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A CD3xCD123 bispecific DART for redirecting host T cells to myelogenous leukemia: Preclinical activity and safety in nonhuman primates

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Current therapies for acute myeloid leukemia (AML) are largely ineffective, and AML patients may benefit from targeted immunotherapy approaches. MGD006 is a bispecific CD3xCD123 dual-affinity re-targeting (DART) molecule that binds T lymphocytes and cells expressing CD123, an antigen up-regulated in several hematological malignancies including AML. MGD006 mediates blast killing in AML samples, together with concomitant activation and expansion of residual T cells. MGD006 is designed to be rapidly cleared, and therefore requires continuous delivery. In a mouse model of continuous administration, MGD006 eliminated engrafted KG-1a cells (an AML-M0 line) in human PBMC (peripheral blood mononuclear cell)-reconstituted NSG/ $\beta 2m^{-/-}$ mice at doses as low as 0.5 $\mu\text{g/kg}$ per day for ~7 days. MGD006 binds to human and cynomolgus monkey antigens with similar affinities and redirects T cells from either species to kill CD123-expressing target cells. MGD006 was well tolerated in monkeys continuously infused with 0.1 $\mu\text{g/kg}$ per day escalated weekly to up to 1 $\mu\text{g/kg}$ per day during a 4-week period. Depletion of circulating CD123-positive cells was observed as early as 72 hours after treatment initiation and persisted throughout the infusion period. Cytokine release, observed after the first infusion, was reduced after subsequent administrations, even when the dose was escalated. T cells from animals with prolonged *in vivo* exposure exhibited unperturbed target cell lysis *ex vivo*, indicating no exhaustion. A transient decrease in red cell mass was observed, with no neutropenia or thrombocytopenia. These studies support clinical testing of MGD006 in hematological malignancies, including AML.

INTRODUCTION

Acute myeloid leukemia (AML) is a disease with abnormal hematopoiesis resulting from rapid proliferation of immature myeloid cells in the bone marrow. Treatment of AML has changed little in recent decades and consists of standard induction chemotherapy whose outcome has been disappointing. The interleukin-3 (IL-3) receptor α chain CD123 is overexpressed on cancerous cells in a wide range of hematological malignancies including AML (1, 2) and is associated with poor prognosis (3), possibly owing to its expression on leukemia stem cells (LSCs) (4–6). The IL-3/CD123 autocrine axis may play a role in sustaining leukemogenesis, as shown by the ability of a CD123-blocking monoclonal antibody (mAb) to reduce LSC engraftment and improve survival in a mouse model of AML (5). In a phase 1 study in high-risk AML patients, however, a CD123-blocking mAb, CSL-360, did not demonstrate considerable antileukemic activity (7), spurring an interest in alternate CD123-targeting approaches, including cell-depleting strategies. Such depleting strategies appear feasible because CD123, despite being expressed by a subset of normal hematopoietic progenitor cells, shows little, if any, expression by hematopoietic stem cells (HSC) (4, 8), suggesting that reconstitution of normal hematopoiesis should occur after depletion. Enabling the patient's own T lymphocytes to target leukemic cells for elimination represents a promising immunotherapeutic strategy for the treatment of hematological malignancies, whose potential has been demonstrated by blinatumomab, an antibody-based bispecific T cell engager (BiTE) against CD3 and CD19, as well as by

chimeric antigen receptor-transduced T cells (CAR-T cells) in patients with B cell lymphomas and leukemias (9–12).

The dual-affinity re-targeting (DART) scaffold is a bispecific, antibody-based modality that offers improved properties of stability and manufacturability and compared favorably to the BiTE format in terms of potency (13, 14). A CD3xCD123 DART protein, MGD006 (also referred to as Les Laboratoires Servier's compound S80880), was engineered with antibodies that cross-react with both human and cynomolgus monkey targets. Here, we report the engineering and *in vitro* evaluation of MGD006, as well as its pharmacology in a mouse xenograft model that mimics clinical delivery and in cynomolgus macaques, an appropriate primate species for ascertaining preclinical safety and activity.

RESULTS

Engineering, physicochemical characterization, and binding properties of MGD006

MGD006 was engineered by incorporating the humanized anti-CD123 and anti-CD3 variable domains into a disulfide-linked heterodimer (13), driven by oppositely charged coiled-coil sequences (E/K coils) (Fig. 1A). Short linkers between the V_L (variable region of immunoglobulin light chain) and V_H (variable region of immunoglobulin heavy chain) segments promote “diabody”-type association, and oppositely charged coiled-coil sequences (E/K coils) promote heterodimeric association with the disulfide bond stabilizing the structure (15). The purified MGD006 is a homogeneous heterodimer with a molecular mass of 58.9 kD (fig. S1, A to C) that was stable at 2° to 8°C for at least 12 months in phosphate-buffered saline (PBS) (fig. S1D). MGD006

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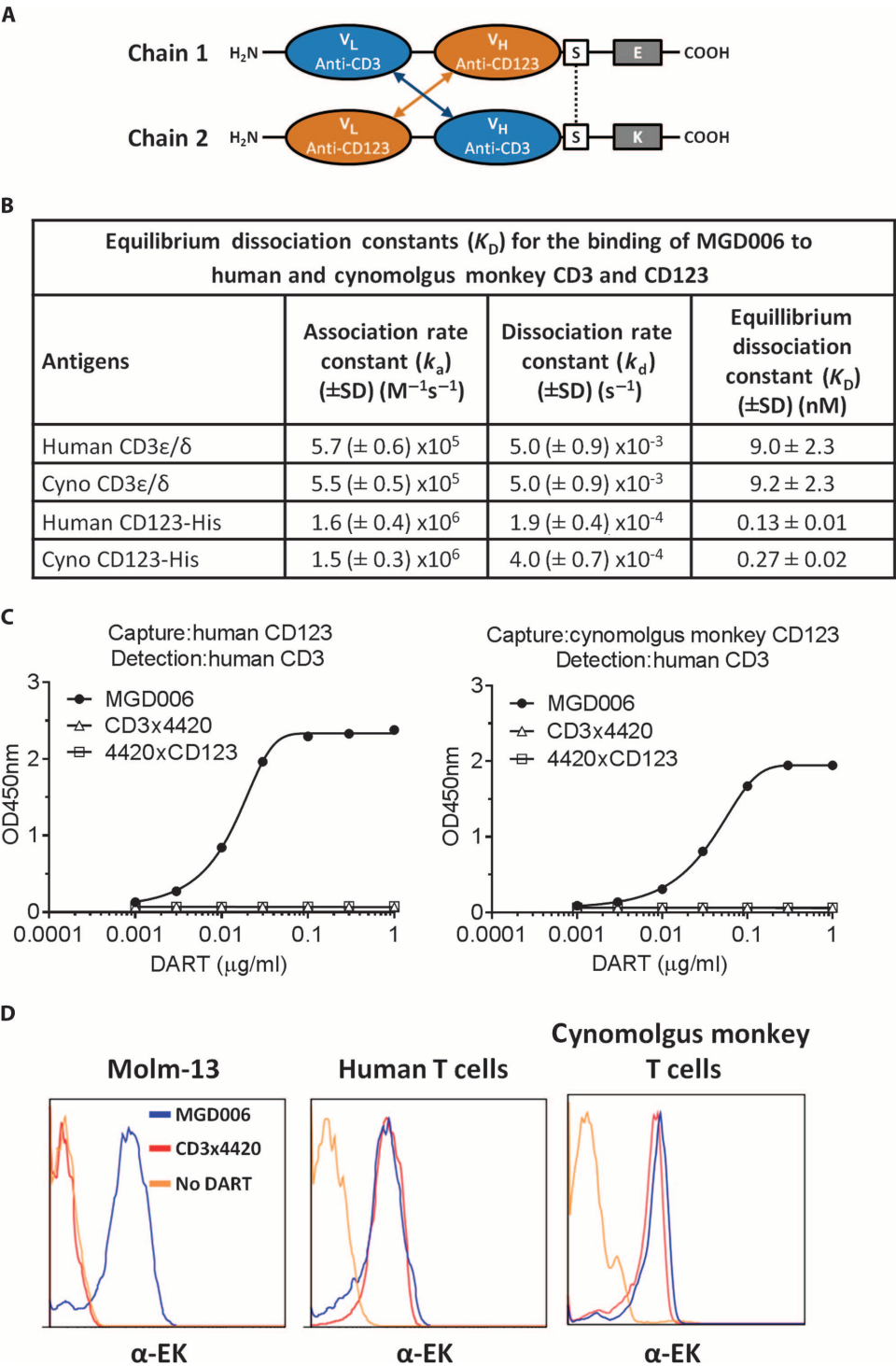


Fig. 1. MGD006 binding to human and cynomolgus monkey CD3 and CD123. (A) Schematic representation of MGD006. (B) Equilibrium dissociation constants (K_D) for the binding of MGD006 to human and cynomolgus (Cyno) monkey CD3 and CD123 determined by surface plasmon resonance analysis. (C) Bi-functional ELISA demonstrates simultaneous engagement of both target antigens by MGD006. ELISA plates were coated with human CD123 (left) or cynomolgus monkey CD123 (right). DART at a range of concentrations was added, and binding was detected with human CD3-biotin. OD, optical density. (D) Cell surface binding of MGD006 to CD123⁺ Molm-13 target cells (left), human T cells (middle), and cynomolgus T cells (right) was detected by fluorescence-activated cell sorting (FACS) with a mAb specific to E-coil and K-coil regions of the DART (α -EK).

demonstrated similar binding affinities and kinetics to human and cynomolgus monkey CD3 and CD123 antigens (Fig. 1B and fig. S1E). Furthermore, MGD006 simultaneously bound both antigens in a bispecific enzyme-linked immunosorbent assay (ELISA) format that used human or monkey CD123 for capture and CD3 for detection (Fig. 1C) and exhibited similar cell surface binding to human and monkey T lymphocytes (Fig. 1D).

MGD006 activity in primary AML patient samples

Leukemic blasts from an AML patient sample were identified as CD45^{med+}/CD33⁺ cells in the peripheral blood mononuclear cells (PBMCs) and demonstrated robust CD123 expression (Fig. 2A and fig. S2). Treatment with MGD006 over a 6-day period resulted in a dose-dependent depletion of leukemic blasts accompanied by a concomitant expansion of autologous T cells, up-regulation of the proliferation marker Ki-67, and a proportionally greater expansion of CD8⁺ cells. Greater activation (CD25 expression) of CD4⁺ than CD8⁺ cells was observed, with IFN- γ (interferon- γ) and IL-6 being the predominant cytokines produced. Expression of granzyme B and perforin, however, was elevated in CD8⁺ cells with only a modest elevation in CD4⁺ cells (Fig. 2A). Similar data were obtained with a bone marrow sample from a separate AML patient whose blasts expressed moderate levels of CD123 (fig. S3). These data show that MGD006 is capable of expanding and redirecting autologous T cells from AML patients toward leukemic blast cell killing.

Antitumor activity in human PBMC-reconstituted tumor-bearing mice continuously exposed to MGD006

We previously demonstrated that redirected T cell tumor killing can be recapitulated by systemic DART protein injections in xenograft mouse models in which human PBMCs are provided as a source of effector cells (14). On the basis of its design, the short circulating half-life of MGD006 allows close regulation of the exposure in patients but requires continuous infusion to ensure prolonged and adequate exposure. To ascertain MGD006 antitumor activity under conditions recapitulating

the clinical settings, MGD006 was delivered continuously for up to a week via peritoneally implanted osmotic pumps in NSG/ $\beta 2m^{-/-}$ mice reconstituted with human PBMCs and grafted intradermally with KG-1a cells, an AML-M0 line. KG-1a cells express moderate levels of CD123 and can be efficiently killed ex vivo by primary human T cells in the presence of MGD006 (fig. S4). Xenograft tumors of implanted AML cell lines have been previously used to evaluate the potency of AML therapeutics (16–19). Treatment was initiated after tumors were allowed to grow to an average size of $\sim 100 \text{ mm}^3$. Significant tumor regression was observed in mice treated with MGD006 at all doses tested

($P < 0.005$), whereas no activity was observed in animals treated with the CD3x4420 control DART (Fig. 2B). Although a dose response was not established, 500 ng/kg per day was sufficient to completely eliminate the tumor cells.

The cynomolgus macaque as a target-appropriate species for MGD006 pharmacology

The tissue distribution of CD3 and CD123 in cynomolgus monkeys was comparable to that in humans (1, 8), as determined by immunohistochemistry with precursor mAbs and MGD006. CD123-positive staining

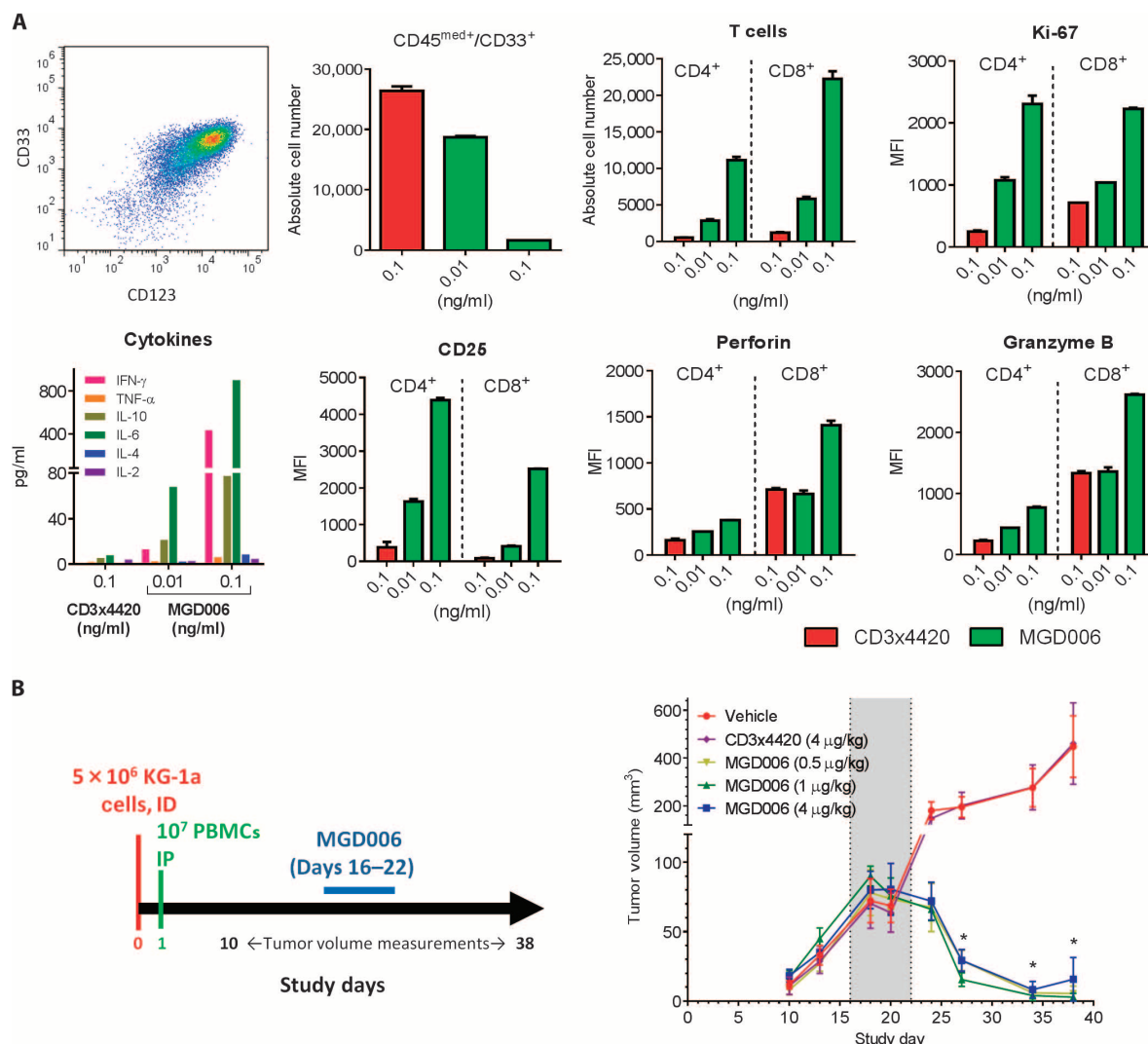


Fig. 2. MGD006-mediated killing of blasts and expansion and activation of T cells in primary AML patient PBMCs. (A) CD123 and CD33 expression on ungated AML patient primary PBMCs. Primary AML sample was incubated with MGD006 for 144 hours, and the absolute numbers of CD45^{med+}/CD33⁺ leukemic blast cells and CD4 and CD8 T cells and the mean fluorescence intensity (MFI) of CD25 in T cells and of granzyme B, perforin, and Ki-67 on T cells were determined by FACS. Cytokines were measured in the culture supernatants after 144 hours of the indicated treatments. TNF- α , tumor necrosis factor- α . **(B)** NSG/ $\beta 2m^{-/-}$ mice were intradermally implanted with the KG-1a (AML-M0) cells on day 0 and intraperitoneally (IP) injected with human

PBMCs on day 1. MGD006 was administered via continuous infusion with peritoneally implanted mini-osmotic pumps from days 16 to 22. Tumor volumes (means $\pm$ SEM) were measured in human PBMC-reconstituted mice ($n = 8$ per group) implanted intradermally with KG-1a cells and treated with the indicated doses of MGD006. The filled gray area indicates continuous delivery of MGD006 or controls using osmotic pumps. Relative to the vehicle control group, tumor growth was significantly inhibited (* indicates significant difference; P values are provided in table S2) from day 27 in the MGD006-treated groups. Statistical analyses used two-way analysis of variance (ANOVA). Data are representative of two independent experiments. ID, intradermally.

was limited to the cytoplasm and membrane of hematopoietic bone marrow cells and cytoplasm of the endothelium in multiple tissues in both species. CD123 was also expressed by subsets of circulating leukocytes, including CD14⁺/CD123⁺ cells [encompassing plasmacytoid dendritic cells (pDCs) and basophils] and CD14⁺/CD123⁺ cells (encompassing classical monocytes) (Fig. 3A). CD14⁺/CD123⁺ cells expressed CD123 at levels that were comparable between humans and monkeys. CD123 expression in the CD14⁺ cell population was lower than in CD14⁺/CD123⁺ cells and comparatively less in monkeys than in humans. Incubation of human or monkey PBMCs with MGD006 resulted in a dose-dependent depletion of CD14⁺/CD123⁺ cells (Fig. 3B). Furthermore, MGD006 redirected monkey effectors to kill Kasumi-3 cells with potency consistent with that of human effector cells (Fig. 3C). Together, these data indicate that the cynomolgus monkey represents a cross-reactive, target-appropriate species for testing MGD006.

MGD006 toxicology and pharmacodynamics in cynomolgus macaques

To ascertain the tolerability, potential toxicity, pharmacokinetics, and pharmacodynamic effects of MGD006 and to guide dose selection in humans, we conducted a toxicology study in cynomolgus monkeys consisting of six treatment groups (four males and four females per group) comparing various dose levels and two dosing schedules (Table 1). The design was aimed at ascertaining tolerability and was informed by a series of small studies that established starting dose ranges and preliminary pharmacokinetics. A continuous infusion paradigm was used, with percutaneous ports surgically implanted in the femoral and subclavian veins. All groups received vehicle for the first infusion, followed by vehicle or MGD006 for four weekly cycles. Group 1 animals received vehicle for all four subsequent infusions, whereas group 2 received the same weekly dose (100 ng/kg per day); groups 3 to 5 received escalating doses of MGD006 continuously for 4 days a week (4-day-on/3-day-off) for all subsequent infusions. Group 6 animals were treated with 7 days of uninterrupted (7-day-on) weekly escalating doses of MGD006 for all infusions. The 4-day-on/3-day-off and 7-day-on schedules were implemented to distinguish between durable and transient effects associated with MGD006 administration and guide human dosing with respect to reversibility of these effects. Two males

and two females per group were necropsied at the end of the treatment phase (day 36), and the remaining monkeys were necropsied after a 4-week recovery (day 65).

Pharmacokinetics of MGD006 in cynomolgus macaques

Concentration analysis of MGD006 in serum samples collected at the end of each infusion from group 6 animals showed a dose-dependent increase (fig. S5A). Pharmacokinetic parameters (two-compartment

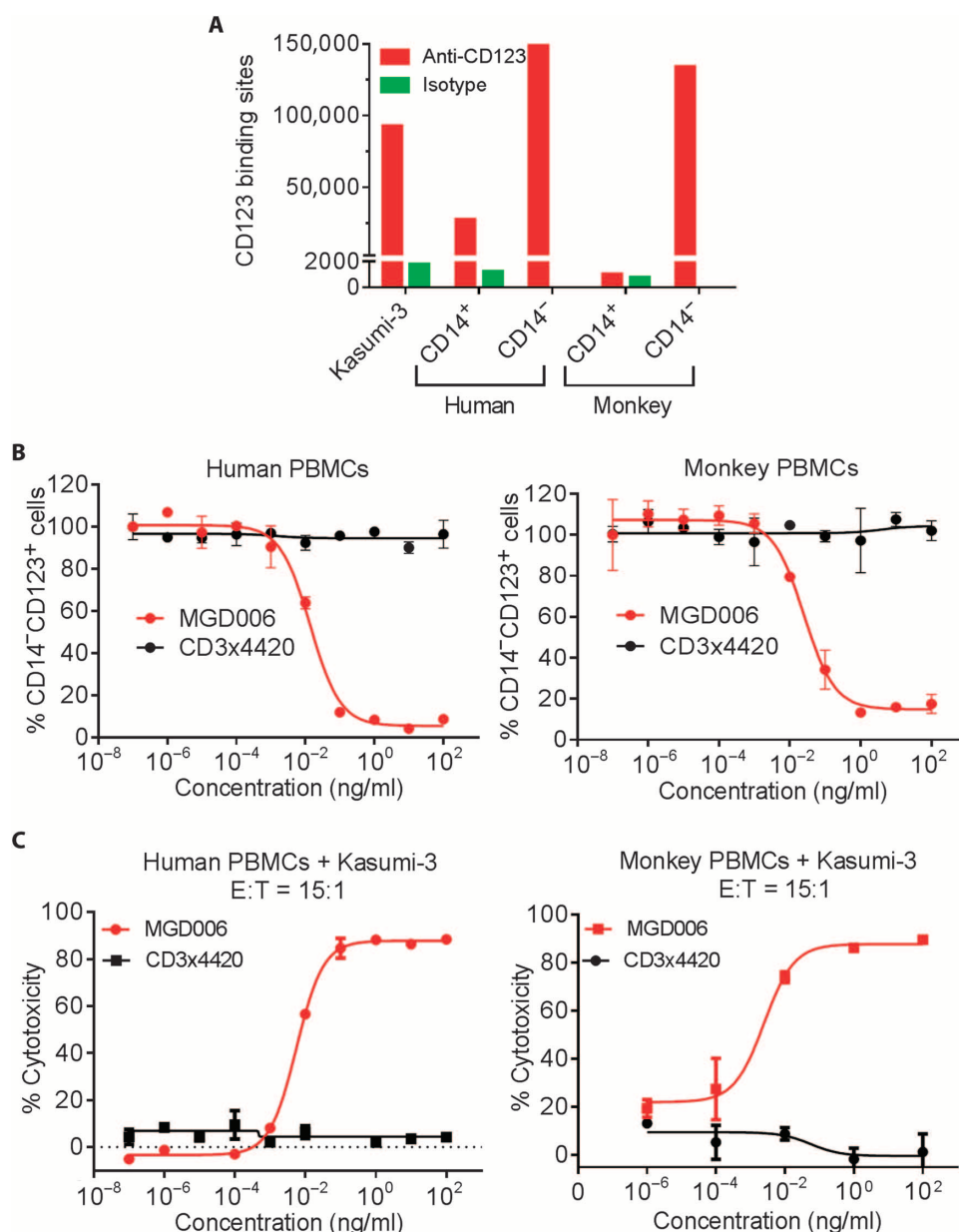


Fig. 3. Comparison of human and cynomolgus macaque cell populations and effector function. (A) CD123 mAb binding sites on Kasumi-3, CD14⁺/CD123⁺, and CD14⁺/CD123⁺ cells ("monocytes") in human and cynomolgus monkey PBMCs determined by quantitative FACS analysis. (B) Percentage of CD14⁺/CD123⁺ cells in human or cynomolgus monkey PBMCs incubated for 24 hours with MGD006 or CD3x4420-DART. (C) MGD006 and CD3x4420-DART were evaluated for cytotoxicity against the Kasumi-3 AML cell line using human PBMCs or cynomolgus PBMCs. Cytotoxicity was determined by FACS using CMTMR-labeled target cells.

Table 1. MGD006 pharmacology and toxicology in cynomolgus monkeys: Study design.

Infusion no.	Study day	Vehicle*	MGD006 [†] (4-day-on/3-day-off) ng/kg per day [ng/kg per 4 days]					MGD006 [†] (7-day-on) ng/kg per day [ng/kg per 7 days]
			Group 1 (4 M/4 F)	Group 2 (4 M/4 F)	Group 3 (4 M/4 F)	Group 4 (4 M/4 F)	Group 5 (4 M/4 F)	Group 6 (4 M/4 F)
1	1	Vehicle		Vehicle	Vehicle	Vehicle	Vehicle	Vehicle
2	8	Vehicle		100 [400]	100 [400]	100 [400]	100 [400]	100 [700]
3	15	Vehicle		100 [400]	300 [1200]	300 [1200]	300 [1200]	300 [2100]
4	22	Vehicle		100 [400]	300 [1200]	600 [2400]	600 [2400]	600 [4200]
5	29	Vehicle		100 [400]	300 [1200]	600 [2400]	1000 [4000]	1000 [7000]
Recovery	36–65	—	—	—	—	—	—	—

*PBS (pH 6.0) containing recombinant human albumin (0.1 mg/ml), polysorbate 80 (PS-80; 0.1 mg/ml), and 0.24% benzyl alcohol was used for all vehicle infusions during the first 4 days of each infusion week. [†]MGD006 was administered at the required concentration for the indicated amounts of time as a continuous intravenous infusion in a solution of PBS (pH 6.0) containing recombinant human albumin (0.1 mg/ml), PS-80 (0.1 mg/ml), and 0.24% benzyl alcohol.

model) were estimated for the 300 and 600 ng/kg per day doses (groups 3 and 4 on days 15 and 22, respectively), when frequent sampling was used (before infusion; 4 and 24 hours after start of infusion; end of infusion; and 0.25, 1, 3, 8, 24, and 48 hours after end of infusion) (fig. S5B). $T_{1/2\alpha}$ (distribution half-life) was short (~4 min) and, together with the volume of distribution (~1 to 2 liters/kg), suggests rapid and extensive binding to target cells in tissues. MGD006 was rapidly cleared after the end of infusion, with a mean residence time of only 7 to 9 hours, as expected for a molecule of this size (59 kD), which is likely subject to renal filtration.

Detectable antidrug antibodies (ADAs) developed in 70% (28 of 40; 4 of 8 in group 2, 5 of 8 in group 3, 6 of 8 in group 4, 8 of 8 in group 5, and 5 of 8 in group 6) of MGD006-treated animals within 2 to 3 weeks of the first administration; however, exposure continued despite the presence of ADA, with all animals showing circulating MGD006 levels through at least two MGD006 infusion cycles and with 60% (24 of 40) of the animals being exposed during all four infusion cycles. Persistent CD123⁺ cell or CD303⁺ cell depletion also generally confirmed that most animals continued to be exposed for four cycles (Fig. 4A and fig. S6). The range of ADA titers was broad and predominantly directed to the humanized Fv sequences of MGD006 (fig. S7), as might be expected in monkeys. The presence of ADA was not associated with exaggerated pharmacology, such as increased cytokine production. Given the humanized complementarity-determining regions and because linker and E/K-coil sequences were not immunodominant in monkeys, undue immunogenicity of MGD006 in humans is not anticipated.

Persistent depletion of CD123-positive leukocytes in MGD006-treated cynomolgus macaques

The circulating CD14⁺/CD123⁺ cell numbers were explored as a pharmacodynamic end point in preliminary primate studies of MGD006 at doses ranging from 0.1 ng/kg per day to 5 µg/kg per day, with depletion observed as early as 4 hours from the start of infusion and at doses ≥3 ng/kg per day (Fig. 4B for representative data); no effect was observed in vehicle-treated monkeys or monkeys that received control DART

at doses of up to 100 µg/kg per day. As predicted by these results, MGD006 treatment in the toxicology study was associated with extensive depletion of circulating CD14⁺/CD123⁺ cells at the first time point after the start of the first infusion (100 ng/kg per day) in all animals across all active treatment groups, whereas the number of CD123-positive cells in control group 1 animals remained stable over time (Fig. 4A). The depletion persisted during the 3-day-off period in groups 2 to 5 and generally returned to baseline during the recovery period; in several monkeys, however, an earlier recovery was noted, which correlated with the appearance of ADA. To eliminate the possibility of MGD006 masking or modulating CD123 (an unlikely scenario, given the low circulating levels), pDCs were enumerated by a different marker, CD303, as an orthogonal measure. Consistent with the CD123 data, CD303-positive pDCs were similarly depleted in monkeys treated with MGD006 (fig. S6).

PD-1 expression on T lymphocytes in MGD006-treated cynomolgus macaques, with no evidence of exhaustion

In contrast to the persistent depletion of circulating CD123⁺ cells, MGD006 administered on the 4-day-on/3-day-off schedule (groups 2 to 5) was associated with fluctuations in circulating CD4 and CD8 T cells. Administration of MGD006 as continuous 7-day infusions resulted in decreased circulating T cell numbers after the first administration; these did not fluctuate and slowly recovered even during the subsequent dosing periods (Fig. 5A). The differences in circulating T lymphocytes observed with the two dosing strategies suggest that the effect of MGD006 on T lymphocytes may be the result of MGD006-mediated trafficking and/or margination, rather than depletion. After the cessation of dosing, T cells rebounded to levels about two-fold higher than baseline for the duration of the recovery period. Similar T cell kinetics were observed in humans after the administration of a CD3xCD19 BiTE molecule, blinatumomab (9). Infusion of MGD006 was associated with an exposure-dependent, progressive increase in the frequency of T cells expressing the late activation marker PD-1 (programmed cell death protein 1) particularly in CD4⁺ cells, with group 6 animals displaying

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the highest overall levels (Fig. 5B and fig. S8). TIM-3 (T cell immunoglobulin and mucin domain-3), a marker associated with T cell exhaustion, was not detected on CD4⁺ cells and only at low frequency among CD8⁺ cells (5.5 to 9.7%), with a 20.5 to 35.5% frequency among the CD8⁺/PD-1⁺ double-positive cells. There was no consistent change in the early T cell activation marker CD69 and only modest variations in CD25 expression among circulating cells (Fig. 5B and fig. S8). This contrasts with the observation of CD25 up-regulation on T cells after MGD006 exposure of human PBMCs in vitro (Fig. 2A) and may relate to selective margination or tissue trafficking of the CD25⁺ cells.

To rule out T cell exhaustion after in vivo exposure, the cytotoxic potential of effector cells isolated from cynomolgus monkeys after four weekly infusions of MGD006 was compared to that of cells from naïve monkeys. PBMCs isolated from MGD006-treated monkeys or naïve untreated monkeys show similar levels of MGD006-mediated cytotoxicity ex vivo against Kasumi-3 cells (Fig. 5C), indicating that in vivo exposure to MGD006 does not negatively affect the cytotoxic activity of T cells. Furthermore, Kasumi-3 cells also express PD ligand 1 (PD-L1

(fig. S9), suggesting that MGD006-mediated T cell activation might overcome some amount of PD-1/PD-L1 inhibitory signals.

Cytokine release in MGD006-treated cynomolgus macaques as a first-dose effect

Given the T cell activation properties of MGD006 after target and T cell coengagement, an increase in circulating cytokines accompanying the infusion was anticipated, as previously reported for other T cell-targeted interventions (11, 20). Because cytokine release is often a first-dose phenomenon, the toxicology study was designed to ascertain the effectiveness of an intra-subject dose escalation strategy in limiting this effect while achieving a larger overall drug exposure. Of the cytokines tested, serum IL-6 concentration increased the most upon administration, including small increases after vehicle infusions, indicating a sensitivity of IL-6 responses to manipulative stress in the animals. MGD006-dependent increases (<80 pg/ml) in IL-6 concentration, however, were seen in several animals after the first DART infusion (100 ng/kg per day), with considerable inter-animal and inter-group

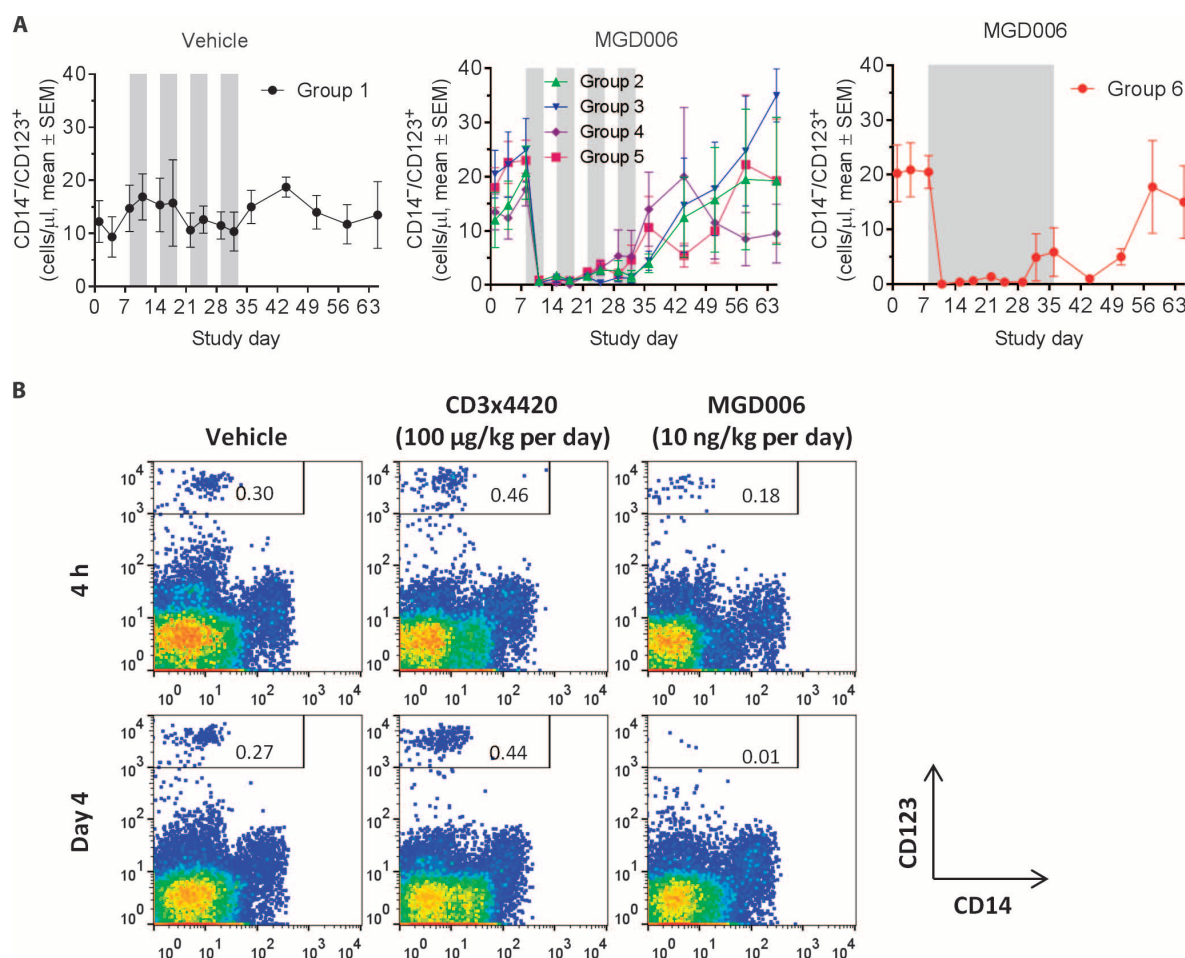


Fig. 4. Pharmacodynamic activity of MGD006 in cynomolgus monkeys. (A) Mean $\pm$ SEM of the amount of circulating CD14⁺/CD123⁺ leukocytes by study day and group. Cynomolgus monkeys were treated with vehicle control starting on day 1 for the first week, followed by four weekly infusions of either vehicle (group 1) or MGD006 administered as 4-day weekly infusions starting on days 8, 15, 22, and 29 (groups 2 to 5) or as a 7-day/week infusion

for 4 weeks starting on day 8 (group 6). Treatment intervals are indicated by the filled gray bars. (B) Cynomolgus monkeys were treated with a continuous infusion of vehicle, control DART, or MGD006. Peripheral blood samples were collected at the indicated time points from the start of the infusion and analyzed by flow cytometry, as indicated. CD123-positive cells are shown in the gate.

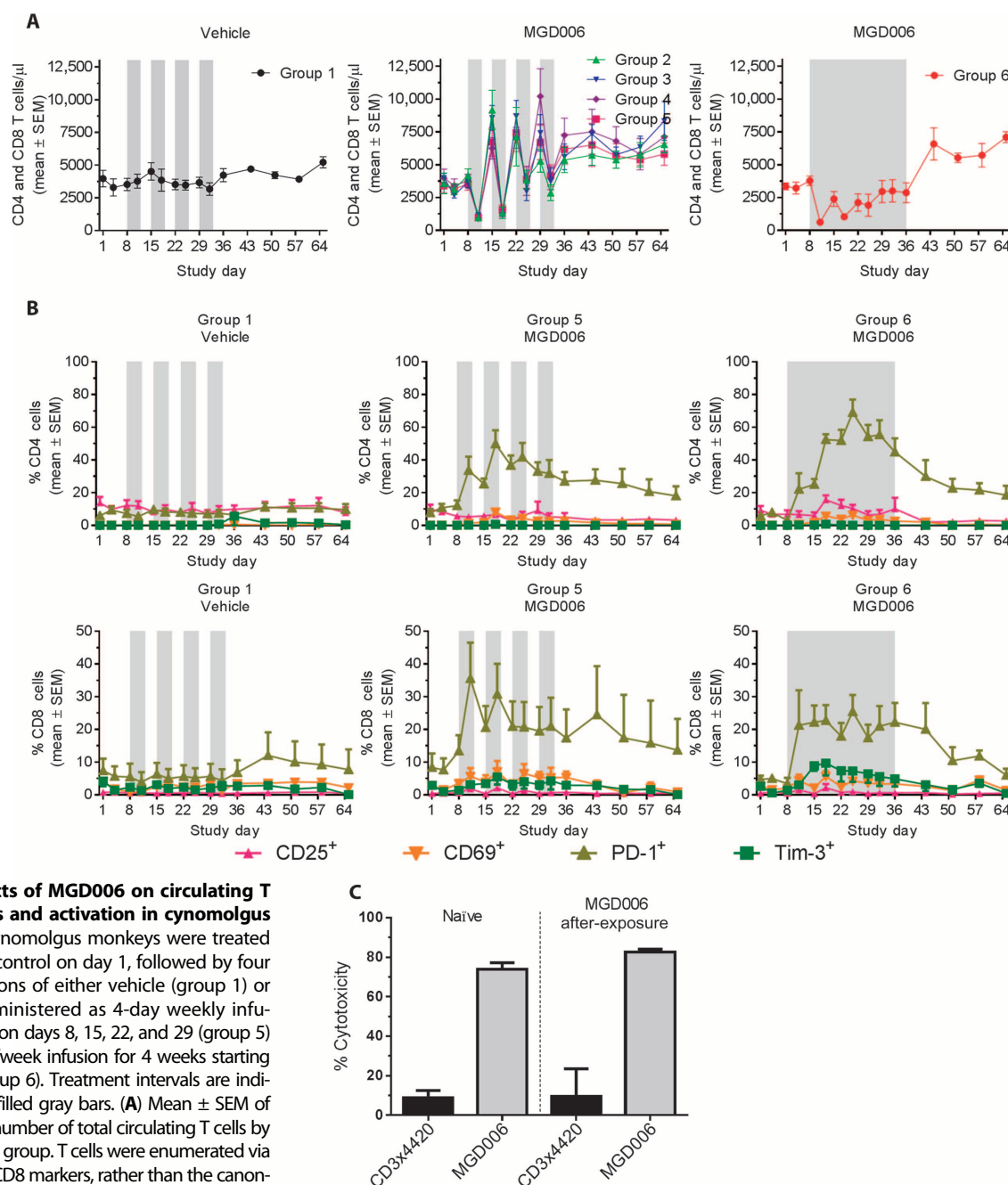


Fig. 5. Effects of MGD006 on circulating T cell numbers and activation in cynomolgus monkeys.

Cynomolgus monkeys were treated with vehicle control on day 1, followed by four weekly infusions of either vehicle (group 1) or MGD006 administered as 4-day weekly infusions starting on days 8, 15, 22, and 29 (group 5) or as a 7-day/week infusion for 4 weeks starting on day 8 (group 6). Treatment intervals are indicated by the filled gray bars. **(A)** Mean $\pm$ SEM of the absolute number of total circulating T cells by study day and group. T cells were enumerated via the CD4 and CD8 markers, rather than the canonical CD3, to eliminate possible interference of MGD006. **(B)** Relative amounts (mean percentage $\pm$ SEM) of CD25⁺, CD69⁺, PD-1⁺, and TIM-3⁺ cells among CD4 (top) or CD8 T cells (bottom) by study day and group. **(C)** MGD006-mediated cytotoxicity against Kasumi-3 cells with PBMCs from either naïve monkeys or monkeys treated with weekly escalating doses of MGD006 for 4 weeks.

variations (Fig. 6, A to C). The effect was transient, with IL-6 concentration returning to baseline by 72 hours. Furthermore, the magnitude of IL-6 response decreased with each successive MGD006 infusion, even when the dose was increased to up to 1000 ng/kg per day. Minimal and transient MGD006-related increases in serum tumor necrosis factor- α (<10 pg/ml) were also observed after the first infusion. There were no MGD006-related changes in concentrations of IL-5, IL-4, IL-2, or IFN- γ throughout the study (fig. S10) when compared with the control group.

Overall, cytokine release in response to MGD006 treatment in monkeys was minimal, transient, and consistent with a first-dose effect.

MGD006-mediated reduction in CD123⁺ bone marrow precursors and effects on hematopoiesis in cynomolgus macaques

White blood cell counts, neutrophils, and platelets (Fig. 7, A to C) showed transient fluctuations and a modest decrease during treatment,

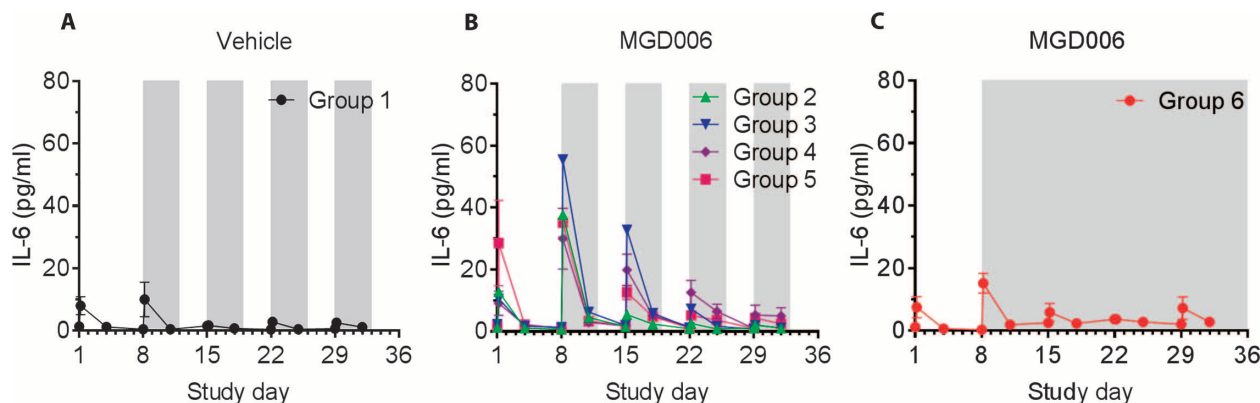


Fig. 6. MGD006-induced cytokine production in cynomolgus monkeys. Serum IL-6 concentrations (means $\pm$ SEM) in monkeys infused with MGD006 are shown by treatment group. (A to C) Cynomolgus monkeys were treated with vehicle control on day 1, followed by four weekly infusions of either

vehicle (group 1) (A) or MGD006 administered as 4-day weekly infusions starting on days 8, 15, 22, and 29 (groups 2 to 5) (B) or as a 7-day/week infusion for 4 weeks starting on day 8 (group 6) (C). Treatment intervals are indicated by the filled gray bars.

but remained within normal range throughout the entire study, except for a single time point on day 25 in group 6 (7-day-on), when mean platelet counts were just below the lower normal limit (261,000/ μ l). However, in group 6 (7-day-on), recovery was apparently slower than in other groups, and neutrophils did not completely return to baseline, although they remained in the normal reference range at all times. Reversible reductions in hematocrit and red blood cell (RBC) mass were observed at the highest doses (Fig. 7D and fig. S11A). Frequent blood sampling was a contributing factor because vehicle-treated animals also exhibited a modest decline in red cell mass. An increased magnitude in treated animals, however, indicated that MGD006 further mediated this effect. A reticulocyte response was observed in all animals; the response, however, appeared slightly less robust for a similar decrease in red cell mass at the highest exposure (group 6) (fig. S11B). Morphological analysis of bone marrow smears from the highest dose groups (groups 4 to 6) throughout the study was unremarkable (table S1).

Flow cytometric analysis of bone marrow aspirates, however, revealed that the frequency of CD123⁺ cells within the immature lineage-negative (Lin⁻) bone marrow populations was significantly decreased in MGD006-treated animals compared to vehicle-treated group 1 ($P < 0.005$) at the end of the treatment, returning to baseline levels by the end of the recovery period (Fig. 7E, left panel). The bone marrow cell subset defined as Lin⁻/CD45RA⁻/CD38⁻/CD34⁺/CD90⁺ cells [a population encompassing the HSC in humans (21) and referred to as “HSCs” hereafter] showed large inter-group variability; although all groups appeared to show reduction compared to the corresponding pre-dose levels, no statistically significant differences were seen when compared to vehicle-treated animals at the same time point (Fig. 7E, right panel). Similar inter-group variability with no significant change was observed for the Lin⁻/CD34⁺/CD38⁻/CD45RA⁻/CD90⁺ cell subset (multipotent progenitors, MPP in humans), as well as for the Lin⁻/CD34⁺/CD38⁺/CD45RA⁻/CD123^{low} cell subset (common myeloid progenitors, CMP in humans), the latter showing a nonstatistically significant numerical decrease (fig. S12, A and B). Consistent with the reversible effect of MGD006 on CD123⁺ immature hematopoietic precursors, these data indicate that the population of HSCs, which express little or no CD123, is less susceptible to targeting by MGD006. Except for the hematopoietic effects described, MGD006 was otherwise well tolerated at all doses tested.

DISCUSSION

We provide preclinical activity, safety, pharmacokinetic, and pharmacodynamic data supporting MGD006, a CD3 $\times$ CD123 bispecific DART capable of redirecting host T cells to kill CD123⁺ targets, as a potential therapeutic agent for the treatment of CD123⁺ hematological malignancies. We focus on ascertaining the safety and activity of MGD006 under treatment conditions that can be translated to the clinical setting.

MGD006 was engineered with humanized antibody arms displaying proportionally greater affinity for CD123 than CD3 to provide for preferential binding to target cells. An evolution of a previously reported DART structure (13), MGD006 preserves the original C-termini stabilizing disulfide linkage and adds opposing E/K-coil sequences that improve heterodimer formation. Together with its cynomolgus monkey cross-reactivity, these structural features distinguish MGD006 from other CD3-targeted bispecific formats, including single-chain BiTEs, and contribute to its improved homogeneity and stability, the latter a critical feature for delivery under continuous infusion over several days. When administered to cynomolgus macaques under continuous intravenous infusion, MGD006 demonstrated on-target activity with depletion of circulating CD123-positive cells at doses as low as 3 ng/kg per day. MGD006 was safe and well tolerated when administered at starting doses as high as 100 ng/kg per day, followed by stepwise weekly escalation to 300, 600, or 1000 ng/kg per day on either a 4-day-on/3-day-off weekly infusion schedule or a 7-day-on schedule. These dose ranges translate into circulating MGD006 concentrations of up to 100 pg/ml, consistent with those at which antitumor activity was observed in AML patient samples in vitro (10 to 100 pg/ml); furthermore, they encompass the continuous administration dose (500 ng/kg per day for 7 days) at which complete tumor regression was observed in the human PBMC-reconstituted mouse model.

CD123 is overexpressed in several hematological malignancies, including AML, myelodysplastic syndromes, and more rare diseases such as hairy cell leukemia, blastic pDC neoplasms, and systemic mastocytosis (1, 2, 22–24). Certain subsets of B-precursor acute lymphoblastic leukemia and chronic lymphocytic leukemia, as well as Hodgkin’s disease Reed-Sternberg cells, may also express CD123 (24). In leukemia, a low effector/target (E/T) cell ratio may limit the effectiveness of redirected cytotoxicity; MGD006, however, was capable of eliminating leukemic

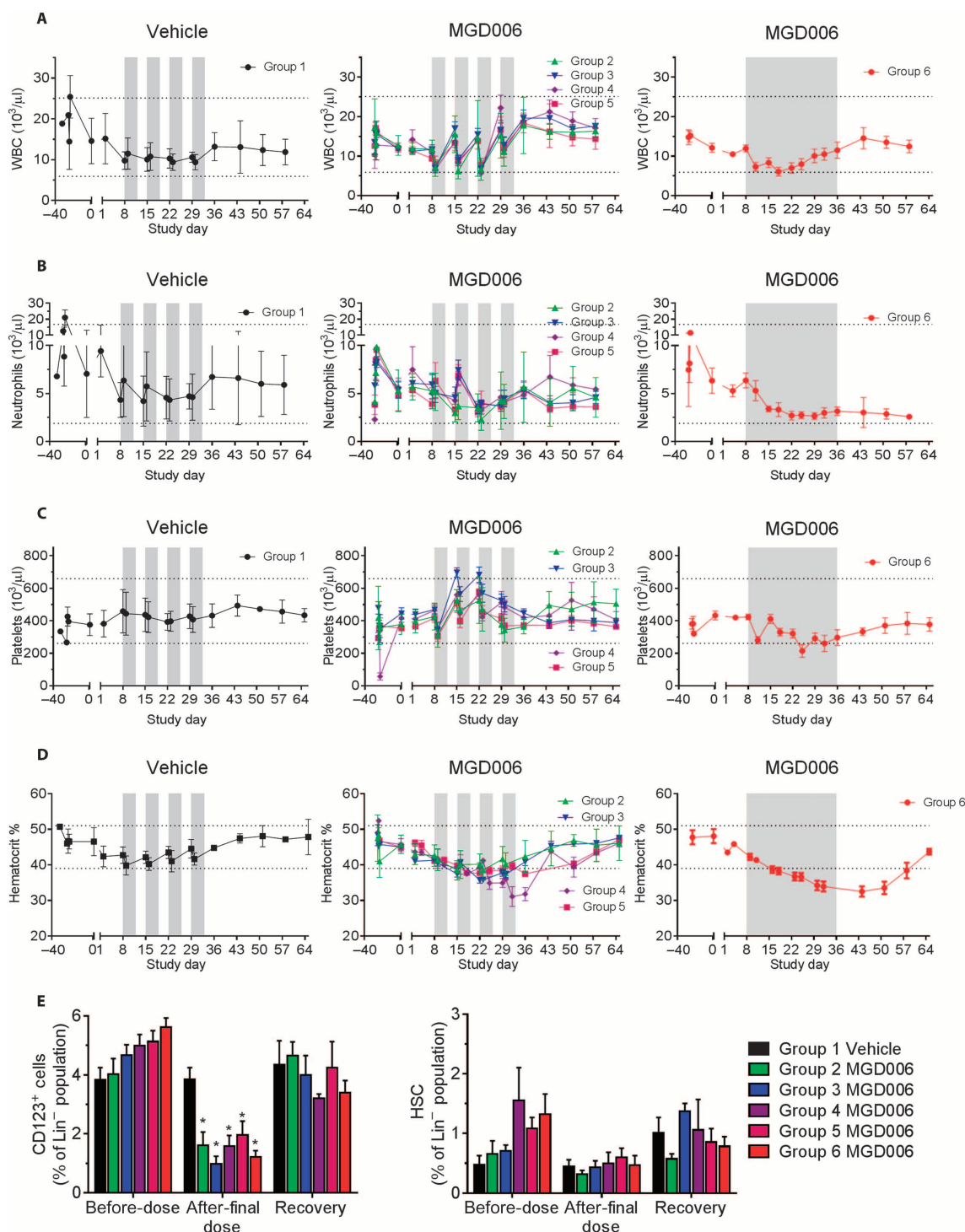


Fig. 7. Effects of MGD006 on hematopoiesis and bone marrow. (A to D) Mean $\pm$ SEM of white blood cells (WBC) (A), neutrophils (B), platelets (C), or hematocrit (D) in peripheral blood samples collected at the indicated time points from monkeys treated with vehicle or MGD006. Cynomolgus monkeys were treated with vehicle control on day 1, followed by four weekly infusions of either vehicle (group 1) or MGD006 administered as 4-day weekly infusions starting on days 8, 15, 22, and 29 (groups 2 to 5) or as a 7-day/week infusion for 4 weeks starting on day 8 (group 6). Treatment intervals are indicated by the filled gray bars. Horizontal dotted lines

indicate the reference range in cynomolgus monkeys. (E) The frequency (mean percentage $\pm$ SEM) of CD123⁺ cells or HSC (defined as CD34⁺/CD38⁻/CD45RA⁻/CD90⁺ cells) within the Lin⁻ cell population in bone marrow samples collected at the indicated time points from monkeys treated with MGD006. Relative to the vehicle-treated group 1, CD123⁺ cells were significantly depleted (* indicates significant difference; *P* values are provided in table S3) in the MGD006-treated groups after the final dose, whereas no significant difference was observed for HSCs. Statistical analyses used two-way ANOVA.

blasts in AML samples by arming and expanding the patient's residual T cells in vitro under unfavorable E/T ratios and at doses (10 to 100 pg/ml) well within the range of circulating levels observed in cynomolgus monkeys treated with MGD006. These data also confirm that AML T cells can be imbued with efficient effector functions, as previously reported (25, 26), even if they have been shown to poorly polarize actin to form immune synapses with autologous blasts (27).

Adverse effects associated with MGD006 administration included cytokine release and reversible decrease in RBCs. Cytokine release is a general concern with T cell-directed therapies, further heightened by the report of a CD123-bispecific single-chain Fv (scFv) immunofusion protein with bivalent CD3 recognition that demonstrated not only antileukemic activity in vitro but also target-independent T cell activation and IFN- γ secretion (28). The monovalent nature of the binding arms of MGD006 ensures that T cell activation depends exclusively on target cell engagement: consistently, no T cell activation was observed in the absence of target cells or with a CD3x4420-DART that includes only the CD3-targeting arm. In MGD006-treated animals, cytokine release was minimal and occurred as a first-dose phenomenon that was manageable through repeated or escalating doses. The rapid depletion of CD123⁺ cells after the first MGD006 dose eliminates a source of CD3 ligation and likely explains the transient nature of cytokine release observed in this model.

MGD006 administration reduced bone marrow CD123⁺ precursor cells but not Lin⁻/CD38⁻/CD45RA⁻/CD34⁺/CD90⁺ cells, a subset encompassing HSC in humans (21), and did not change bone marrow cellularity or erythroid/myeloid ratio. This effect was associated with a mild anemia and a modest reduction (within the normal range) of both white blood cell counts and neutrophils at the highest doses, but no thrombocytopenia. After treatment cessation, the hematopoietic parameters returned to baseline, consistent with repopulation from spared CD123^{low}/negative HSC; however, in continuously treated animals, recovery was slower. These observations confirm the attractiveness of CD123 as a target, which rests in large part on the ability of a cytolytic modality to discriminate HSC from LSC.

Limitations of the study include a discrepancy in monocytes' CD123 expression between humans and monkeys. The lower CD123 expression on monkeys' monocytes may limit the ability of the preclinical model to adequately predict cytokine responses in humans. Another potential limitation of the approach relates to the up-regulation of PD-1, an activation marker with co-inhibitory function, which was observed in MGD006-treated monkeys. PD-1 expression may also implicate T lymphocyte exhaustion; however, the ex vivo cytotoxic potential of T cells from monkeys exposed to MGD006 was unaffected, including their ability to kill cells that express PD-L1. Furthermore, PD-1 up-regulation was not accompanied by TIM-3 expression, a hallmark of exhaustion when T cells are exposed to protracted stimulation or chronic infections (29, 30). Therefore, MGD006 administration did not appear to induce functionally exhausted T cells; nonetheless, a counter signal via PD-1/PD-L1 will need to be carefully considered when assessing MGD006 in the clinic, and additional studies will be required to further investigate the effects of the PD-1/PD-L1 pathway on MGD006-mediated T cell functions.

The reversibility of the adverse effects of MGD006 distinguishes this antibody-based approach of redirected T cell killing from the genetic transduction of T cells with CD123-specific chimeric antigen receptors (CD123-CAR T cells), an alternate modality for targeting CD123⁺ leukemic cells. CD123-CAR T cells have exhibited potent leukemic blast

cell killing in vitro and in vivo (31, 32), but concerns have arisen with the observation of hematopoietic ablation in NSG mice engrafted with human CD34⁺ cells after CD123-CAR T cell transfer (31). Although this phenomenon has not been universally observed (33, 34), the possibility that CAR T cells may not spare cells that express very low amounts of antigen may limit their applicability against this target.

Other CD123-targeted approaches include an anti-CD123 Fc-enhanced mAb (7, 35); a trispecific scFv directed against CD123, CD33, and CD16 (36); and an IL-3/diphtheria immunotoxin (37), all with demonstrated antileukemic activity in vitro and/or in vivo. The first two modalities rely on Fc receptor-mediated cell depletion, whereas the latter is a cytotoxic payload-driven, effector cell-independent strategy. These modalities are welcomed additions to the arsenal of potential tools filling a void in unmet clinical needs in myeloid leukemia. The potent antitumor activity that T cells have demonstrated in various models may provide an advantage to MGD006 as well as to a recently reported CD3xCD33 BiTE approach (25). The studies reported here provide evidence of safety, activity, and a favorable therapeutic window that support clinical testing of MGD006 for the treatment of CD123-positive hematological disorders. MGD006 is currently undergoing phase 1 testing in patients with relapsed/refractory AML (<http://clinicaltrials.gov/show/NCT02152956>).

MATERIALS AND METHODS

Study design

The objective of the in vivo efficacy studies was to evaluate the activity of MGD006 on the established human xenograft tumors of KG-1a cell line in NSG/ $\beta 2m^{-/-}$ mice reconstituted with human PBMCs. Sample sizes ($n = 8$ per group) were determined on the basis of consistency and homogeneity of tumor growth in the selected model and were sufficient to determine statistically significant differences in tumor size between groups. Animals were randomly assigned to groups on the basis of tumor size and were sacrificed at the end of the study. All tumor measurements were included in the analysis. Tumor volumes were calculated with the following formula: $[(\text{length} \times \text{width}^2)/2]$. Mean tumor volume with SEM was plotted.

The objective of the nonhuman primate study was to evaluate the safety, pharmacokinetics, and pharmacodynamics of MGD006 administered by continuous intravenous infusion to cynomolgus monkeys. Animals were randomly assigned to experimental groups to achieve a similar group mean body weight. The sample size chosen ($n = 8$ per group) is standard for safety and pharmacodynamic assessment in nonhuman primates to minimize animal use. Study design is provided in Table 1, and full experimental details are provided below.

Antibody humanization and expression

V_H and V_L sequences from a murine anti-human CD3 antibody were humanized as previously reported (38). V_H and V_L sequences from the murine anti-CD123 mAb 7G3 were humanized using the respective germline V_H1-18 (V_H1-69 FR3) and V- κ B3 sequences. V regions were assembled into a DART format, cloned into EE13.4 vector, and transfected into Chinese hamster ovary cells, and the protein was purified as described previously (13). CD3x4420-DART (14) and 4420xCD123-DART were constructed in a similar manner, with variable domain sequences of anti-fluorescein mAb 4-4-20 replacing one or the other specificity. The approximate size and homogeneity of purified proteins were analyzed with TSKgel G3000SWxl size exclusion high-performance liquid

chromatography column (Tosoh Bioscience) and SDS–polyacrylamide gel electrophoresis (NuPAGE Bis-Tris system, Life Technologies). Molecular mass was analyzed in a Waters Xevo G2-S ESI QToF mass spectrometer using Acquity UPLC BEH300 C4, 2.1 × 100 mm, 1.7-μm particle column, and elution with 90% acetonitrile/0.1% formic acid.

Enzyme-linked immunosorbent assay

A MaxiSorp ELISA plate (Nunc) coated with soluble human or cynomolgus IL-3 receptor α (0.5 μg/ml) in bicarbonate buffer was blocked with 0.5% bovine serum albumin, 0.1% Tween 20. DART molecules were applied, followed by sequential addition of human CD3 ϵ /δ–biotin and streptavidin–horseradish peroxidase (HRP) (Jackson ImmunoResearch). HRP activity was detected using tetramethylbenzidine (BioFX) as substrate.

Surface plasmon resonance analysis

Binding of MGD006 to human and cynomolgus monkey CD3 or CD123 proteins was analyzed by Biacore 3000 biosensor (GE Healthcare) as previously described (13, 14). Soluble CD3 or Penta-His mAb (capturing CD123-His) was injected over the activated CM5 surface in 10 mM sodium acetate (pH 5.0) at a flow rate of 5 μl/min, followed by 1 M ethanolamine for deactivation. Binding experiments over immobilized CD3 or captured CD123-His surfaces were performed in 10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% P20 surfactant. Regeneration of immobilized receptor surfaces was performed by pulse injection of 10 mM glycine (pH 1.5). K_D values were determined by a global fit of binding curves to the Langmuir 1:1 binding model (BIAevaluation software version 4.1).

Cell killing assays

CD123 cell surface density was determined using Quantum Simply Cellular beads (Bangs Laboratories Inc.). Target cells labeled with CellTracker Orange CMTMR (Life Technologies) were treated with serial dilutions of DART proteins in the presence of PBMCs at 37°C overnight. Cytotoxicity was evaluated by flow cytometry. Cryopreserved AML patient primary PBMCs and bone marrow mononuclear cells (BMMNCs) were purchased from AllCells, LLC, which obtains biological specimens from a donor program subject to Institutional Review Board's or Human Subject Committee's approval, with all donors giving informed consent. AML PBMCs and BMMNCs were cultured in the presence of MGD006 or CD3x4420 (0.01 or 0.1 ng/ml) for 144 hours in RPMI 1640 supplemented with 10% fetal bovine serum, and leukemic blast cells and T cells were measured by flow cytometry. Absolute cell counts were determined using Liquid Counting Beads (BD Biosciences).

Cynomolgus monkey studies

The in vivo portion of all nonhuman primate experiments was performed at Charles River Laboratories, according to the guidelines of the Institutional Animal Care and Use Committee (IACUC). Purpose-bred, naïve cynomolgus monkeys (*Macaca fascicularis*) of Chinese origin (age range, 2.5 to 9 years; weight range, 2.7 to 5 kg) received vehicle or MGD006 via intravenous infusion through femoral or jugular ports via battery-powered programmable infusion pumps (CADD-Legacy, SIMS Deltec Inc.). MGD006 in serum samples was measured using a sandwich immunoassay with electrochemiluminescence detection (Meso Scale Diagnostics, MSD). Cell surface phenotype analyses in the peripheral blood were performed with a LSRFortessa analyzer (BD Biosciences) equipped with 488-, 640-, and 405-nm lasers, using the following antibodies: CD4-V450, CD8-V450, CD123-PE (phycoerythrin)–Cy7, CD45-

PerCP (peridin chlorophyll protein), CD4-APC (allophycocyanin)–H7, CD8-FITC (fluorescein isothiocyanate), CD25-PE-Cy7, CD69-PerCP, PD-1-PE, TIM-3-APC, CD3-Pacific Blue, CD95-APC, CD28-BV421, CD16-FITC, CD3-Alexa 488, CD38-PE, CD117-PerCP-Cy5.5, CD34-APC, CD90-BV421, CD45RA-APC -H7, and CD33-APC (BD Biosciences). The absolute number of cells was determined with TruCOUNT (BD Biosciences). Serum levels of cytokines were measured with Cytometric Bead Array Kits (BD Biosciences).

In vivo efficacy studies in xenograft model

In vivo studies were reviewed and approved by IACUC. NSG/B2m^{-/-} female mice (Taconic Farm), 7 to 8 weeks old, were randomly divided into five groups and were implanted intradermally with KG-1a cells (5 × 10⁶ cells per mouse on day 0), followed by intraperitoneal injection of 10 × 10⁶ PBMCs per mouse on day 1. MGD006 was continuously delivered through peritoneally implanted osmotic pumps from days 16 to 22. Individual tumor volumes were measured with calipers throughout the study.

Statistical analysis

In vitro assays were repeated more than three times. Nonlinear regression analyses were used to fit curves in GraphPad Prism. For in vivo studies, inter-group differences were assessed by two-way ANOVA in GraphPad Prism. *P* values of ≤0.05 were considered statistically significant. Individual data for in vivo experiments are provided in table S4.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Physicochemical characterization of MGD006.

Fig. S2. MGD006 activity in primary AML patient PBMCs.

Fig. S3. MGD006 activity in a primary AML patient bone marrow specimen.

Fig. S4. MGD006-mediated redirected killing of KG-1a cells ex vivo.

Fig. S5. Pharmacokinetics of MGD006 in cynomolgus monkeys.

Fig. S6. PDCs in MGD006-treated monkeys.

Fig. S7. Determination of ADA reactivity to MGD006 components.

Fig. S8. Effects of MGD006 treatment on circulating T cell activation markers and subtypes in cynomolgus monkeys (group 2 to 4 data).

Fig. S9. PD-L1 expression on KG-1a and Kasumi-3 cells.

Fig. S10. MGD006-induced cytokine production in cynomolgus monkeys.

Fig. S11. Effects of MGD006 treatment on RBCs and reticulocytes in cynomolgus monkeys.

Fig. S12. Effect of MGD006 on bone marrow progenitors.

Table S1. Myeloid/erythroid ratio in smears of bone marrow aspirates from monkeys treated with MGD006.

Table S2. *P* values comparing tumor volumes in mice treated with MGD006 or control DART.

Table S3. *P* values comparing CD123⁺ cells in bone marrow samples from cynomolgus monkeys treated with MGD006 or vehicle.

Table S4. Individual data for in vivo experiments (provided as a separate Excel file).

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R.A. designed and performed research and collected, analyzed, and interpreted data. S.J. designed and engineered the DART proteins used in the study. L. Huang, H.L., S.B., L. He, Q.T., L.J., S.G., V.C., and F.C. performed research, collected and analyzed data, and performed statistical analysis. M.S. and S.K. analyzed and interpreted data. P.A.M. designed research and analyzed and interpreted data. E.B. designed research, analyzed and interpreted data, and wrote the manuscript. **Competing interests:** MacroGenics is developing MGD006 as a clinical compound. All authors are employees of MacroGenics Inc., the company developing MGD006, and receive salary and stocks as compensation for their employment. **Data and materials availability:** Materials are available from MacroGenics under a material transfer agreement.

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Editor's Summary

Killing leukemia with DARTs

Acute myeloid leukemia, one of the most common types of leukemia, is very difficult to treat, and most current therapies are ineffective for this disease, so new therapeutic approaches are urgently needed. Now, Chichili *et al.* have designed a DART, or dual-affinity re-targeting protein, to force T cells to recognize and kill the leukemia cells. To do this, the DART combines a portion of antibody recognizing CD3, a T cell marker, with a portion of antibody that recognizes CD123, a molecule found on the cells of some blood cancers. The researchers showed that the DART is effective in a mouse model of acute myeloid leukemia and that it is safe in primates, paving the way for human clinical trials.

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