SUPPLEMENTARY MATERIALS

This appendix has been provided by the authors to give readers additional information about their work. Supplement to: Fanton C, Furie R, Chindalore V, et al. Selective expansion of regulatory T cells by NKTR-358 in healthy volunteers & patients with systemic lupus erythematosus: two randomized phase 1 studies

Contents

Supplementary material & methods

Study populations

In the SAD study, healthy volunteers, enrolled between March 2017 and November 2018, were required to have a body mass index of 18–32 kg/m^2 and normal blood pressure (systolic: 90–140 mmHg; diastolic: 50–90 mmHg), heart rate (resting: 40–90 bpm), organ function, and hepatic, renal, and hematologic function. Participants were excluded from the SAD study if they had: concurrent active bacterial, viral, or fungal infection; history of organ or bone marrow transplant; positive serology for hepatitis B virus (HBV), hepatitis C virus (HCV), or HIV; previous adverse reaction to subcutaneous medication; history of tuberculosis; clinically significant illness or electrocardiographic abnormalities; use of prescription, over-the-counter, illegal, or investigational drugs, or donation of blood or plasma within 28 days of study start; active and clinically significant renal, cardiovascular, hepatic, metabolic, allergic, dermatologic, hematologic, pulmonary, neurological, or psychiatric illness or disorder; receipt of blood products within 6 months prior to screening; prior administration of any IL-2 product; and administration of an inactivated vaccine within 2 weeks of study drug administration or live attenuated vaccine within 90 days of dosing.

In the MAD study, eligible patients with SLE, enrolled between May 2018 and June 2019, were aged 18–70 years with a body mass index of 18–32 kg/m^2, a normal blood pressure (as per the SAD study), and a resting heart rate of 40–100 bpm. If the patient was taking any of the following medications, they must have been administered for at least 12 weeks, with a stable dose of at least 8 weeks: azathioprine ≤200 mg/day, antimalarials (e.g. chloroquine, hydroxychloroquine, quinacrine), mycophenolate mofetil ≤2 g/day or mycophenolic acid ≤1.44 g/day, or methotrexate ≤15 mg/week. Patients were excluded if they had current active bacterial, viral, or fungal infection; significant hematologic, liver, or kidney dysfunction; clinically significant non-SLE-related vasculitis syndrome; active severe or unstable neuropsychiatric SLE; active severe SLE-driven renal disease or history of severe active lupus nephritis with persisting proteinuria levels >0.5 g/24 hours; diagnosis (<1 year) of mixed connective tissue disease or any history of overlap syndromes of SLE; inflammatory joint or skin disease other than SLE; history of non-SLE disease that required treatment with oral or parenteral corticosteroids for >2 weeks within the last 24 weeks; active or evidence of latent tuberculosis; known history of a primary immunodeficiency, splenectomy, or any underlying condition that predisposed the patient to infection; positive serology for HBV,

HCV, or HIV; any severe herpes infection or opportunistic infection requiring hospitalization or intravenous antimicrobial treatment within 3 years; major surgery within the last 12 weeks; clinically significant electrocardiographic abnormalities or history of significant cardiovascular disease or cancer; receipt of blood products within 6 months prior to screening, or donated blood or plasma in the last 28 days; or history of organ or bone marrow transplant. Patients should not have received any investigational product within 4 weeks or 5 half-lives; belimumab or blood products in the last 6 months; parenteral glucocorticoids in the last 6 weeks; rituximab or ocrelizumab (or other B cell depleting agent) in the last 6 months; or cytotoxic medication in the last 12 months.

Biomarker assay methods

Flow cytometry immunophenotyping

For flow cytometry of peripheral whole blood samples, fluorescent antibodies against CD45, CD3, CD4, CD8, CD25, FoxP3, CD56, CTLA4, ICOS, and Ki67 were obtained from BD Biosciences (San Jose, CA); and against Helios from Biolegend (San Diego, CA). Absolute cell counts were determined by inclusion of AccuCheck Counting Beads from ThermoFisher (Waltham, MA) and calculation of molecules of equivalent soluble fluorochrome (MESF) was done using the SPHERO Ultra Rainbow Calibration Particles kit from SpheroTech, Inc (Lake Forest, IL). Flow cytometry data were acquired using a FACSCanto II (Becton Dickinson) and instrument settings were set with machine software in conjunction with calibration beads. Data were analyzed using BD FACSDiva software (San Jose, CA). After gating on lymphocytes, positive populations were identified based on either isotype controls or fluorescence minus one control. Total Tregs were defined as $CD45^{+}CD3^{+}CD4^{+}CD25^{+}FoxP3^{+}$; $CD25^{bright}$ Tregs were defined as $CD45^{+}CD3^{+}CD4^{+}CD25^{+++}FoxP3^{+}$; $CD4^{+}$ T cells as $CD45^{+}CD3^{+}CD4^{+}$; $CD8^{+}$ T cells as $CD45^{+}CD3^{+}CD8^{+}$; and total NK cells as $CD45^{+}CD3^{-}CD56^{+}$. Ki67 was gated off the $CD25^{bright}$ Treg population and all additional markers, Helios, ICOS, and CTLA-4, were gated off the total Treg population. Immunophenotyping results were enumerated as absolute cell counts (cells/µL), percent relative (%) values for each phenotype, and/or MESF. To ensure comparability across longitudinal samples, the baseline total Treg gate was set to approximately 5% (4–6%) of $CD4^{+}$ T cells for each individual, and this gate position was maintained across the individual's timepoints. For $CD25^{bright}$ Tregs, the

baseline gate was set at approximately 0.5% (0.4–0.6%) of $CD4^+$ T cells for each individual. An example of the gating strategy for the $CD25^{bright}$ Treg population is in Supp Fig 1.

Pharmacokinetic methods

Sample collection schedules

In the SAD study, heparin plasma for PK analysis was collected at the following time points after dose administration: 4, 8, 12, 16, 20 (cohorts 0.3–1 µg/kg), 24, 30 (cohorts 0.3–1 µg/kg), 36, 42, 48, 60, 72, and 84 hours (±15 minutes), and on Days 4, 5, 6, 7, 8 (pre-discharge), 10, 12 (cohorts 4–28 µg/kg), 15, 20, 25 (cohorts 4–28 µg/kg), 30, 40, and Day 50/early termination visit. In the MAD study, plasma for PK analysis was collected on the following days: 3, 5, 7 ± 1, 11 ± 1, 15, 21 ± 1, 29, 31, 34, 36 ± 1, 38 ± 1, 43, 50 ± 1, 60 ± 1, 70 ± 1, and 79/early termination visit.

Bioanalytics

NKTR-358 was measured in human plasma samples with an indirect sandwich ligand binding assay with electrochemiluminescence detection. The ligand binding assay used a combination of anti-IL-2 monoclonal antibodies for capture and an anti-PEG monoclonal antibody for detection. The method was validated according to the US Food and Drug Administration Guidance for Industry: Bioanalytical Method Validation (May 2018). The lower limit of quantitation (LLOQ) was 1.01 ng/mL. Accuracy and precision were ± 25% over the quantitative range and ± 30% at the LLOQ.

Parameter calculations

PK parameters were calculated by standard noncompartmental analysis methods. PK data from the SAD study were analyzed with the PKNCA package (v0.8.5) in R Software v3.5.2 (R Foundation for Statistical Computing; Vienna, Austria), and PK data from the MAD study were analyzed using Phoenix® WinNonlin® 8.1 (Certara USA, Inc; Princeton, NJ).

Supplementary Table 1

Pharmacokinetic parameters after a single dose of NKTR-358 in the SAD study.

Cohort (µg/kg)	Statistic	C_{max} (ng/mL)	T_{max} (days)	AUC_{last} (ng*day/mL)	CL/F (L/day)	$t_{½}$ (days)
	N[a]	5	5	5	2	2
0.3	Mean	2.32	5.1	15.7	0.565	7.4
	%CV	38.4	24.4	73.5	5.96	59.8
	N	9	9	9	8	8
1	Mean	10	5.7	157	0.686	8.7
	%CV	40.7	26.6	60.6	125	48.8
	N	9	9	9	7	7
3	Mean	36.8	7.3	649	0.375	8.6
	%CV	48.8	118	52.3	45.8	19
	N	9	9	9	8	8
6	Mean	77.9	5	1170	1.4	8.3
	%CV	51.7	40	71.6	164	59.2
	N	9	9	9	9	9
9	Mean	89.5	6.5	1690	0.485	9.1
	%CV	33.4	35.7	41.7	67.9	38.1
	N	8	8	8	7	7
13.5	Mean	135	6.1	1620	0.546	8.8
	%CV	27.9	37.5	32.9	37.4	33.1
	N	12	12	12	12	12
20	Mean	203	5.7	3710	0.398	11
	%CV	26.2	36.2	21.7	31.6	20.9
	N	9	9	9	8	8
28	Mean	241	6.8	4030	0.511	11.4
	%CV	32	40.6	39.1	34	18.2

Summary statistics for CL/F and $t_{½}$ excluded values where there was an unsuitable terminal log-linear phase in the concentration-versus-time profile.

Abbreviations: AUC_{last}: area under the plasma drug concentration–time curve up to the last measurable plasma concentration; BLQ: below the limit of quantitation; CL/F: apparent clearance; C_{max}: maximum observed concentration; CV: coefficient of variation; N: number of patients; SAD: single ascending dose; $t_{½}$: terminal elimination half-life; T_{max}: time to C_{max}.

[a]Four patients with BLQ observations were not included in summary statistics.

Supplementary Table 2

Pharmacokinetic parameters after multiple doses of NKTR-358 in the MAD study.

Cohort (µg/kg)	Statistic	First dose			Third dose			
		C_{max} (ng/mL)	T_{max} (day)	$AUC_{0–14d}$ (day*ng/mL)	C_{max} (ng/mL)	T_{max} (day)	$AUC_{0–14d}$ (day*ng/mL)	$t_{½}$ (day)
3	N	9	9	8	9	9	9	9
	Mean	31.6	6.52	330	50.1	5.52	553	10.6
	%CV	28.0	56.8	26.9	40.9	45.1	36.9	38.1
6	N[a]	9	9	9	8	9	8	9
	Mean	41.0	6.47	423	78.2	6.07	921	10.5
	%CV	45.0	24.7	45.6	30.3	25.3	28.9	19.0
12	N	8	8	8	8	8	8	7
	Mean	107	5.97	1060	157	4.22	1790	10.7
	%CV	25.7	49.1	24.9	25.2	59.4	25.2	11.7
24	N[b]	9	9	9	5	6	5	6
	Mean	189	5.72	2100	302	4.44	3530	12.9
	%CV	30.7	34.7	32.1	26.8	49.5	23.3	17.0

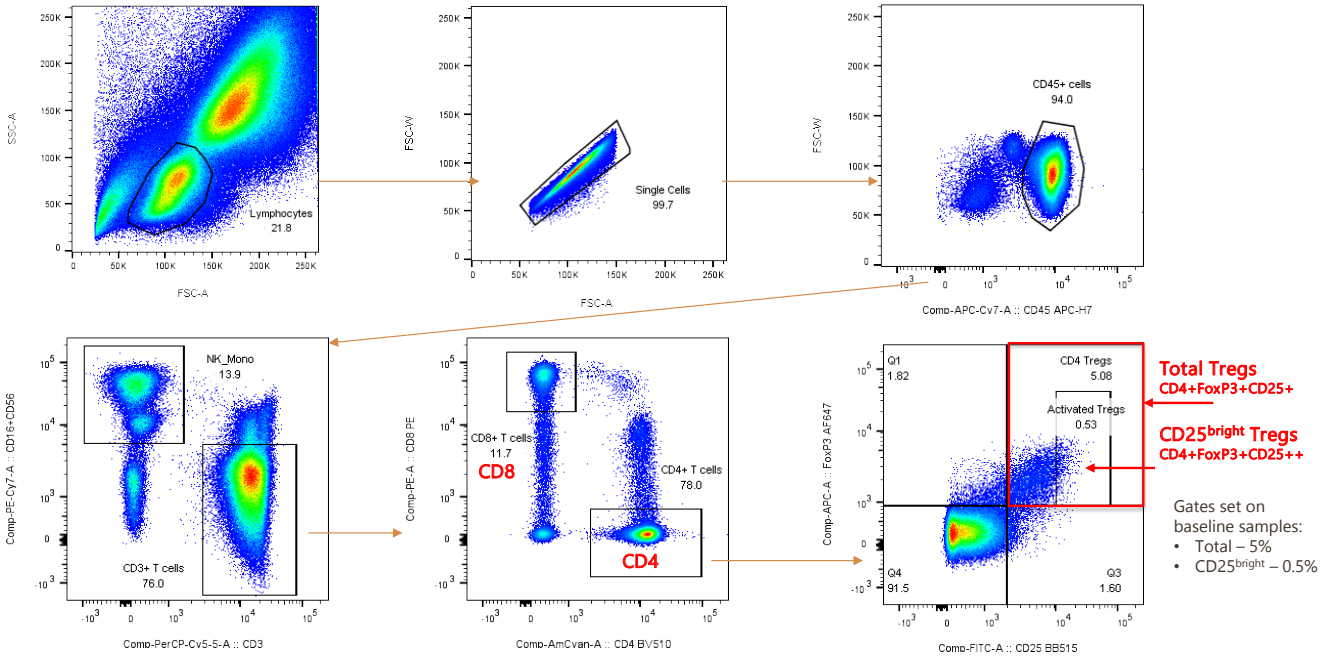
Summary statistics for $t_{½}$ excluded values where there was an unsuitable terminal log-linear phase in the concentration-versus-time profile.

Abbreviations: $AUC_{0–14d}$: area under the plasma drug concentration–time curve during one dosing period (i.e. 14 days after the dose); C_{max}: maximum observed concentration; CV: variability; MAD: multiple ascending dose; N: number of patients; $t_{½}$: terminal elimination half-life; T_{max}: time to C_{max}.

[a]One patient received the third dose of study drug ≥5 days late and was excluded from all summary statistics for Dose 3, except for T_{max} and $t_{½}$.

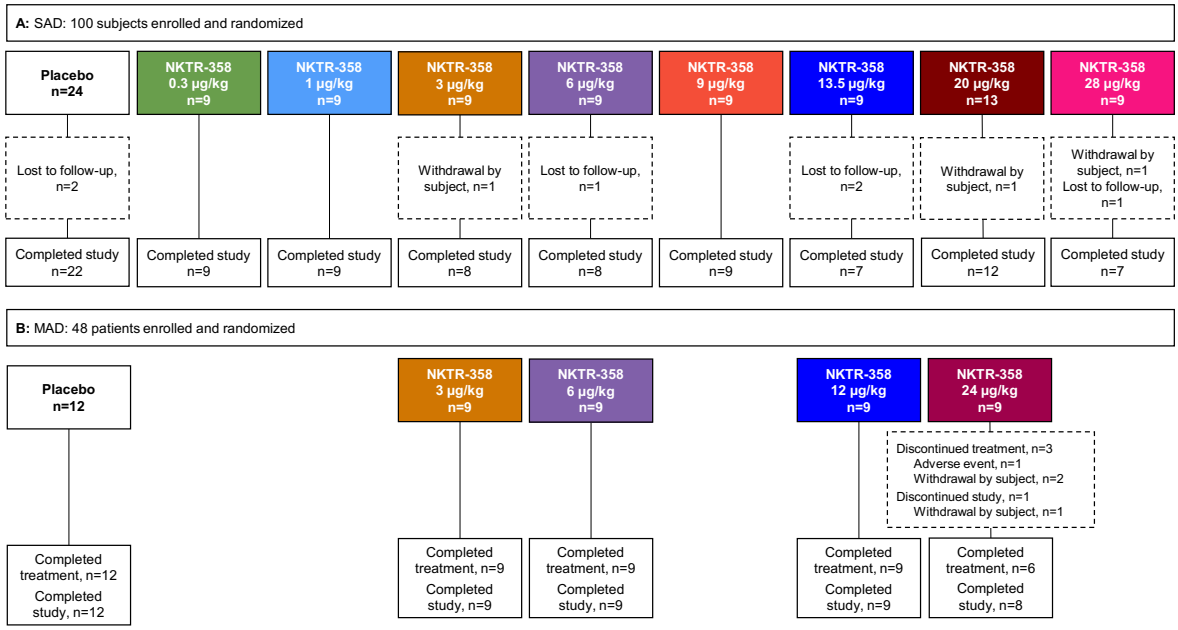
[b]Four patients were excluded from summary statistics for Dose 3 for the following reasons: three did not receive the third dose of study drug and one received the third dose of study drug ≥5 days late and was excluded from all summary statistics for Dose 3, except for T_{max} and $t_{½}$.

Supplementary Fig. 1. Gating scheme for enumeration of Tregs, CD25^{bright} Tregs, and CD4^{+} and CD8^{+} T cells



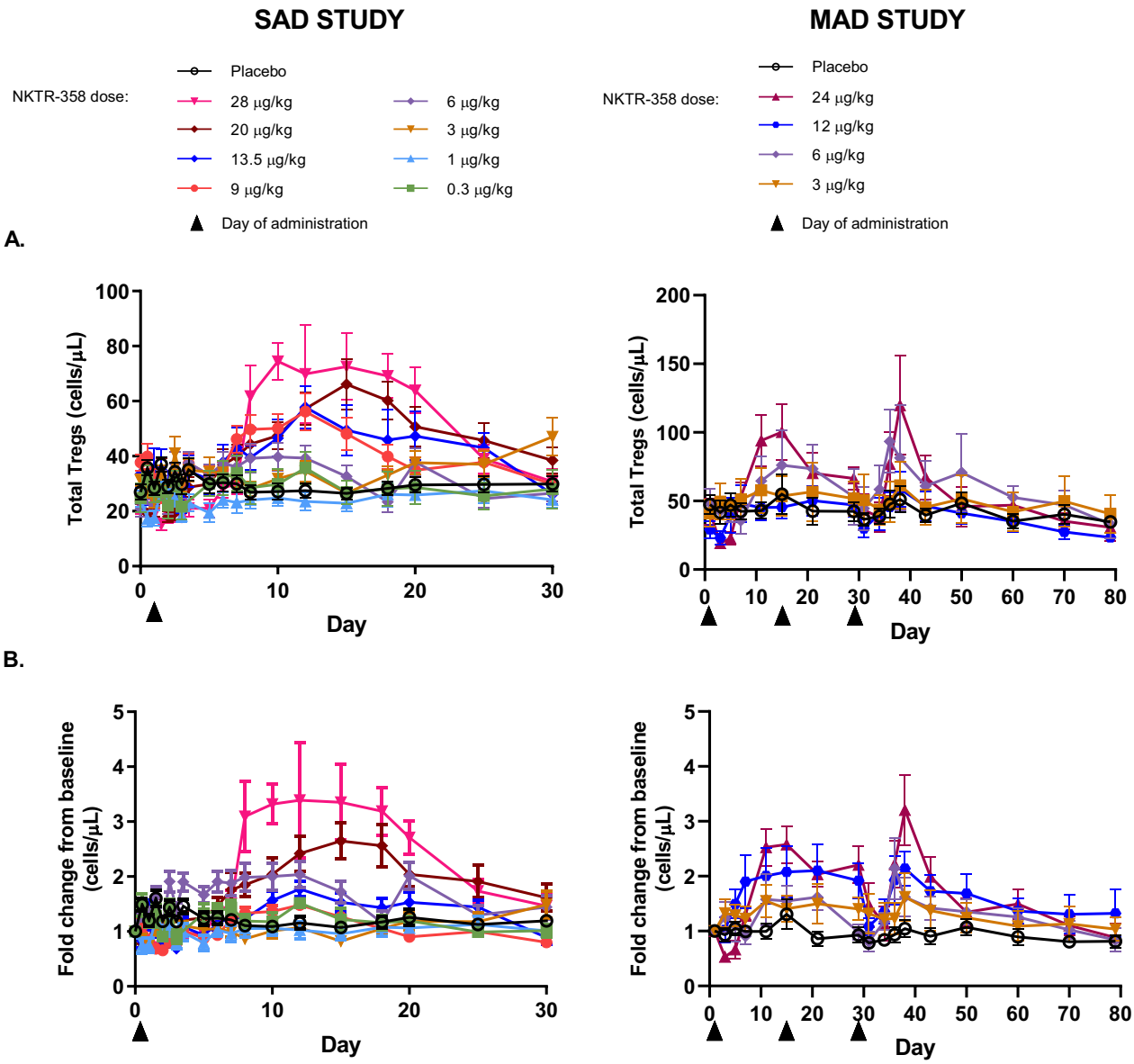
Abbreviations: FOXP3, forkhead box protein P3; NK, natural killer; FSC-W, forward light scatter width; SSC-A, side scatter parameter; Treg: T regulatory cell.

Supplementary Fig. 2. Participant disposition in A) the SAD study and B) the MAD study.



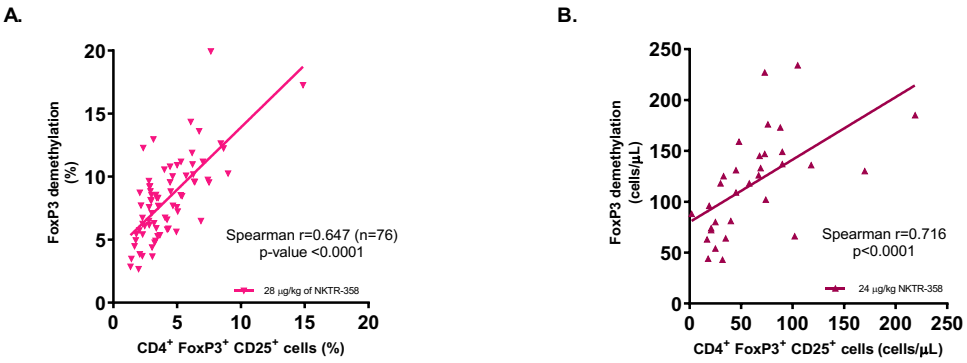
Abbreviations: FU: follow-up; MAD: multiple ascending dose; SAD: single ascending dose.

Supplementary Fig. 3. Changes from baseline in the numbers and percentages of total Tregs in peripheral blood after a single dose of NKTR-358 in the SAD study and with multiple doses of NKTR-358 in the MAD study. A) absolute number of total Tregs; B) fold-change in the absolute number of total Tregs.



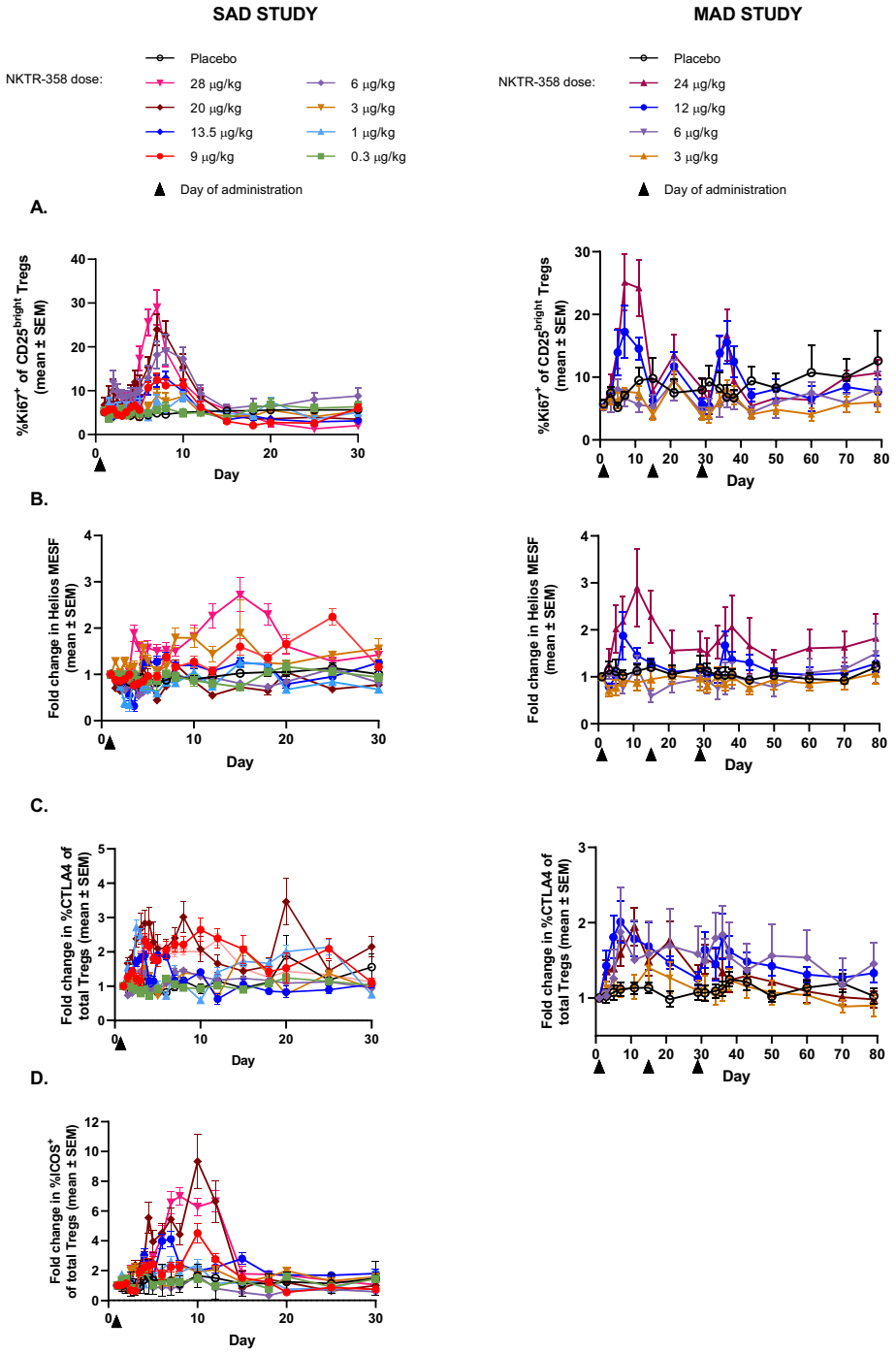
Abbreviations: MAD: multiple ascending dose; SAD: single ascending dose; Treg: regulatory T cell.

Supplementary Fig. 4. Correlation of Tregs with FoxP3 demethylated at A) 28 µg/kg of NKTR-358 (SAD study) and B) 24 µg/kg of NKTR-358 (MAD study).



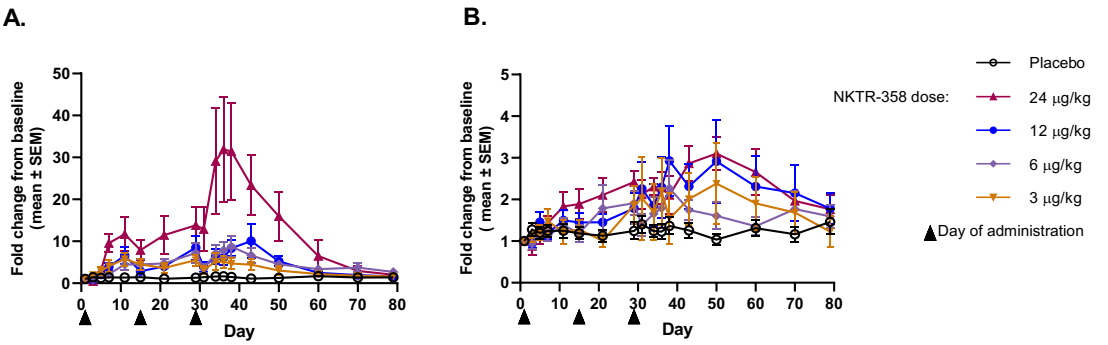
Each data point represents an individual blood sample from nine NKTR-358-treated patients where data were available for both flow cytometry and demethylation assays.
Abbreviations: MAD: multiple ascending dose; SAD: single ascending dose; Treg: regulatory T cell; TSDR: Treg-specific demethylated region.

Supplementary Fig. 5. Changes in the percentage or expression of Treg markers in peripheral blood after a single dose of NKTR-358 in the SAD study and with multiple doses of NKTR-358 in the MAD study. A) percentage of Ki67 on CD25^{bright} Tregs; B) Helios MESF on total CD4^{+} Tregs; C) percentage of CTLA4 on total CD4^{+} Tregs and D) percentage of ICOS on total CD4^{+} Tregs



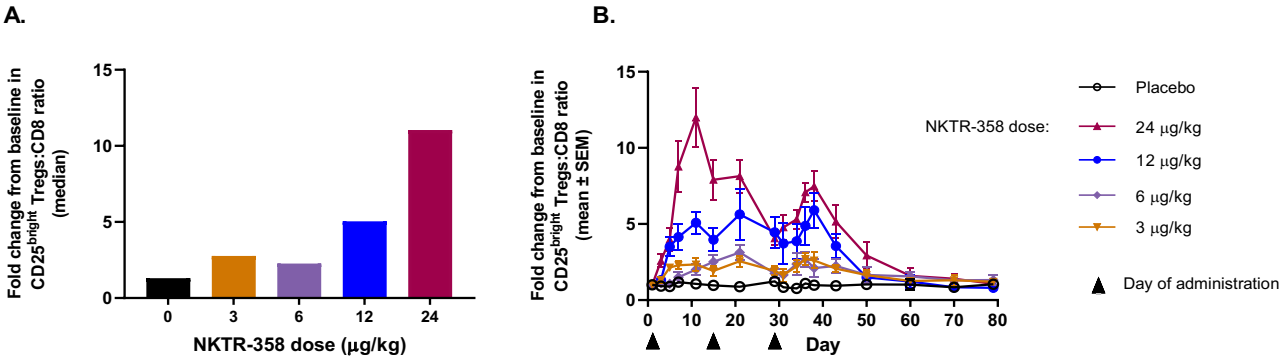
Abbreviations: CTLA4: cytotoxic T-lymphocyte-associated protein 4; ICOS: inducible T cell costimulatory; MAD: multiple ascending dose; MESF: molecules of equivalent soluble fluorochrome; SAD: single ascending dose; SEM: standard error of the mean; Treg: T regulatory cell.

Supplementary Fig. 6. Fold-change in frequency (percentage CD45) of A) $CD56^{bright}$ $CD16^{-}$ NK cells and B) in $CD56^{dim}$ $CD16^{+}$ NK cells following multiple doses of NKTR-358 in the MAD study.



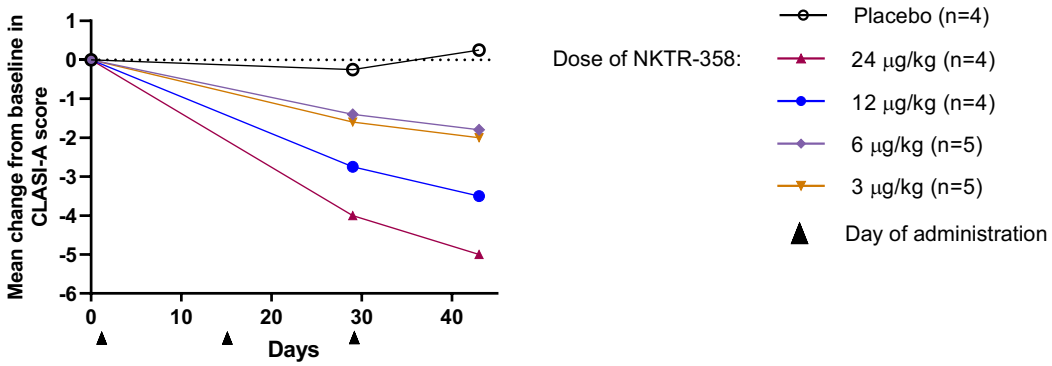
Abbreviations: MAD: multiple ascending dose; NK: natural killer; SEM: standard error of the mean.

Supplementary Fig. 7. Changes in Treg expansion after multiple administrations of NKTR-358 in the MAD study. A) Peak level of fold-change in CD25^{bright} Tregs:CD8^{+} ratio after first dose and B) fold-change in CD25^{bright} Tregs:CD8^{+} ratio after each dose



Abbreviations: MAD: multiple ascending dose; SEM: standard error of the mean; Treg: T regulatory cell.

Supplementary Fig. 8. Mean change in CLASI-A score from baseline with NKTR-358 in the 22 patients with SLE with a CLASI-A score of ≥4 at baseline in the MAD study.



Abbreviations: CLASI-A: cutaneous lupus erythematosus disease area and severity index–activity; MAD: multiple ascending dose; SLE: systemic lupus erythematosus.