetes [41, 42]. This implies a novel potential therapeutic strategy for human type 1 diabetes based on the control of IL-17 immunity. However, the results presented in the present study indicate that a single blockade of an effector cytokine such as IL-17 or IFN-γ readily compensates for the Th shift from Tregs to effector Th lineage through multiple networks of cytokines. The appropriate timing or therapeutic strategy for inhibiting the Treg/Teff conversion—such as a combination blockade of multiple cytokines or transcriptional factors such as the Janus kinase (JAK)–signal transducers and activator of transcription (STAT) pathway, Runx3 and IRF-4—should be carefully considered with the goal of preventing or delaying the development of type 1 diabetes [36].

Acknowledgements We thank O. Kanagawa for providing IFN-γR-deficient NOD mice and M. Motomura and Y. Kataoka for their technical assistance. G. S. Eisenbarth, who was executive director of the Barbara Davis Center for Diabetes, contributed greatly to the early experiments of the study before his untimely death.

Funding This work was supported by research grants from the Japan Society for the Promotion of Science (Nos 24591334, 21591143, 23791036 and 22790865).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement All authors contributed to the conception and design of the study, acquisition, analysis and interpretation of data, and drafting and editing of the manuscript. All of the authors approved the final version of the manuscript. GK, MK and NA had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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