SUPPLEMENTAL APPENDIX

Supplemental methods

Actual administration of the treatment regimen in practice:

- Drug name: sutimlimab
- Dose: 10 mg/kg test dose followed by 60 mg/kg 4 times at weekly intervals (in the future, this will likely change to a flat dose of approximately 5.5 g every other week, after 2-weekly infusions)
- Route: parenteral, no central venous access required unless poor peripheral venous access
- Type and volume of diluent: 500 mL of NaCl 0.9% over 1 hour; rate of administration
- Cycle length and number of cycles, or criteria for discontinuation: not applicable
- Premedications and concurrent medications: no premedication required
- Patient-monitoring parameters: routine checks for hemolytic anemia

Flowcytometry of erythrocytes

Cell-bound C3 fragments were detected by staining erythrocytes (10 µL; 1:5 diluted) at 4°C for 45 minutes with a murine mAb recognizing human C3/C3b/iC3b (50 µL; Pierce™ complement C3b Antibody (6C9), 1 mg/mL; Thermo Fisher Scientific, Waltham, MA). After 3 washes in fluorescence-activated cell sorter (FACS) buffer (PBS plus 0.5% bovine serum albumin plus 0.1% NaN3), Alexa Fluor 488 goat anti-mouse IgG1 (1.5 mg/mL; Molecular Probes by Life Technologies, Eugene, OR; diluted 1:1000 in FACS buffer) was added at room temperature for 30 minutes. Cells were washed 3 times in FACS buffer and were analyzed by flow cytometry (FACScan, Becton Dickinson, Vienna, Austria). Lysis II software was used for analysis. Non-labeled erythrocytes and erythrocytes treated with the second step antibody alone were used to exclude any background; 30,000 events were acquired for each sample. Serum concentrations of sutimlimab were measured with a validated immunoassay by a GLP-certified laboratory (Vela Laboratories, Vienna, Austria).

N-of-1 trials in named patient program

Background: The first-in-human clinical trial examined the safety and efficacy of sutimlimab in patients with cold agglutinin disease. Four weekly doses of sutimlimab completely corrected hemolytic anemia in several patients and caused a complete arrest of hemolysis and hemoglobin rise of >4 g/dL, particularly in patients who had no concurrent lymphoma or mixed autoimmune hemolytic anemia. After completion of washout of protocol treatment, each of the responding patients suffered a relapse of hemolytic anemia with a drop-in hemoglobin to pre-treatment levels.

Rationale: Although the primary objective of named patient drug support was simply to give these patients the access to treatment with sutimlimab that was otherwise unavailable to them, this also provided the occasion: 1) to explore lower dose regimens and alternative dosing schedules in individual N-of-1 trials; 2) to try to recapitulate the initial success of complete remission under protocol treatment; 3) to test the durability of response with a longer-term maintenance treatment with sutimlimab.

Methods: All clinical procedures were conducted in the same manner and at the same investigational site where the sutimlimab-01 trial was conducted.

Dosing:

45 mg/kg sutimlimab dose regimen (4 weekly doses, then every other week; n=3)

After finishing the sutimlimab-01 trial, the first 2 relapsing patients and patient C1008 received a modestly reduced dose regimen consisting of 4 weekly doses of 45 mg/kg sutimlimab, followed by 45 mg/kg every other week until breakthrough.

60 mg/kg sutimlimab dose regimen (2 weekly doses, then every other week; n=5)

Based upon the experience of re-treating the first 2 patients, a simplified dosing regimen was then tried for the named patients, using a single priming dose of 60 mg/kg on day 0 followed by 60 mg/kg every other week (biweekly) thereafter beginning on day 7. This biweekly maintenance dose of 60 mg/kg sutimlimab was continued until biochemical breakthrough or washout for determination of drug-anti-drug antibodies.

A fixed dose of 5.5 g every other week was eventually started (n=3).

Supplemental results of the N-of-1 trials

All infusions of 45mg/kg were well tolerated without premedication and without relevant adverse effects by all three patients. As expected, sutimlimab re-exposure immediately decreased CH50 activity and increased complement C4 levels. Sutimlimab rapidly abrogated extravascular hemolysis, normalizing bilirubin levels in all patients within 1 week. Infusion of sutimlimab swiftly blocked the destruction of reticulocytes. Sutimlimab increased hemoglobin levels by 2.8-5.8 g/dL (range). Haptoglobin levels started to normalize within 14 days in all patients. Hemolysis recurred 2 weeks after the sixth infusion of 45mg/kg in all three patients as indicated by a decrease in haptoglobin and a rise in CH50, bilirubin, and lactate dehydrogenase.

All infusions of 60 mg/kg were well tolerated without premedication and without relevant adverse effects by all five patients. Sutimlimab increased hemoglobin levels by 3.0-4.4g/dL (range) except in one patient who started off with the highest hemoglobin (11.1 g/dL) immediately after the breakthrough event. Sutimlimab also normalized haptoglobin levels in those patients who achieved a normal haptoglobin in a previous period of treatment with sutimlimab. The dose of 60 mg/kg every other week appeared to maintain responses better than 45 mg/kg, but laboratory evidence of biochemical breakthrough events also occurred in 2 patients after 8 or 9 doses of 60 mg/kg, respectively.

Three patients received the fixed dose of 5.5g every other week. Patient C1001 reached a maximum of 12.5 g/dL within 2 months and her haptoglobin levels increased 10-fold above baseline. Patient C1002 also responded with a rapid stop of hemolysis, but she had two extended periods of vacation resulting in breakthrough hemolysis 18 or more days after the last infusions, respectively. In both cases the patient returned to the research ward with hemoglobin values of ~6.5 g/dL confirming her dependence on sutimlimab. The patient reached normal

hemoglobin levels again after re-starting 5.5g sutimlimab every other week. In total, the patient has so far experienced 7 on/off periods with sutimlimab. Statistically there is a 1:128 likelihood that she would respond similarly just by chance. This N-of-1 trial therefore demonstrates that sutimlimab significantly improved anemia in this patient.

Potential benefit of additional erythropoietin treatment in case of inadequate production

Patient C1004 was supported with erythropoietin 5000 U 3 times per week in addition to sutimlimab, which hastened the hemoglobin increase as compared with the main study. She received 10 infusions of 60 mg/kg sutimlimab. The patient's hemoglobin increased from 7.9 to 9.0 g/dL within 1 week, and hemoglobin reached 13.4 g/dL after 9 weeks. Due to the diagnosis of a massive inoperable uterine cancer with unilateral hydronephrosis, which required radiotherapy, the patient's participation was terminated in the named patient program and the patient became transfusion dependent again, requiring regular transfusions every 1–2 weeks.

Patient C1010 patient had developed renal insufficiency and bone marrow aplasia (severe neutropenia) after the last chemotherapy (R-ESHAP for a 60% bone marrow infiltration with lymphoplasmacytic lymphoma). As sutimlimab quickly stopped hemolysis, but a further increase in hemoglobin appeared to have halted, we evaluated the patient for relative erythropoietin deficiency. Once we started the patient on erythropoietin, the patient quickly responded with an increase in the highly fluorescent fraction of reticulocytes. Erythropoietin further boosted the patient's hemoglobin levels to 11.6 g/dL. We continued erythropoietin treatment (10,000 U 3 times per week) even after the sutimlimab-01 trial when she washed out. This was done to confirm that the hemolysis resolution and subsequent improvement in hemoglobin was mainly due to sutimlimab treatment and that erythropoietin simply gave sufficient capacity to regenerate new erythrocytes. Indeed, the patient had a breakthrough event at the end of trial visit (day 53), when hemoglobin dropped from a peak of 11.6 g/dL to 9.6 g/dL within 1 week. Further hemoglobin checks showed a relatively slow decline to 7.3 g/dL despite continuing erythropoietin treatment, proving that erythropoietin alone was insufficient to maintain a response. We then administered the fixed dose of 5.5 g sutimlimab, which induced a remarkable 4.7 g/dL increase in hemoglobin to 12 g/dL after only 28 days, while the patient continued additional erythropoietin therapy.

Overall conclusions

(i) Re-treatment of responders with sutimlimab unequivocally confirmed its good safety and efficacy profile. Some of the patients have now been on and off sutimlimab for 2.5 years without relevant adverse effects and very good responses. All patients have achieved hemoglobin levels of 12 g/dL at least once.

(ii) Bilirubin appears to be a very sensitive and early marker of treatment response to sutimlimab and relapse of patients with cold agglutinin disease, but bilirubin also decreases in patients where the hemoglobin response is less pronounced (eg, due to IgG antibodies or tumor load).

(iii) Patients can have normal hemoglobin levels despite undetectable haptoglobin levels, showing that haptoglobin levels are a less clinically relevant endpoint compared with hemoglobin.

Supplemental Table S1. Comparison of treatment responses in the first-in-human trial and the maximal absolute changes in hemoglobin (g/dL) during the different periods of the N-of-1 trials

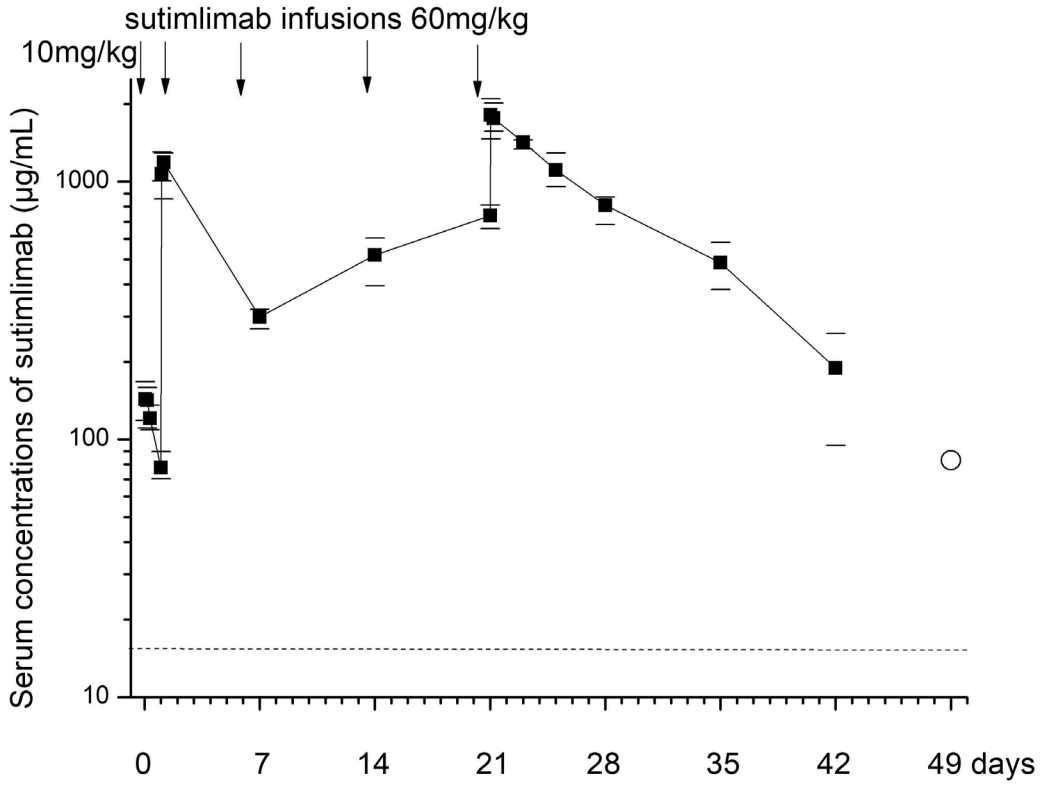
	First-in-human trial	Periods of the N-of-1 trials		
	60 mg/kg weekly	45 mg/kg qw/eow	60 mg/kg LD/eow	5.5 g LD/eow
Patient				
C1001	8.3 / +3.8	8.8 / +2.8	8 .9 / +3.0	9.0 / +3.5
C1002	7.5 / +4.8	7.1 / +4.1	11.1 / +0.1	6.4 / +5.0
C1003	7.9 / +0.5		7.7 / +4.5	
C1004	6.8 / +4.0		7.9 / +5.5	
C1006	7.7 / +4.5		8.1 / +4.4	
C1008	8.2 / +5.0	7.4 / +5.8		
C1009	6.1 / +3.7			
C1010	7.6 / +4.0			7.3 / +4.7
C1011	9.3 / +1.3			
C1013	10.4 / +0.9			
Median baseline hemoglobin	7.8	7.4	8.1	7.3
Median change in hemoglobin	/ +3.9	/ + 4.1	/ + 4.4	/ + 4.7
95% confidence intervals for change	2.0-4.5	0.5-8.0	1.0-6.2	2.4-6.4

Patients received 10 mg/kg followed by weekly doses (qw) of 60 mg/kg for 4 weeks in the first-in-human trial. Period 1: The N-of-1 trials started with 45 mg/kg every week, followed by 45 mg/kg every other week (eow). Period 2: Then patients received a 60 mg/kg loading dose (LD) on days 1 and 8 followed by 60 mg/kg eow. Period 3: Patients received a 5.5 g loading dose on

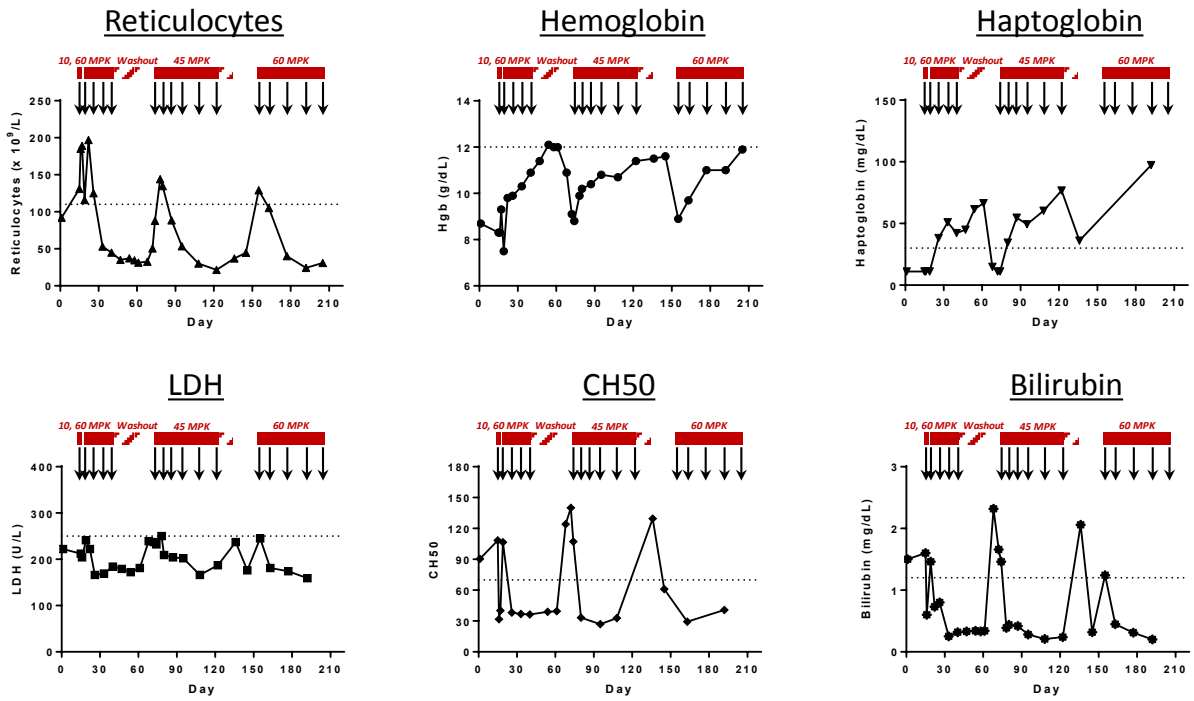
days 1 and 8 followed by 5.5 g eow in period 3. Individual participation in periods 1 and 2 was terminated when there was biochemical evidence of breakthrough hemolysis, indicating that the doses were insufficient to support infusion intervals of 14 days.

Supplemental Table S2. Safety results

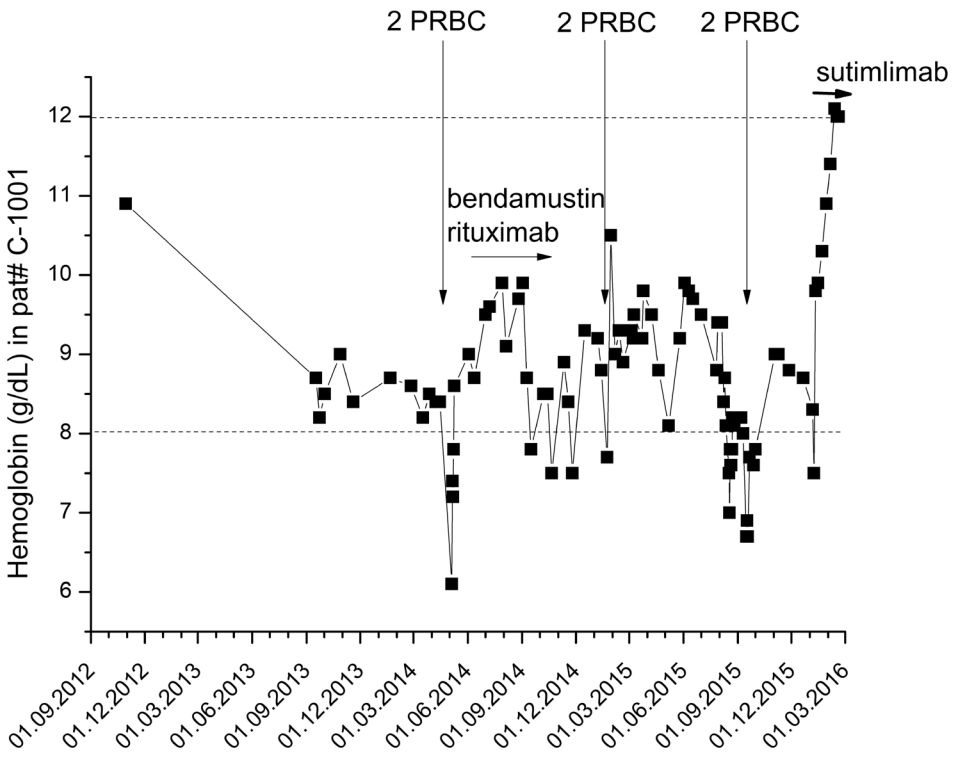
System Organ Class	AE term	Severity	Causal relationship to study drug	n
Ear and labyrinth disorders				
	VERTIGO	Grade 1 – mild	Unrelated	1
Gastrointestinal disorders				
	EMESIS REPEATEDLY	Grade 1 - mild to moderate	Unlikely	1
	DIARRHEA	Grade 2 – moderate	Unlikely	1
General disorders and administration site conditions				
	SHIVERING	Grade 1 – mild	Unlikely	1
	FATIGUE	Grade 1 – mild	Unlikely	1
Infections and infestations				
	DENTAL ABSCESS	Grade 2 - moderate	Unlikely	1
	HERPES LABIALIS	Grade 1 - mild	Unrelated	1
Injury, poisoning, and procedural complications				
	VERTEBRAL BONE FRACTURE	Grade 2 - moderate	Unrelated	1
	CONTUSED WOUND LEFT THUMB	Grade 2 - moderate	Unrelated	1
	FALLS REPEATEDLY (PRE-EXISTING NEUROLOGICAL DISORDER)	Grade 1 - mild	Unrelated	1
Nervous system disorders				
	DIZZINESS	Grade 1 - mild	Unlikely	2
	FAINTING (AFTER WORKING IN THE COLD)	Grade 1 - mild	Unlikely	1
Renal and urinary disorders				
	HEMATURIA REPEATEDLY	Grade 1 - mild to moderate	Unlikely	1
Skin and subcutaneous tissue disorders				
	NIGHT SWEATS	Grade 1 - mild	Unlikely	1
	PRURITIC EXANTHEMA UPPER ARMS	Grade 2 - moderate	Unlikely	1
	PURPURAL RASH BOTH HANDS	Grade 1 - mild	Possible	1
	NIGHT SWEAT	Grade 1 - mild	Unlikely	1
	HAIR LOSS	Grade 2 - moderate	Possible	1
Vascular disorders				
	RAYNAUD SYMPTOMS (ACROCYANOSIS REPEATEDLY)	Grade 2 - moderate	Unlikely	1



Supplemental Figure 1. Pharmacokinetics of sutimlimab. Pharmacokinetic profile of sutimlimab in patients with cold agglutinin disease (n = 10; median and interquartile ranges). Patients received 4 weekly infusions of the anti-C1s antibody sutimlimab (60 mg/kg on days 1, 7, 14, and 21) following a test dose of 10 mg/kg 1 day (first patient's data is not presented off-set for clarity) or 4 days earlier. At the last visit, sutimlimab concentrations were below the detection limit in all except 1 patient (open circle). The dotted line represents the pharmacodynamic threshold of 15.5 µg/mL observed in healthy volunteers (compare also Figure 3 in main manuscript).



Supplemental Figure S2. Biochemical response pattern upon repeat administration of sutimlimab. The temporal link between complement blockade (suppressed CH50 activity) and response during the crossover between periods on-drug and off-drug is depicted for patient C1001. The 45 mg/kg dose of sutimlimab every other week was insufficient and induced a breakthrough (rise in CH50 and bilirubin, but fall in haptoglobin).



Supplemental Figure S3. Comparison of historical hemoglobin values to sutimlimab response in patient C1001. PRBC, packed red blood cell,

CONSORT

TRANSPARENT REPORTING of TRIALS

CONSORT 2010 Flow Diagram