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Low-dose interleukin-2 treatment selectively modulates CD4+ T cell subsets in patients with systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a potentially life-threatening autoimmune disease characterized by altered balance of activity between effector and regulatory CD4+ T cells. The homeostasis of CD4+ T cell subsets is regulated by interleukin (IL)-2, and reduced production of IL-2 by T cells is observed in individuals with SLE. Here we report that treatment with low-dose recombinant human IL-2 selectively modulated the abundance of regulatory T (T_{reg}) cells, follicular helper T (T_{FH}) cells and IL-17-producing helper T (T_H17) cells, but not T_H1 or T_H2 cells, accompanied by marked reductions of disease activity in patients with SLE.

SLE is an autoimmune disease triggered by the breakdown of self–nonself discrimination and the resultant autoreactive cellular and humoral immune responses[1]. A loss of the healthy balance of activity between effector and regulatory CD4+ T cells correlates with the development of SLE[2,3]. As compared to healthy individuals, patients with SLE contain more T_{FH} cells, which support autoantibody production[4,5], and more T_H17 cells, which contribute to the pro-inflammatory milieu that enables innate immune cells to cause tissue damage[6–8]. Impaired function of T_{reg} cells in patients with SLE has also been reported[7]. Therefore, suppressing the function of T_{FH} and T_H17 cells and promoting the function of T_{reg} cells might reinstate immune balance in individuals with SLE[3].

IL-2 regulates the homeostasis of CD4+ T cells. In mice, IL-2 enhances T_{reg} cell development and survival, and it suppresses the differentiation of the T_{FH} and T_H17 subsets[9–11]. Recent studies report the benefits of low-dose IL-2 treatment in patients with autoimmune and inflammatory conditions, including in a patient with SLE; increases in the number of T_{reg} cells in blood were observed[12–17]. However, analysis of T cells in patients with graft-versus-host disease (GVHD) that were treated with low-dose IL-2 concluded that low-dose IL-2 treatment had minimal overall effect on effector T cells[18]. Thus, defective IL-2 production[19] in patients with SLE could, in theory, be associated with impaired T_{reg} cell function[7] and/or aberrant activation of T_{FH} and T_H17 cells[4–8].

Here we investigated the effects of low-dose recombinant human IL-2 (rhIL-2) treatment on regulatory and effector CD4+ T cell subsets in patients with active SLE (**Supplementary Table 1**). Three cycles of rhIL-2 were administered subcutaneously at a dose of 1 million IU every other day for 2 weeks, followed by a 2-week break in treatment (**Supplementary Fig. 1**).

Twenty-three of 40 patients studied consented at enrollment to donate blood for comprehensive immunophenotyping during the treatment period, and their peripheral T_{reg}, T_H1, T_H2, T_H17 and T_{FH} cells in blood were analyzed by flow cytometry (**Supplementary Fig. 2**). Over the course of rhIL-2 administration, there was a significant increase in the relative number of $CD25^{high}CD127^{low}$ T_{reg} cells in the CD4+ T cell population ($P < 0.001$) (**Fig. 1a**). We also observed an increase in T_{reg} cell function, as measured by an *ex vivo* suppressive assay (**Supplementary Fig. 3**). In contrast, the relative number of $CXCR5^+PD\text{-}1^+CCR7^{low}$ T_{FH} cells in the CD4+ T cell population fell from a median of 3.93% (range: 1.27–15.14) to 1.99% (range: 0.69–7.55) ($P < 0.001$), and the relative number of $CCR6^+CXCR3^-CCR4^+CCR7^{low}$ T_H17 cells decreased from 4.08% (range: 1.69–11.18) to 2.67% (range: 1.28–8.87) ($P = 0.001$) (**Fig. 1b**,**c**). Consequently the ratio of '(T_{FH} + T_H17) cells/T_{reg} cells' fell significantly over the course of rhIL-2 administration (**Fig. 1d**). In contrast, the relative numbers of $CXCR3^+CCR6^-CCR4^-CCR7^{low}$ T_H1 and $CXCR3^-CCR6^-CCR4^+CCR7^{low}$ T_H2 cells were not significantly affected by rhIL-2 administration (**Fig. 1e**,**f**). We also observed a significant reduction of $CD4^-CD8^-$ (double-negative; DN) αβ T cells during low-dose rhIL-2 treatment (**Supplementary Fig. 4**). These cells can produce IL-17 in patients with SLE[20]. Notably, no such changes in CD4+ T cell subsets were observed in patients with SLE who were treated with conventional therapy (**Supplementary Fig. 5** and **Supplementary Tables 2** and **3**).

To understand the dose sensitivity of T_{reg}, T_{FH} and T_H17 cells to IL-2, we examined these T cell subsets in wild-type (WT) mice that had been immunized with ovalbumin and were treated subcutaneously with a low-dose range of rhIL-2 (10,000, 30,000 or 100,000 IU) daily for 1 week (**Supplementary Fig. 6**). Low doses of rhIL-2 led to the expansion of the T_{reg} cell population and the suppression of T_{FH} and T_H17 cell numbers, without affecting splenocyte numbers (data not shown). At the lowest dose, however, IL-2 had negligible effects on T_{reg} cell numbers, although T_{FH} and T_H17 cell numbers, as well as

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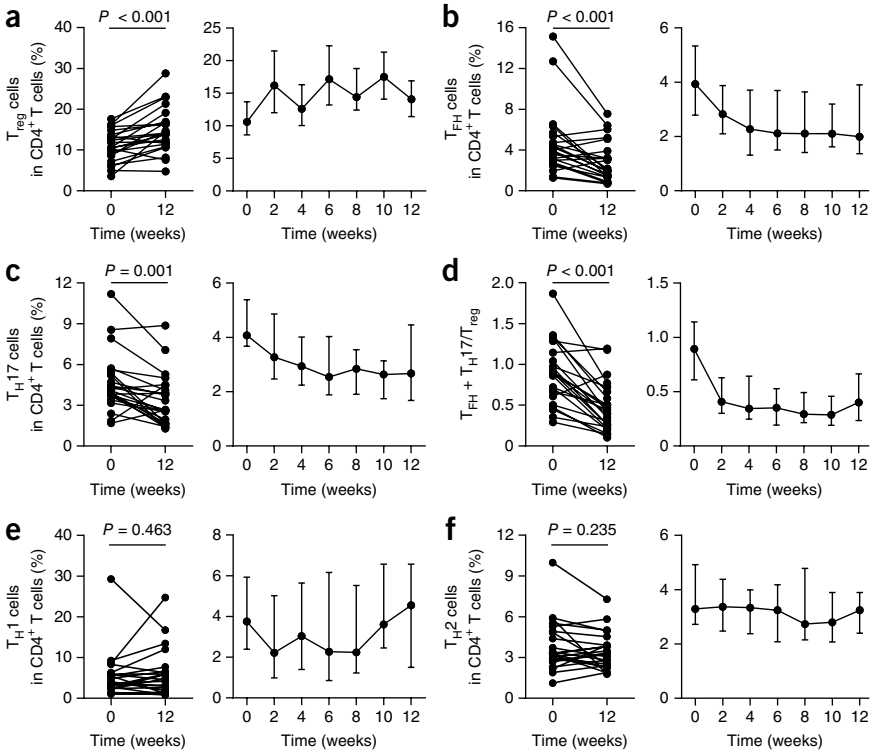

Figure 1 Immunological responses to low-dose rhIL-2. Immunological responses are shown for each of the three courses of treatment (each course consisted of a dose of 1 million IU every other day for 2 weeks (a total of seven doses), followed by a 2-week break) ($n = 23$ patients who consented for comprehensive immunological analysis). Relative proportions of T_{reg}, T_{FH}, T_H17, T_H1 and T_H2 cell subsets in peripheral blood were analyzed by flow cytometry. (**a**–**c**) Changes in the percentages of T_{reg} cells (**a**), T_{FH} cells (**b**) and T_H17 cells (**c**) within the $CD4^+$ T cell population at the indicated time points (right) and comparison between amounts of cells at baseline and at week 12 (left) are shown ($n = 23$ human subjects). Data are mean ± s.e.m. $P < 0.001$ by paired-sample *t*-test. (**d**) Changes in the ratio of (T_{FH} + T_H17) cells to T_{reg} cells at the indicated time points (right) and comparison of the ratio between baseline and week 12 (left) ($n = 23$ human patients). Data are mean ± s.e.m. $P < 0.001$ by paired-sample *t*-test. (**e**,**f**) Changes in percentage of T_H1 and T_H2 cells within $CD4^+$ T cells ($n = 23$ human patients). Data are mean ± s.e.m. $P > 0.05$ by paired-sample *t*-test. Data is presented as median and interquartile range, and the median values at each indicated time are linked by the trend line. Throughout, paired-sample *t*-tests were used to compare immunological features between baseline and week 12 after the rhIL-2 administration.

germinal center formation, were suppressed (**Supplementary Fig. 7**). These observations suggest that the differentiation of T_{FH} and T_H17 cells *in vivo* is probably more sensitive to IL-2 than that of T_{reg} cells. Moreover, low-dose IL-2 can suppress T_{FH} and T_H17 cells independently of the expansion of T_{reg} cells in WT mice infected with virus[10,11]. Similar to the observation made in patients with SLE, low-dose IL-2 increased the suppressive function of T_{reg} cells (**Supplementary Fig. 8**) but did not affect T_H1 cell numbers, again suggesting a selective effect of IL-2 on T_{reg}, T_{FH} and T_H17 cells.

Thirty-eight patients completed three cycles of IL-2 treatment (**Supplementary Table 4**). Two patients withdrew before completion of therapy because of nonmedical reasons. No serious adverse events were observed (**Supplementary Tables 5** and **6**), and injection-site reactions (13.2%) and influenza-like symptoms (5.3%) resolved without any intervention. Notably, neither microbiologically confirmed nor antibiotic-treated infection was observed during the 12-week period of IL-2 treatment (**Supplementary Table 5**).

All 38 patients who completed therapy showed decreased disease activity at the end of study, as compared to baseline disease activity. The proportion of patients who achieved an SLE response index (SRI) with a 4-point drop in the SLE disease activity index (SLEDAI) (SRI-4 response rate) was 31.6% (95% confidence interval (CI); range: 17.5%–48.7%) at week 2, 71.1% (95% CI; range: 54.1%–84.6%) at week 4 and 89.5% (34/38 patients; 95% CI; range: 75.2%–97.1%) at the end of 12 weeks, and accordingly, significant reductions in the SLEDAI were observed across the duration of the study

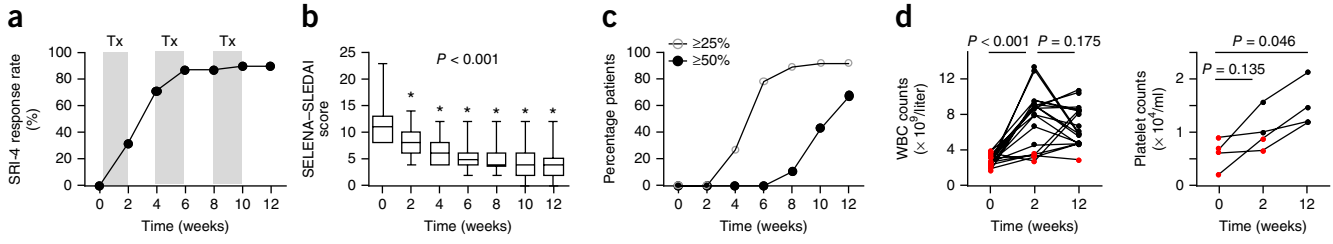

Figure 2 Clinical responses to low-dose rhIL-2. (**a**,**b**) Responses to rhIL-2 during a period of 12 weeks, as assessed by SRI-4 (**a**) and the Safety of Estrogens in Lupus Erythematosus National Assessment version of the systemic lupus erythematosus disease activity index (SELENA–SLEDAI) score (**b**) ($n = 38$ patients). In **b**, data boxes denote median, minimum (bottom edge) and maximum (top edge). SELENA–SLEDAI score gradually decreased during the rhIL-2 administration. Tx, treatment. *$P < 0.001$ by paired-sample *t*-test to compare SELENA–SLEDAI scores between baseline and each indicated time point during the rhIL-2 administration. (**c**) Effects of rhIL-2 on glucocorticoid reduction by ≥25% and ≥50% from baseline to 12 weeks. Percentage of patients with glucocorticoid reduction by ≥25% or ≥50%, as compared to the doses at baseline before rhIL-2 treatment, is shown ($n = 37$). (**d**) Changes in white blood cell (WBC) (left) and platelet (right) counts at weeks 0, 2 and 12 in patients with leukopenia and thrombocytopenia, respectively (leukopenia, $n = 19$ patients; thrombocytopenia, $n = 4$ patients). $P < 0.001$ by paired-sample *t*-test for differences in WBC counts at baseline and week 2 for patients with leukopenia (left), and $P < 0.05$ by paired-sample *t*-test for differences in platelet counts at baseline and week 12 in patients with thrombocytopenia (right).

(**Fig. 2a**,**b**). Reductions in corticosteroid use during the study were permitted at the physician's discretion. At week 12, 34/37 corticosteroid-treated patients (91.9%) were receiving prednisone at a dose reduced by ≥25% as compared to that at baseline, and 25 patients (67.6%) were receiving prednisone at a dose reduced by ≥50% than that at baseline (**Fig. 2c**). Almost all lupus-related manifestations improved in a majority of the patients during rhIL-2 treatment: rash (20/24), alopecia (13/14), arthritis (10/11), fever (3/3) and serositis (5/5) ($P < 0.05$). Laboratory measures also demonstrated improvement (**Supplementary Table 4**). Resolution of leukopenia and thrombocytopenia was observed in 94.7% (18/19) and 100% (4/4) of patients, respectively, at week 12 (**Fig. 2d**). Resolution of discoid and malar rashes was observed within 2 weeks of rhIL-2 administration (data not shown). The 23 patients who underwent comprehensive immunological analysis had baseline clinical characteristics comparable to those of the other patients who did not undergo such analysis, and clinical responses were not different between these two groups (**Supplementary Tables 7–9**), suggesting that conclusions drawn from the immunological analysis are representative. Clinical responses did not differ according to treatments, other than those for low-dose IL-2 treatment (**Supplementary Fig. 9**). The sample size was insufficient to compare the magnitude of clinical effect among subsets of immunological responders, and no significant correlation between change in immunological markers and change in the SLEDAI scores was observed in the overall group. The mean change in the (T_{FH} + T_H17) cell/T_{reg} cell ratio between week 0 and week 12 did not differ significantly between low and high responders ($P > 0.05$). However, the (T_{FH} + T_H17) cell/T_{reg} cell ratio was significantly lower at week 12 than at week 0 among high responders; this reduction in the (T_{FH} + T_H17) cell/T_{reg} cell ratio was not statistically significant among low responders (**Supplementary Fig. 10**). These findings suggest that low-dose IL-2 affects immune function in SLE to promote T_{reg} cell–mediated suppression relative to that in T_{FH} cell–mediated and T_H17 cell–mediated pathogenic responses.

In summary, this study provides evidence suggesting that administration of low-dose rhIL-2 to patients with active SLE changes the proportions of effector and regulatory T cells and reduces disease activity. Future randomized trials of low-dose IL-2 in the treatment of SLE are required to further validate the potential therapeutic and corticosteroid-sparing effects of this therapy.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.H. and Xia Zhang participated in literature searches, study design, patient recruitment, data collection, data analysis, data interpretation and writing of the paper; Y.W., X.S., X.H., Z.H. and Y.C. analyzed the biological data; R.J., C.X., L.Z., J.F., Y.A., C.L., X.L., H. Ye, Y. Jia, L.R., R.L., S.C., Xuewu Zhang, Y.S., Y. Jin, H. Yao and Y.L. participated in patient recruitment and data collection; J.D., Y.G. and Y.A.L. participated in animal experiments; J.G., N.S. and E.F.M. participated in data interpretation and in writing the report; Z.L. and D.Y. participated in study design, data interpretation and writing of the paper; and all authors contributed to the critical revision of the manuscript and approved the final version.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mouse experiments. For induction of T_{FH} and T_H17 immune responses, C57BL/6 mice (male, 8–12 weeks old) were intraperitoneally immunized with 50 μg ovalbumin in complete Freund's adjuvant (CFA). Different doses (10,000 IU, 30,000 IU or 100,000 IU) of rhIL-2 (Teceleukin; from the National Cancer Institute Biological Resource Branch (NCI–BRB) Preclinical Repository) or PBS were administrated subcutaneously daily from day 3 to day 9. Five mice for each group were chosen, based on our experience and estimation. All mice were in same genetic background and allocated into each experimental group by randomization. Immunized mice were analyzed at day 10, and investigators were blinded to the experimental groups. Splenocytes were incubated with fluorophore-conjugated monoclonal antibodies and analyzed by flow cytometry. The phenotypic characterization of T_{reg} ($FOXP3^+CD25^+CD4^+$), T_{FH} ($BCL6^+CXCR5^{high}FOXP3^-CD4^+$), T_H17 ($IL\text{-}17^+CD44^{high}CD4^+$), T_H1 (interferon (IFN)-$\gamma^+CD44^{high}CD4^+$) and germinal center B ($BCL6^+GL\text{-}7^+B220^+$) cells is shown in **Supplementary Figure 6**. For examination of T_{reg} cell function, mice expressing green fluorescent protein gene (*GFP*) under the *Foxp3* promoter (*Foxp3-GFP* reporter mice) (male, 8–12 weeks old) were subcutaneously injected with 30,000 IU rhIL-2 daily for 7 d. T_{reg} cells were isolated by using flow cytomety to sort for GFP^+ cells. Splenocytes from naive C57BL/6 mice were labeled with CellTrace Violet dye (Thermo Fisher Scientific) and stimulated by Mouse T-Activator CD3/CD28 Dynabeads (Thermo Fisher Scientific) in the absence or presence of T_{reg} cells at the indicated ratios for 3 d. T cell proliferation was measured by flow cytometry. Percentages of divided cells and division indices (the average number of cell divisions that a cell in the original population has undergone) were calculated as per instructions (FlowJo). The results represent two independent experiments. Preliminary experiments were performed using five mice to determine the expected means and sample distributions of the control and experimental groups. Animals were of matched sex and age, and no randomization was performed for the groupings of the animals. Blinding was not performed. All mouse experiments were approved by the Monash University Animal Ethics Committee.

Clinical study design. We conducted a prospective, open-label study to evaluate the effects of low-dose rhIL-2 (recombinant human interleukin-2^{Ser125}, Beijing SL Pharma) in patients with active SLE. The rhIL-2, produced in *Escherichia coli,* is approved by the State Food and Drug Administration, China, and has comparable bioactivity to Proleukin (aldesleukin). Three cycles of rhIL-2 were administered subcutaneously at a dose of 1 million IU every other day for 2 weeks (a total of 7 doses), followed by a 2-week break. After the initiation of IL-2 treatment, no increase in any other treatments for SLE was permitted. Clinical and laboratory data were measured at baseline and every 2 weeks thereafter until week 12 (**Supplementary Fig. 1a**). During the study, corticosteroid doses were tapered at the discretion of the treating physician, according to European League Against Rheumatism (EULAR) evidence-based recommendations on the management of corticosteroid therapy[21]. Briefly, patients' disease activity was evaluated every 2 weeks, as assessed by two rheumatologists, and patients were advised to taper 5–10% of the current dose of corticosteroids if disease activity was reduced.

This study was approved by Peking University People's Hospital Ethics Committee. All patients provided written informed consent. The trial is registered at https://clinicaltrials.gov (NCT02084238). Subject recruitment commenced in August 2013, and the study was concluded at the end of the 12-week observation period for the last patient recruited, in May 2014.

Participants. The study recruited 3 male and 37 female patients with SLE aged 18–65 years, who were diagnosed according to the 1997 revised classification criteria of the American College of Rheumatology[22] with moderate-to-severe disease activity (Safety of Estrogens in Lupus Erythematosus National Assessment version of the systemic lupus erythematosus disease activity index (SELENA–SLEDAI)[23] ≥ 8), despite at least 4 weeks of stable background treatment with corticosteroids (≤1.0 mg per kg body weight per day (mg/kg/d) prednisone or equivalent) and/or with antimalarials, nonsteroidal anti-inflammatory drugs or immunosuppressants (**Supplementary Table 6**). Exclusion criteria included were active severe neuropsychiatric manifestations of SLE, a history of treatment with rituximab or other biologics, use of high-dose corticosteroids (>1.5 mg/kg/d) in the preceding month, severe comorbidities including heart failure (≥grade III NYHA), renal insufficiency (creatinine clearance ≤30 ml/min) or hepatic insufficiency (alanine aminotransferase or aspartate aminotransferase ≥2 times of the upper limit of the normal range), active infection (hepatitis B or C virus, Epstein-Barr virus, human immunodeficiency virus or *Mycobacterium tuberculosis*) or history of chronic infection, malignancy, and pregnancy or lactation in females. Consecutive patients managed by the investigators between August 2013 and August 2014 who met the inclusion and exclusion criteria and provided informed consent were recruited. We aimed to recruit 30–40 patients, based on our earlier study in which we were able to detect a significant difference in T_{FH} signature between 26 patients with SLE and 30 healthy control subjects[4]; no formal sample size calculation was undertaken.

A separate group of patients with SLE who fulfilled the above inclusion and exclusion criteria, but who were treated with conventional therapies, was subsequently recruited from October 2015 to January 2016 (**Supplementary Fig. 1a** and **Supplementary Table 9**). This control group comprised consecutive patients who met the inclusion and exclusion criteria and who consented to donate blood for immunological analysis before and after a 12-week period of conventional therapy.

To examine the function of T_{reg} cells after low-dose IL-2 treatment, ten additional patients with SLE who fulfilled the above inclusion and exclusion criteria were recruited for a single cycle of low-dose IL-2 administration. Eight of ten patients donated blood for immunological assays to evaluate T_{reg} cell function before and after 2 weeks of low-dose IL-2.

End points. The primary end points were response-measured by the SLE responder index (SRI)[24] and safety. SRI-4 response was defined as: (i) a ≥4-point reduction in SELENA–SLEDAI score; (ii) no new British Isles lupus Assessment Group 2004 (BILAG) A (representing very active disease) score or ≤1 new BILAG B (representing moderate active disease) score; and (iii) no deterioration from baseline in the physician's global assessment by ≥0.3 points. Other clinical assessments included change in SELENA–SLEDAI score, change in corticosteroid dose, resolution of organ involvement present at the time of enrollment, and laboratory variables including immunoglobulin G, complement C3 and C4, double-stranded-DNA-specific antibodies, nucleosome-specific antibodies and urinary protein excretion. Twenty responder patients, as defined by SELENA–SLEDAI scores <8 after 12 weeks of low-dose IL-2 administration, were classified into a low-responder group (final SLEDAI score ranging from 4 to 7; $n = 10$) and a high-responder group (final SLEDAI score < 4; $n = 10$).

Immunological analysis. Twenty-three patients consented to donate blood for comprehensive immunological analysis, including enumeration of T_{reg} and effector $CD4^+$ T cell subsets (T_H1, T_H2, T_H17 and T_{FH} cells). Protocol-specific immunophenotypic analysis of peripheral blood leukocyte subsets was performed at baseline and every 2 weeks thereafter until week 12 (weeks 0, 2, 4, 6, 8, 10 and 12). Relative proportions of T_{reg}, T_{FH}, T_H1, T_H2 and T_H17 cell subsets were analyzed by flow cytometetry using a FACSAria III (BD) instrument and FlowJo software (TreeStar). T_{reg} cells were defined as $CD3^+CD4^+CD25^{high}CD127^{low}$ (refs. 25,26), T_{FH} cells were defined as $CD3^+CD4^+CXCR5^+PD1^{high}CCR7^{low}$[4], and T_H17 cells were defined as $CD3^+CD4^+CXCR3^-CCR6^+CCR4^+CCR7^{low}$ (ref. 27). The detailed gating strategy for these subsets is outlined in **Supplementary Figure 2**. The clone and catalog numbers for all of the antibodies used in this study are provided in **Supplementary Table 10**.

Human T_{reg} cell–mediated suppression assay. To evaluate the suppressive activity of $CD4^+CD25^{hi}CD127^{low}$ T_{reg} cells before and after low-dose IL-2 administration, carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD25^-CD4^+$ T cells were sorted from healthy donors and were stimulated with anti-CD3- and anti-CD28-coated beads from the T_{reg} Suppression Inspector Kit (Miltenyi Biotec), in the absence or presence of T_{reg} cells sorted from patients with SLE at a 1:1 ratio. On day 7, the proliferation of effector cells was determined by flow cytometry.

Statistical analysis. All statistical analyses were carried out using GraphPad Prism (version 5.0, GraphPad Software) or Statistical Package for the Social

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Sciences (SPSS) software (version 17.0, IBM). A per-protocol analysis was undertaken, excluding patients who did not complete treatment. Paired-sample *t*-test was used for paired comparison of differences in clinical characteristics, laboratory parameters and immunological features between values at baseline and those at the indicated time points during the IL-2 administration. Confidence intervals for proportions were calculated by using the Clopper–Pearson exact confidence interval. The Mann–Whitney *U* test was used to evaluate the influence of background therapy on clinical and laboratory measures during rhIL-2 administration. Two-tailed *t*-tests were used to compare T_{reg} cell function in groups of immunized mice that were treated with PBS or different doses of IL-2, as well as to compare immunological features at baseline between IL-2-treated and conventional-therapy-treated patients with SLE. $P < 0.05$ was considered statistically significant.

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