# First-in-Human Phase I Study of a CD16A Bispecific Innate Cell Engager, AFM24, Targeting EGFR-Expressing Solid Tumors

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## ABSTRACT

**Purpose:** Innate immune cell–based therapies have shown promising antitumor activity against solid and hematologic malignancies. AFM24, a bispecific innate cell engager, binds CD16A on NK cells/macrophages and EGFR on tumor cells, redirecting antitumor activity toward tumors. The safety and tolerability of AFM24 were evaluated in this phase I/IIa dose-escalation/dose-expansion study in patients with recurrent or persistent, advanced solid tumors known to express EGFR.

**Patients and Methods:** The main objective in phase I was to determine the MTD and/or recommended phase II dose. The primary endpoint was the incidence of dose-limiting toxicities during the observation period. Secondary endpoints included the incidence of treatment-emergent adverse events and pharmacokinetics.

**Results:** In the dose-escalation phase, 35 patients received AFM24 weekly across seven dose cohorts (14–720 mg). One patient experienced a dose-limiting toxicity of grade 3 infusion-related reaction. Infusion-related reactions were mainly reported after the first infusion; these were manageable with premedication and a gradual increase in infusion rate. Pharmacokinetics was dose-proportional, and CD16A receptor occupancy on NK cells approached saturation between 320 and 480 mg. Paired tumor biopsies demonstrated the activation of innate and adaptive immune responses within the tumor. The best objective response was stable disease in 10/35 patients; four patients had stable disease for 4.3 to 7.1 months.

**Conclusions:** AFM24 was well tolerated, with 480 mg established as the recommended phase II dose. AFM24 could be a novel therapy for patients with EGFR-expressing solid tumors, with suitable tolerability and appropriate pharmacokinetic properties for further development in combination with other immuno-oncology therapeutics.

## Introduction

NK cells are innate immune cells that are crucial components of a multipronged approach to fighting cancer, with many sophisticated features that provide substantial potential compared with T cells (1). Significant ongoing efforts are attempting to engage the innate immune system for the purpose of redirecting immune effector cells to eliminate tumor cells (2–4). Novel antibody constructs are under development to enhance and prolong NK cell–mediated antitumor responses through targeting of different activating NK-cell receptors and tumor cell targets (4–6). Antibodies that can engage cells of the innate immune system, such as NK cells and macrophages, thus triggering antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP), respectively, toward target tumor cells have shown promising clinical activity (7, 8). Despite favorable preclinical findings across a broad spectrum of solid tumors and hematologic malignancies (9, 10), the complexities of the tumor microenvironment mean the potential of NK-cell engagers in orchestrating an antitumor response has yet to be fully realized.

AFM24 is a first-in-class, bispecific, tetravalent, Fc-silenced EGFR/CD16A innate cell engager (ICE; refs. 2, 11); details of the molecular structure have been previously published (11). AFM24 was designed to engage CD16A on innate cells to harness the cytotoxic properties of NK cells and macrophages and to the extracellular domain of EGFR on the surface of target cells, triggering ADCC and ADCP directed toward EGFR-expressing solid tumors (2, 11). The unique mode of action of AFM24 has previously been discussed (11), but in brief, AFM24 binds to a distinct region of CD16A that does not overlap with the Fc-binding site (12), and preclinical data show that AFM24 effectively targets tumors expressing varying levels of EGFR and binding occurs regardless of the mutational status; notably, the presence of *BRAF* and *KRAS* mutations does not affect the activity and ADCC in tumor cell lines (11).

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## Translational Relevance

AFM24 is a first-in-class, bispecific, tetravalent, innate cell engager designed to bind CD16A on innate cells to harness the antitumor activity of NK cells and macrophages, triggering antibody-dependent cell-mediated cytotoxicity and antibody-dependent cell-mediated phagocytosis directed toward EGFR-expressing solid tumors. Importantly, AFM24 does not rely on inhibition of the EGFR-signaling pathway that differentiates it from current EGFR-targeting treatment options. AFM24 monotherapy is well tolerated and demonstrated modest activity in an unselected and heavily pretreated patient population. Promising correlative biomarker data, with evidence of cytotoxic NK cell and CD8 T cells present in the tumor microenvironment, provide strong support for AFM24 treatment to be used in conjunction with other immunotherapies to enhance the innate and adaptive immune systems as a potential combination strategy to target EGFR-expressing solid tumors.

Therefore, AFM24-mediated ADCC is only dependent on the docking of AFM24 to the EGFR extracellular domain of tumor cells—a motif is conserved irrespective of signaling cascade downstream of the receptor, thus differentiating AFM24 from other approved mAbs that rely on signal inhibition (11). Similarly, AFM24 triggers ADCP activity of all subtypes of human macrophages in cell lines with high and low EGFR expressions (12, 13). Preclinical data show more potent cytotoxicity across a range of EGFR-expressing tumor cell lines derived from various solid tumors [including colorectal cancer, non–small cell lung cancer (NSCLC), pancreatic adenocarcinoma, glioblastoma, and epidermoid cancers] compared with Fc-enhanced anti-EGFR IgG1, classical EGFR-targeting IgG1, or a monovalent-binding bispecific engager (cetuximab; ref. 11). In addition, the dose-dependent reduction in EGF-mediated EGFR phosphorylation shown with AFM24 occurs at >1,000 fold lower potency than observed with cetuximab; therefore, this may correspond to lower EGFR signaling inhibition and less effect on the EGFR cascade at clinically relevant doses (11). AFM24 monotherapy was observed to be well tolerated in cynomolgus monkeys; notably, no skin or organ toxicities were identified up to the highest dose levels tested (11).

Elevated EGFR expression is seen across a broad range of solid tumors, including but not limited to colorectal cancer, NSCLC, clear-cell renal cell carcinoma, head and neck squamous cell carcinoma, and triple-negative breast cancer (14, 15). EGFR expression is also a prognostic indicator of poor relapse-free and overall survival outcomes (14). The high expression of EGFR on tumor cell surfaces presents an ideal therapeutic target, with several approved targeted therapies currently available (16). These include humanized mAbs directed against the receptor extracellular domain as well as small-molecule inhibitors (e.g., gefitinib, erlotinib, osimertinib, and afatinib) that are designed to target the tyrosine kinase domain of the receptor (17).

AFM24 could be a novel therapeutic option for a broad range of EGFR-expressing tumors; based on the unique mechanism of action, AFM24 induces ADCC toward tumor cells independent of EGFR signaling and at doses that do not interfere with signaling in normal tissues. Therefore, AFM24 has the potential to overcome treatment resistance in tumors that have become addicted to EGFR signaling and tumors that harbor mutations downstream of EGFR signaling, such as *KRAS* or *BRAF* (11). In April 2020, a phase I/IIa open-label, nonrandomized, first-in-human, multicenter study was initiated to evaluate AFM24 in patients with treatment-refractory, advanced solid tumors. In this study, we present the findings of the phase I, dose-escalation portion of the study, which aimed to establish the MTD and/or the recommended phase II dose (RP2D; ref. 18).

## Patients and Methods

### Study design

This was a phase I/IIa, open-label, multicenter, dose-escalation/dose-expansion study, aiming to assess the safety, tolerability, pharmacokinetics (PK), immunogenicity, and preliminary efficacy of AFM24 in patients with advanced solid cancers (**Fig. 1**). The protocol was registered under the ClinicalTrials.gov number NCT04259450. The phase I part of the study was conducted at four sites across the United States, Spain, and the United Kingdom. The study was approved by the Institutional Review Board/ethics committee at each participating center and performed in accordance with good clinical practice and local regulatory requirements. The informed consent form contained all the essential elements of informed consent set forth in 21 CFR, part 50, the International Conference on Harmonization Guideline for Good Clinical Practice, and the terms of the Declaration of Helsinki. Written informed consent was obtained from all patients or their legal representative, and consent could be withdrawn at any time.

### Patient population

Patients aged ≥18 years with advanced or metastatic solid tumors known to express EGFR were eligible; determination of tumor EGFR expression via IHC was not required for enrollment in the dose-escalation phase.

Patients were required to have been previously treated with one or more lines of anticancer therapy and have documented radiological disease progression during or after their most recent line of anticancer therapy and to have no further standard-of-care therapy options available to them. An Eastern Cooperative Oncology Group performance status of 0 or 1 was required, in addition to adequate organ function assessed within 7 days before the first AFM24 infusion, including adequate bone marrow function (based on absolute neutrophil count $\geq 1.5 \times 10^9$/L, platelet count $\geq 100 \times 10^9$/L, and hemoglobin ≥8 g/dL), as well as sufficient hepatic (total bilirubin ≤1.5 × upper limit of normal), and renal (serum creatinine concentration ≥1.5 × upper limit of normal) function. Patients were required to have at least one tumor site amenable to biopsy and be considered sufficiently low risk to undergo a minimum of two biopsies by the investigator. Exclusion criteria included receiving systemic anticancer therapy within the last 4 weeks (6 weeks with mitomycin C or nitrosoureas) or within five half-lives, whichever was longest, before AFM24 treatment; have received radiotherapy within the 2 weeks prior to the first AFM24 infusion; or had an unresolved (Common Terminology Criteria for Adverse Events v5.0 grade >1) radiotherapy-related toxicity from a prior therapy. The full list of the phase I study eligibility criteria is provided in Supplementary Methods S1, and an overview of the study representativeness is presented in Supplementary Table S1.

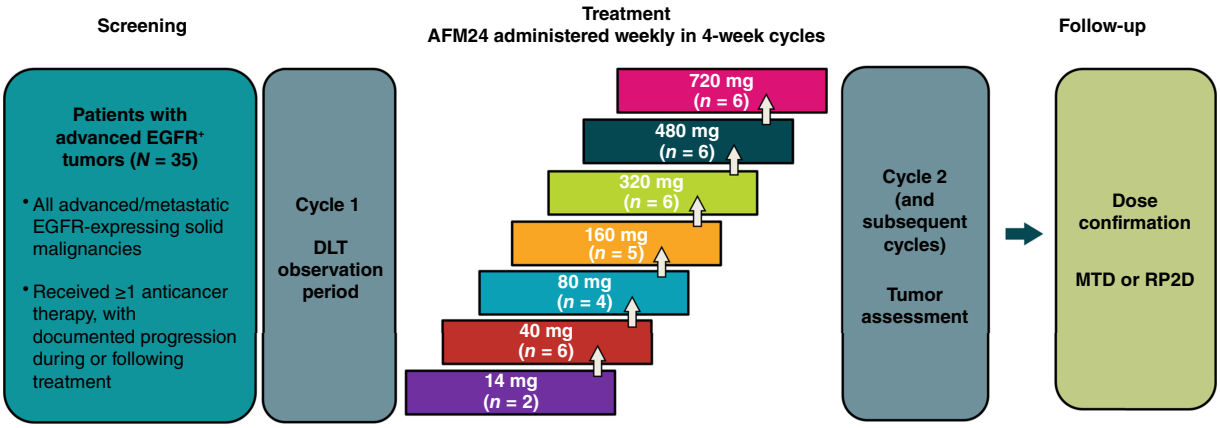


**Figure 1.**

Phase I dose-escalation study design of the Phase I/IIa study of AFM24 in patients with EGFR-expressing solid tumors (NCT04259450). The study design included a 21-day screening period followed by a DLT observation period (cycle 1). Patients received AFM24 administered as a weekly intravenous infusion on day 1, day 8, day 15, and day 22 of a 28-day cycle using flat, fixed dosing. The starting dose of AFM24 was 14 mg, with six incrementally increasing dose levels for the dose escalation (i.e., 40, 80, 160, 320, 480, and 720 mg). Tumor assessment with CT and/or MRI took place at screening and during the last week of cycles 2, 4, 6, and 8, and every three cycles thereafter.

### Study endpoints

The primary endpoint of phase I was to assess the incidence of dose-limiting toxicities (DLT) observed during the first 28-day cycle (the DLT observation period) to determine the MTD and/or select one or more RP2Ds of AFM24 in patients with advanced or metastatic solid tumors. The secondary endpoints were to characterize the safety and tolerability, PK/pharmacodynamics (PD), and preliminary antitumor efficacy of AFM24.

### AFM24 dose-escalation phase

An adaptive two-parameter Bayesian logistic regression model (19), guided by the dose escalation with overdose control, was used in the escalation phase to guide the determination of the MTD and/or RP2D in subjects with advanced or metastatic solid malignancies. DLTs were assessed in treatment cycle 1, and a full list of DLT definitions are provided in Supplementary Table S2.

Following a 21-day screening period, AFM24 was administered as a weekly (QW) intravenous (IV) infusion on day 1, day 8, day 15, and day 22 of a 28-day cycle using flat, fixed dosing. The starting dose of AFM24 was 14 mg, with six incrementally increasing dose levels for the dose escalation (i.e., 40, 80, 160, 320, 480, and 720 mg).

The protocol was amended during the 40-mg dose cohort due to the observation of a grade 3 infusion-related reaction (IRR) and a mandatory premedication regimen that contained corticosteroids, H1 antagonist with or without H2 antagonist, and oral acetaminophen 650 mg or equivalent, for at least the duration of the first cycle was established. AFM24 infusions took place over an approximately 4-hour period, starting with a low infusion rate that could be increased every 30 to 60 minutes, if tolerated, until the final infusion rate had been reached (slow ramp-up). In the higher dosing cohorts, the infusion rate and time were increased to >4 hours up to as long as 2 days (i.e., split schedule dosing required only for patients receiving the highest dose of 720 mg). Patients who had a split dose had to have received premedication on day 1 and day 2; for day 2, corticosteroids could be reduced at the discretion of the investigator. Infusions were followed by a 4-hour observation period. If the first two infusions were well tolerated (defined as no grade >1 IRRs or cytokine release syndrome) then the premedication regimens, as well as the observation period, could be modified (tapered/reduced). For patients who tolerated the initial consecutive infusions, subsequent infusion times could be reduced to <4 hours to a minimum of 1 hour, starting from the third infusion. Only one modification was allowed per infusion.

Patients could continue to receive AFM24 as long as they continued to show clinical benefit, until disease progression by RECIST v1.1 or upon meeting treatment discontinuation criteria, withdrawal of consent or at the discretion of the investigator.

### Safety assessments

All adverse events (AE) were reported up to 30 days after the last administration of AFM24 or until the start of a new anticancer treatment, whichever occurred first. Safety was assessed by periodic vital signs, physical examinations, Eastern Cooperative Oncology Group performance status, 12-lead electrocardiographs, clinical laboratory assessments, and monitoring of AEs. AEs were graded using the NCI Common Terminology Criteria for Adverse Events, v5.0.

### Antitumor activity

Disease responses were assessed by the investigator using local RECIST v1.1. Tumor assessment with CT and/or MRI took place at screening and during the last week of cycles 2, 4, 6, 8, and every three cycles thereafter.

### PK and PD

Serum samples for PK and cytokine assessments as well as peripheral blood mononuclear cells (PBMC) for immunophenotyping were collected at predefined time intervals. More detailed information is given in Supplementary Methods S1.

#### PK assessments and modeling

The PK set included all patients who received ≥1 dose of AFM24 and had ≥1 after dose PK measurement. The PK parameters were estimated by noncompartmental analysis by using

Phoenix WinNonlin professional version 8.3.4 (Certara Inc.; RRID: SCR_024504).

AFM24 serum concentrations were also included in a population PK analysis that suggests that AFM24 is best characterized by a two-compartment model with linear and nonlinear elimination. Due to the nonlinear elimination, no unique terminal elimination half-life ($t_{1/2}$) could be derived for AFM24 but was approximated for doses at which saturation of the nonlinear elimination was reached (≥160 mg) by simulating the time to reach 95% of the trough concentration at steady-state and dividing it by 4.3. Subsequently, the individual simulated AFM24 serum concentrations using the population PK model were linked to CD16A receptor occupancy (RO) on NK cells using a sigmoidal $E_{max}$ equation with Hill coefficient.

### Cytokines

Cytokine levels were assessed in plasma using the V-PLEX Proinflammatory Panel 1 (human) kit by MSD. IL-2, IL-6, IL-10, IL-15, and IFN-γ and TNFα were analyzed before and after the infusion of AFM24.

### CD16A receptor occupancy

The FACS-based assay and data analysis are described in Supplementary Methods S1.

### Immunophenotyping by cytometry time-of-flight

Isolated PBMCs were analyzed by cytometry time-of-flight to identify the relative presence and status of various lymphocyte subsets including NK cells, activated NK cells, T cells, and activated T cells, as previously described (20). When necessary, purified antibodies were conjugated to indicated metal isotopes using Maxpar X8 antibody labeling kits according to the manufacturer's instructions (Standard BioTools Store). Otherwise, directly conjugated antibodies were purchased from Standard BioTools Store and indicated as "Fluidigm".

### IHC

Paired tumor biopsies were collected from patients at screening and cycle 1, day 24 (C1D24) of treatment. Tumor samples were analyzed for the expression of EGFR (clone 5B6, Roche Diagnostics) and immune cells markers (CD45, clone RP2/18, CD3, and clone 2GV6, Roche Diagnostics) by IHC. CD45 and CD3 expressing ($CD45^+$ $CD3^+$) cell density (cell per $mm^3$) was determined by image analysis using the Visiopharm software.

EGFR expression was quantified by a board-certified pathologist using the H-score obtained by the following formula: 3 × percentage of strongly staining nuclei + 2 × percentage of moderately staining nuclei + percentage of weakly staining nuclei, giving a range of 0 to 300.

Additional details about data analysis are provided in Supplementary Methods S1.

### Gene expression profiling

Biopsies from patients who received the higher doses of AFM24 (≥160 mg) had additional gene expression profiling (C1D24 vs. screening). A measure of 250 ng of total RNA, quantified using the NanoDrop 2000 (Thermo Fisher Scientific), was directly hybridized (at 65°C for 18 hours) with the nCounter PanCancer Immune Profiling Panel and the nCounter Tumor Signaling 360TM Panel following manufacturer's instructions. After solution-phase hybridization between target RNA and reporter-capture probe pairs, excess probes were washed away using a two steps magnetic bead–based purification on the nCounter Prep Station. Finally, the RNA/probe complexes were aligned and immobilized in the cartridge for data collection. The cartridge was then transferred to the nCounter Digital Analyzer for image acquisition and counts collection. Quality control was done according to default settings using NanoString nSolver software v4.0. Background correction was conducted with background thresholding by calculating the mean of negative control expression plus double of the standard deviation.

Additional details about data analysis are provided in Supplementary Methods S1.

### Statistical analysis

In phase I (dose escalation) of this study, the first two dose cohorts required at least two subjects to be evaluated. For the remaining cohorts, a minimum of three subjects evaluable for the dose-determining set (DDS) were treated per dose cohort until determination of the MTD and/or one or more RP2Ds. It was estimated that up to 41 subjects would need to be enrolled, taking dropouts and additional subjects enrolled for some of the dose groups into account; the actual number of subjects will depend on the number of dose levels/cohorts that are tested.

The safety set comprised all patients who received at least one dose of AFM24 and was the primary population for all safety-related (except determination of the dose–DLT relationship) and efficacy-related endpoints. The DDS included patients in the safety set who received at least 80% of the assigned AFM24 dose in cycle 1 and completed the 28-day DLT observation period or experienced a DLT any time during cycle 1. The DDS was used in the Bayesian logistic regression model to estimate the dose–DLT relationship. Demographics, baseline characteristics, and prior cancer history were listed and summarized using descriptive statistics. Absolute and relative frequencies were used to summarize the incidence, severity, and type of AEs. Efficacy parameters were visualized using a swimmer plot, showing the RECIST assessments over time and a waterfall plot showing the best percentage change from baseline in sum of the longest diameter.

### Data availability

The data generated in this study are available within the article and its supplementary data files, including RRIDs reported in Supplementary Table S3; additional information is available upon request from the corresponding author.

## Results

### Patient population

In the dose-escalation phase, 38 patients underwent eligibility screening and 35 patients received AFM24 treatment; baseline characteristics are reported in **Table 1**. Among the 35 enrolled patients, the median age was 58.0 years (range = 29–81), and 65.7% were male. The most common tumor types were colorectal cancer ($n$ = 19, 54.3%) and NSCLC ($n$ = 8, 22.9%). Of the patients with colorectal cancer, two patients had tumors with high microsatellite instability; all other patients with colorectal cancer ($n$ = 17) either had microsatellite stable (MSS) disease or their microsatellite instability status was unknown. Patients had received a median of 4 (range = 2–11) previous lines of systemic anticancer therapy, and 28% of patients received prior radiation.

The number of patients included across the dose cohorts were as follows: 14 mg, $n$ = 2; 40 mg, $n$ = 6; 80 mg, $n$ = 4; 160 mg, $n$ = 5;

**Table 1.** Baseline patient characteristics and demographics.

| | Total (*N* = 35) |
|---|---|
| Age (years), *n* (%) | |
| Median (range) | 58 (29–81) |
| 18–64 | 24 (68.6) |
| ≥65 | 11 (31.4) |
| Sex, (male), *n* (%) | 23 (65.7) |
| Race, *n* (%) | |
| White | 27 (77.1) |
| Black or African American | 2 (5.7) |
| Asian | 3 (8.6) |
| Not reported | 3 (8.6) |
| Ethnicity, *n* (%) | |
| Hispanic or Latino | 6 (17.1) |
| Non-Hispanic or Latino | 27 (77.1) |
| Not reported/unknown | 2 (5.7) |
| ECOG performance status, *n* (%) | |
| 0 | 10 (28.6) |
| 1 | 25 (71.4) |
| Tumor type, *n* (%) | |
| Colorectal cancer | 19 (54.3) |
| MSS/unspecified | 17 (48.6) |
| MSI-H | 2 (5.7) |
| Non–small cell lung cancer | 8 (22.9) |
| Other | 8 (22.9) |
| Number of previous therapies, *n* (%) | |
| Median (range) | 4 (2–11) |
| ≥2 | 35 (100) |
| ≥3 | 27 (77.1) |
| ≥4 | 19 (54.3) |

Abbreviations: ECOG, Eastern Cooperative Oncology Group; MSI-H, high microsatellite instability; MSS, microsatellite stable.

320 mg, $n = 6$; 480 mg, $n = 6$; and 720 mg, $n = 6$. The median number of AFM24 doses administered was 8 (range = 1–39). A total of 31 patients completed cycle 1 and were therefore included in the DDS.

### Safety assessments

All 35 patients received at least one dose of AFM24 and were included in the safety set. Safety data for all 35 patients are summarized in **Table 2**.

All patients reported ≥1 treatment-emergent adverse event (TEAE) of any grade, regardless of attribution, with 34 patients (97.1%) reporting at least one AE considered related to AFM24. TEAEs per cohort are summarized in Supplementary Table S4. The most common AFM24-related TEAEs observed were IRRs [27 patients (77.1%)], nausea [9 patients (25.7%)], and dermatitis acneiform [8 patients (22.9%)]. Of the 27 patients experiencing IRRs, 12 (34.3%) experienced maximum grade 1; 13 (37.1%) experienced maximum grade 2; 2 (5.7%) experienced maximum grade 3. Five patients reported AFM24-related grade 3 TEAEs [40 mg: IRR ($n = 1$); 160 mg: IRR ($n = 1$) and hypertension ($n = 1$); 320 mg: lymphocytopenia ($n = 1$); 480 mg: lymphocytopenia ($n = 1$), and dermatitis acneiform ($n = 1$)], and one patient experienced a related grade 4 TEAE (lymphopenia) that subsequently resolved. Four patients discontinued from the study due to TEAEs in cycle 1; three in the 40 mg cohort that included IRR (grade 3), unrelated myocardial infarction (grade 3), and unrelated blood bilirubin increase (grade 2), and in the 160 mg cohort, one patient discontinued due to an IRR (grade 2).

There was only one DLT across all dose levels. This occurred in a patient treated with 40 mg AFM24 who experienced a grade 3 IRR with dyspnea, hypoxia, and hypotension at the beginning of the infusion on C1D1. AFM24 was stopped, and the patient was treated with methylprednisolone, diphenhydramine, epinephrine, meperidine, 100% oxygen, and IV fluids. The patient was not re-challenged.

There were no AFM24-related fatal TEAEs during the dose-escalation period; however, there were two unrelated fatalities in the 80 and 720 mg cohorts; both were due to clinical deterioration from progressive disease. Serious TEAEs occurred in 18 patients (51.4%) with only two patients (5.7%) experiencing a serious event considered related to AFM24 [one was the DLT event described above, and one patient in the 160 mg cohort experiencing hypoxia (Grade 2, possibly related to treatment) that resolved].

IRRs were observed predominantly during C1D1 of AFM24 infusion ($n = 25$, 71.4%), with the symptoms being mild-to-moderate, transient, and reversible. In addition to the patient with DLT described above, another patient treated with 160 mg AFM24 reported a grade 3 IRR. This 63-year-old female, diagnosed with NSCLC and metastases to the bone, liver, and lymph nodes, experienced a grade 3 IRR with shortness of breath, facial flushing, nausea, and tachycardia. The patient was treated, and the event lasted 20 minutes; the patient was re-challenged without further IRRs.

The risk of presenting with an IRR decreased substantially with subsequent infusions ($n = 7$ out of 33 who continued after C1D1, 21.2%), Supplementary Fig. S1. Some of the management strategies included a premedication regimen, limiting the rate of AFM24 infusion, starting at a low infusion rate and a ramp-up, split day dosing and infusion interruption.

### Antitumor activity

Tumor response assessments per RECIST v1.1 are shown in **Fig. 2A**. The best objective response was stable disease (SD) in 10 out of 35 patients. Four patients had SD assessed at least twice with a duration of SD ranging from 4.3 to 7.1 months [MSS colorectal cancer, $n = 3$; NSCLC, $n = 1$]; two patients with SD exhibited tumor regression at best percentage change in sum of the longest diameter from baseline (**Fig. 2B**). One patient, a 64-year-old male with stage 4 MSS colorectal cancer, with liver and lung metastases, demonstrated reduced tumor burden despite disease progression following five prior treatment lines that included immunotherapy.

### Establishing the RP2D

The RP2D of AFM24 of 480 mg QW was selected based on the totality of the safety, PK, and PD data. A dose of 480 mg QW was well tolerated and was comparable to other dose levels, with no split day dosing required. Mild-to-moderate IRRs were the most common AE reported but were mostly confined to first dose and were clinically manageable. Acne-like rash was reported in 9 of 35 patients (26%, one grade 3 event) mainly in the higher dose levels and pronounced at 480 mg (four out of six patients), which indicates distribution to tissue and active dose but was well managed, transient, and not considered a safety concern.

AFM24 serum concentrations for all patients were available for PK analysis. Population PK analysis showed that AFM24 demonstrates both linear and nonlinear elimination. The nonlinear elimination is in line with the target-mediated drug disposition for AFM24. The target-mediated drug disposition seemed to reach saturation at doses ≥160 mg, and the approximated $t_{1/2}$ was 11.3 days at these doses. Approximate dose proportional increase in area under the serum concentration–time curve from time 0 to 168 hours

**Table 2.** Summary of TEAEs in all patients ($N = 35$) and AFM24-related TEAEs by maximum grade.

| | All, *n* (%) | AFM24-related, *n* (%) |
|---|---|---|
| Any TEAE | 35 (100) | 34 (97.1) |
| Serious TEAE | 18 (51.4) | 2 (5.7) |
| TEAE grade ≥3 | 20 (57.1) | 6 (17.1) |
| Fatal TEAE | 2 (5.7) | 0 (0) |
| TEAE leading to discontinuation | 4 (11.4) | 2 (5.7) |

| AFM24-related TEAE by maximum grade, *n* (%) | | | | |
|---|---|---|---|---|
| | Grade 1–2 | Grade 3 | Grade 4 | Overall |
| Overall | 28 (80.0) | 5 (14.3) | 1 (2.9) | 34 (97.1) |
| Blood and lymphatic system disorders | | | | |
| Anemia | 2 (5.7) | 0 (0) | 0 (0) | 2 (5.7) |
| Lymphopenia | 0 (0) | 2 (5.7) | 1 (2.9) | 3 (8.6) |
| Thrombocytopenia | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Cardiac disorders | | | | |
| Palpitations | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Tachycardia | 2 (5.7) | 0 (0) | 0 (0) | 2 (5.7) |
| Gastrointestinal disorders | | | | |
| Diarrhea | 2 (5.7) | 0 (0) | 0 (0) | 2 (5.7) |
| Dry mouth | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Gastro-esophageal reflux disease | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Nausea | 9 (25.7) | 0 (0) | 0 (0) | 9 (25.7) |
| Vomiting | 6 (17.1) | 0 (0) | 0 (0) | 6 (17.1) |
| General disorders and administration site conditions | | | | |
| Chest discomfort | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Chills | 2 (5.7) | 0 (0) | 0 (0) | 2 (5.7) |
| Fatigue | 5 (14.3) | 0 (0) | 0 (0) | 5 (14.3) |
| Feeling of body temperature change | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Pain | 2 (5.7) | 0 (0) | 0 (0) | 2 (5.7) |
| Pyrexia | 4 (11.4) | 0 (0) | 0 (0) | 4 (11.4) |
| Immune system disorders | | | | |
| Cytokine release syndrome | 2 (5.7) | 0 (0) | 0 (0) | 2 (5.7) |
| Infections and infestations | | | | |
| Herpes zoster | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Injury, poisoning, and procedural complications | | | | |
| IRR | 25 (71.4) | 2 (5.7) | 0 (0) | 27 (77.1) |
| Investigations | | | | |
| ALT increased | 4 (11.4) | 0 (0) | 0 (0) | 4 (11.4) |
| TNFα increased | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| AST increased | 4 (11.4) | 0 (0) | 0 (0) | 4 (11.4) |
| Blood alkaline phosphatase increased | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Blood lactate dehydrogenase increased | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| C-reactive protein increased | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Lymphocyte count decreased | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Serum ferritin increased | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Metabolism and nutrition disorders | | | | |
| Decreased appetite | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Hypocalcemia | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Hypophosphatemia | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Musculoskeletal and connective tissue disorders | | | | |
| Bone pain | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Myalgia | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Pain in extremity | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Neoplasms benign, malignant, and unspecified (incl. cysts and polyps) | | | | |
| Acrochordon | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Nervous system disorders | | | | |
| Headache | 6 (17.1) | 0 (0) | 0 (0) | 6 (17.1) |
| Presyncope | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Respiratory, thoracic, and mediastinal disorders | | | | |
| Hypoxia | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Skin and subcutaneous tissue disorders | | | | |
| Dermatitis acneiform | 7 (20) | 1 (2.9) | 0 (0) | 8 (22.9) |
| Erythema | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Pruritus | 3 (8.6) | 0 (0) | 0 (0) | 3 (8.6) |
| Rash macular | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Rash maculopapular | 4 (11.4) | 0 (0) | 0 (0) | 4 (11.4) |
| Vascular disorders | | | | |
| Flushing | 1 (2.9) | 0 (0) | 0 (0) | 1 (2.9) |
| Hot flush | 2 (5.7) | 0 (0) | 0 (0) | 2 (5.7) |
| Hypertension | 0 (0) | 1 (2.9) | 0 (0) | 1 (2.9) |

No grade 5 study drug–related TEAE occurred.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; IRR, infusion-related reaction; TEAE, treatment-emergent adverse event.

($AUC_{0-168}$) was only observed at doses ≥320 mg (Supplementary Fig. S2). Steady state seems to be achieved by day 14 for doses ≤160 mg and by day 28 for doses ≥320 mg. A summary of the PK parameters obtained in the noncompartmental analysis is presented in Supplementary Table S5 (C1D1) and Supplementary Table S6 (C1D22). A summary of the approximated apparent $t_{1/2}$ per dose of AFM24 can be found in Supplementary Table S7.

The relationship of the AFM24 PK to the CD16A RO on NK cells was best described by a sigmoidal $E_{max}$ equation with the Hill coefficient of 1. The $E_{max}$ was estimated at 47.9% and the $EC_{50}$ at 15.1 μg/mL. CD16A RO on NK cells approached saturation at doses >320 mg as shown in Supplementary Fig. S3. Due to the expected loss of noncovalently bound AFM24 during PBMC isolation, as assessed during *in vitro* establishment of the method, the maximally achievable CD16A RO levels were around 50%.

It is therefore assumed that further escalation of the dose would not lead to stronger effects based on these results.

In addition, cytokine data support higher doses of AFM24 compared with lower doses; however, there was minimal differentiation between 320 and 480 mg. The proinflammatory cytokines IFN-γ and TNFα increased slightly between infusions in the pg range at higher doses, as assessed prior to each new dose, which may reflect sustained activation of immune cells. In contrast, increases in IL-6 levels were transient and occurred mainly after the first dose of AFM24, particularly at the higher dose levels (≥160 mg). Following an initial increase after the first AFM24 dose (≥160 mg), all subsequent IL-6 concentrations were <10 pg/mL. NK-cell activation and subsequent T-cell activation was observed in PBMC in doses of 160 mg and higher. However, no significant differences could be observed between higher doses. Thus, such activation is considered as a meaningful biological response that supports 480 mg as RP2D but also other doses of AFM24, such as 320 and 720 mg.

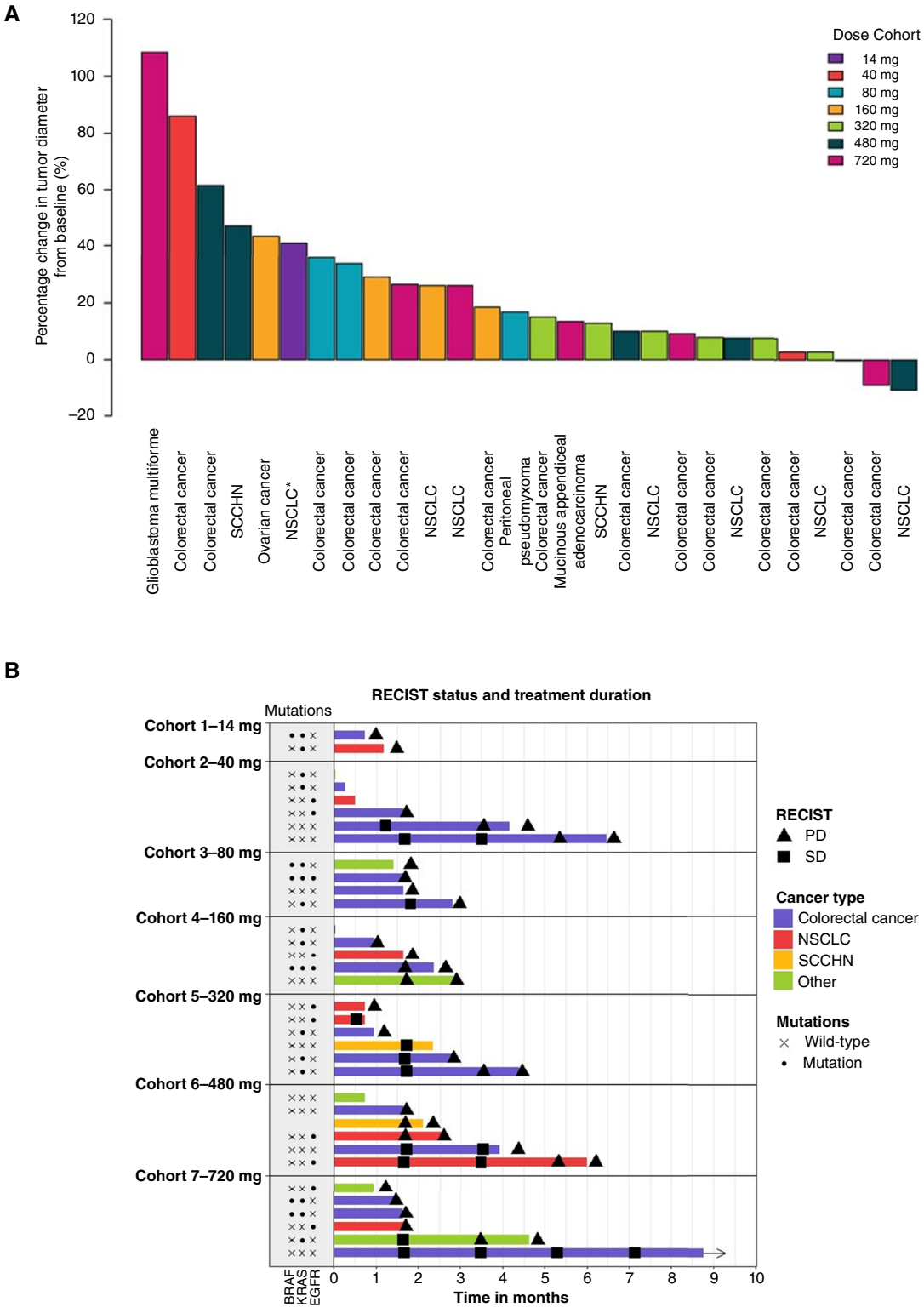


**Figure 2.**

**A,** Tumor status and treatment duration as assessed by the investigator; (**B**) change in tumor diameter from baseline ($N = 28$). **A,** Tumor response assessments per RECIST v1.1 are shown, with the best objective response of SD achieved in 10 of 35 patients. Four patients had SD assessed at least twice, with the duration of SD ranging from 4.3 to 7.1 months. **B,** Two patients with SD exhibited tumor regression at the best percentage change in sum of the longest diameter from baseline. *, The patient died, but their death date is unknown. PD, progressive disease; SCCHN, squamous cell carcinoma of the head and neck.

### AFM24 activates both the innate and adaptive immune system

Tumor biopsies were taken at baseline and on C1D24 (2 days after the third AFM24 dose) of treatment and were available for 18 of 35 patients. All analyzed tumor biopsies were positive for EGFR expression at different levels both at pre-dose (range = 20–285) and C1D24 (range = 40–280), suggesting that EGFR expression is maintained throughout AFM24 treatment (Supplementary Fig. S4). Patients who received higher doses of AFM24 (≥160 mg) showed an increase in infiltrating $CD3^+$ T cells in the tumor area and adjacent tissue upon AFM24 treatment (C1D24; **Fig. 3A** and **B**). Gene expression profiling of immune-related genes revealed an increase in $CD56^{dim}$ NK and CD8 T cell–specific marker genes as well as cytotoxicity-associated genes in tumor biopsies adding further evidence of an increase in NK- and T-cell functions in the tumor upon AFM24 treatment (**Fig. 3C–E**; Supplementary Fig. S5A–S5F).

Serial longitudinal immunophenotyping of PBMCs was performed using cytometry time-of-flight to identify the relative presence and activation status of various hematopoietic cell populations including NK and T cells. $CD16^+$ $CD56^{dim}$ NK cells revealed a reduction in frequency in peripheral blood upon the first dose of AFM24, which was accompanied by an increase in the activation marker Ki-67 and a downregulation of CD16 expression (**Fig. 4A–C**; Supplementary Fig. S6A–S6C). In addition, a decrease in CD8 T-cell frequency and a continuous increase in Ki-67–positive CD8 T cells following AFM24 treatment have been observed, indicating an indirect activation of the adaptive immunity by AFM24 (**Fig. 4D** and **E**). In summary, AFM24 activates cells of the innate immune system and increases NK cells in the tumor area. In addition, T cells, as effector cells of the adaptive immune system, are activated, and their number is increased in the tumor, suggesting broad proinflammatory activity in solid tumors.

## Discussion

AFM24 is a novel, tetravalent bispecific EGFR/CD16A-targeting ICE designed for the treatment of EGFR-positive malignancies. The mechanism of action of AFM24 uses EGFR mainly as a docking site to engage innate immune cells for tumor cell killing through ADCC and ADCP; AFM24 does not rely on inhibition of the EGFR-signaling pathway that differentiates it from current EGFR-targeting treatment options (16). Thus, AFM24 leads to tumor cell killing independent of the mutational status (e.g., *KRAS* and *BRAF*). In addition, as AFM24 does not inhibit signaling through EGFR, the safety profile is distinct from EGFR inhibitors whereby typical toxicities associated with signaling inhibition in normal tissue, such as acne-like rash, mucositis/stomatitis, diarrhea, and electrolyte disturbances, are not observed with AFM24. Notably, AFM24 induces ADCC with higher potency as compared with EGFR-targeting Fc-enhanced mAbs, especially in the presence of physiological concentrations of competing

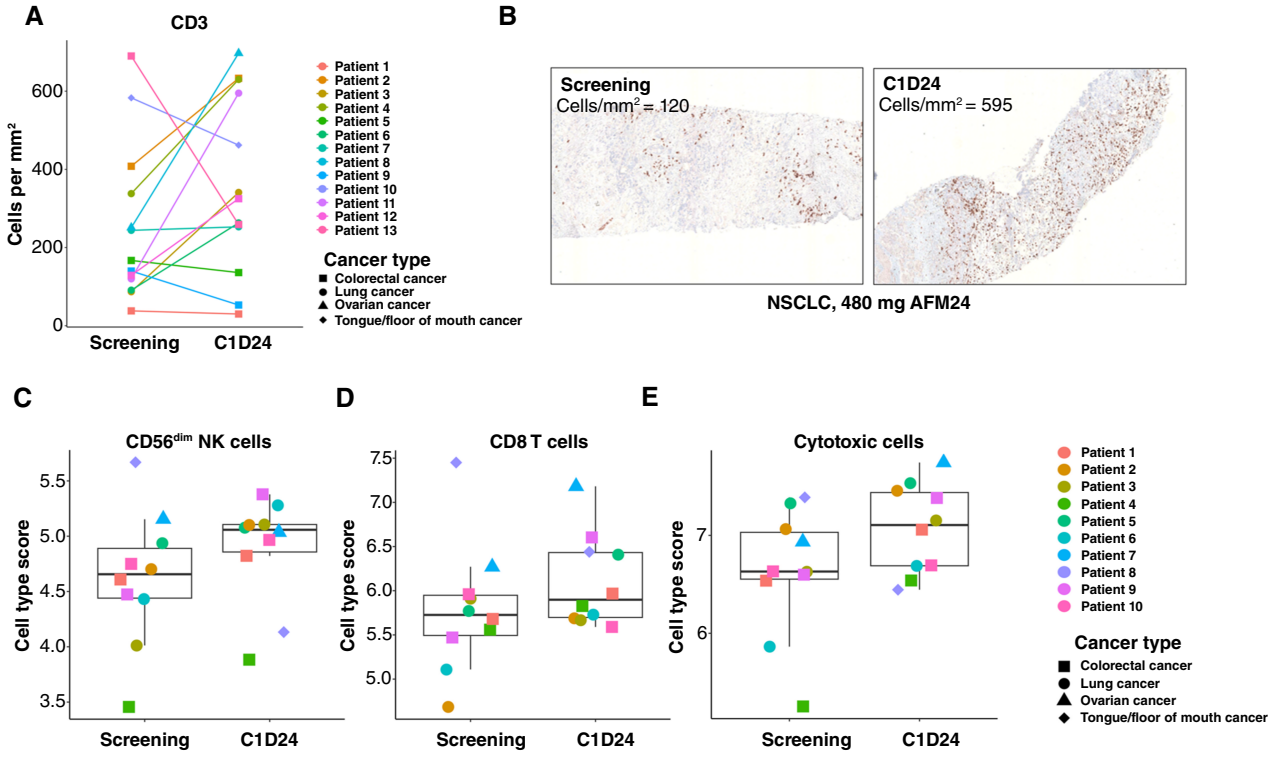


**Figure 3.**

Analysis of tissue biopsies indicates an increase in cytotoxic NK and CD8 T cells in the tumor upon AFM24 treatment. Tumor biopsies were taken at screening and C1D24 of patients treated with AFM24 at doses higher than 160 mg and further analyzed by IHC and gene expression profiling using the NanoString nCounter PanCancer Immune Profiling Panel. **A,** IHC staining of CD3 of tumor biopsies at screening and C1D24, $n = 13$. **B,** Representative CD3 IHC staining of a patient with NSCLC treated with 480 mg AFM24 at screening and C1D24. **C,** Box plot showing $\log_2$ cell type score for $CD56^{dim}$ NK cells, $n = 10$. **D,** Box plot showing $\log_2$ cell type score for CD8 T cells, $n = 10$. **E,** Box plot showing $\log_2$ cell type score for cytotoxic cells (as defined by the NanoString nCounter PanCancer Immune Profiling Panel), $n = 10$.

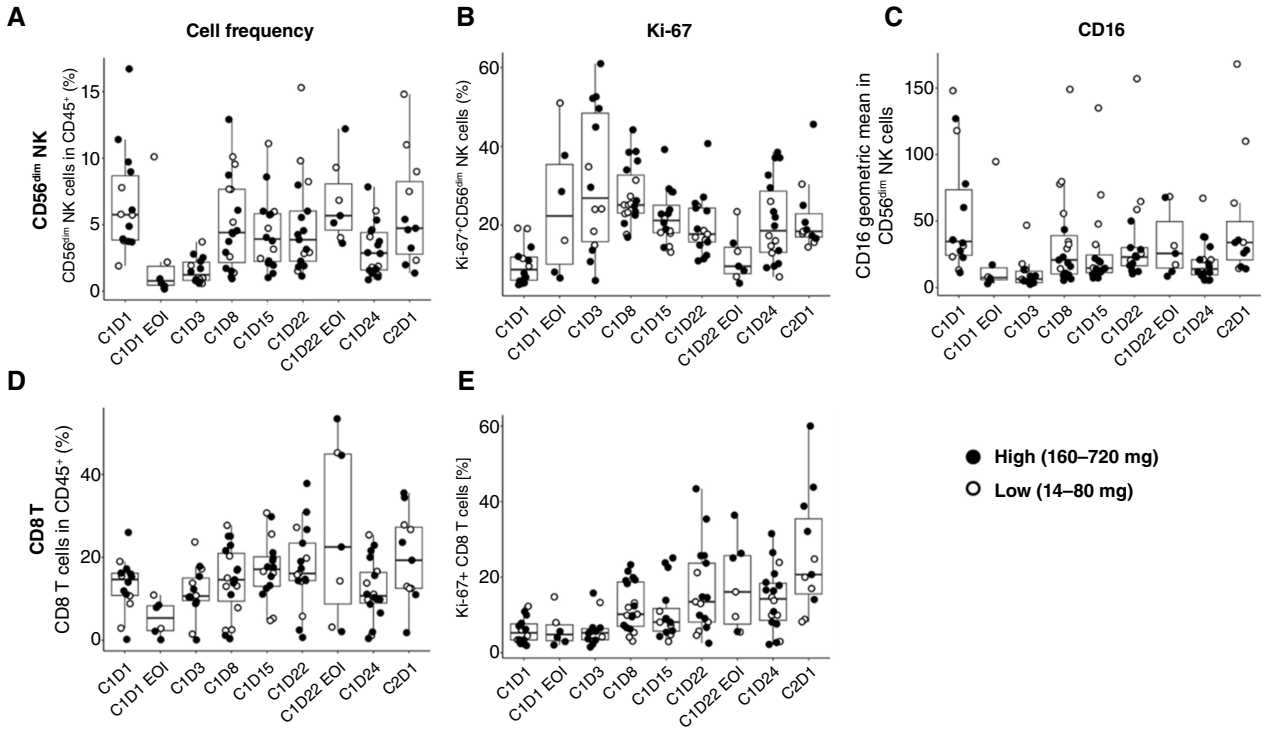


**Figure 4.**

AFM24 activates NK cells in peripheral blood and indirectly triggers adaptive immunity through the activation of CD8 T cells. Immune cell populations in isolated PBMCs and relevant activation markers have been longitudinally analyzed by cytometry time-of-flight. Cell populations have been identified by Boolean gating. Box plots showing (**A**) the CD56$^{dim}$ NK-cell frequency in CD45$^+$ cells, (**B**) the frequency of Ki-67$^+$ CD56$^{dim}$ NK cells, (**C**) the geometric mean of CD16 on CD56$^{dim}$ NK cells, (**D**) the CD8 T-cell frequency in CD45$^+$ cells, and (**E**) the frequency of Ki-67$^+$ CD8 T cells.

IgG (11). In this context, we reported that AFM24-mediated ADCC *in vitro* was observed with comparable potency and efficacy across a broad range of EGFR-positive tumor cell lines, which covered a diverse range of mutations in *BRAF* and *KRAS* as well as against nonmutant EGFR-positive tumor cell lines.

This is the first-in-human study evaluating an ICE in EGFR-expressing tumors. The safety and efficacy of AFM24 monotherapy was investigated in patients with solid tumors known to express EGFR, predominantly colorectal cancer and NSCLC, whose disease had progressed after treatment with multiple standard-of-care and other anticancer therapies. AFM24 demonstrated a well-managed safety profile up to the maximum dose tested of 720 mg QW. There was only one DLT (grade 3 IRR) at the 40 mg dose level, and after this event, the protocol was amended to add steroids to the premedication regimen. IRRs were the most frequently reported TEAE, and additional IRR management methods were established including ramped-rate infusion and split-day dosing. The DLT definition was changed to exclude grade 3 IRR that is well-managed because IRRs occurred independently of the AFM24 dose, and almost all patients could be re-challenged without further IRRs. In addition, most IRRs occurred during the first AFM24 infusion and were associated with mild-to-moderate symptoms. Transient and reversible grade ≥3 AFM24-related TEAEs were infrequent and reported in six patients (17.1%), only two patients (5.7%) had serious events, and importantly, there were no AFM24-related deaths. The MTD was not reached, and therefore, the RP2D of 480 mg QW was selected based on the tolerable safety profile, dose proportional increases in PK, and peripheral CD16A RO reaching a plateau.

Following treatment with AFM24, NK-cell activation in peripheral blood and an increase in NK cell–specific marker genes in tumor biopsies were observed. Activation of CD3$^+$ cells in peripheral blood and increased infiltration of these cells into the tumor area were also observed, suggesting stimulation of anticancer immunity beyond the innate immune system, possibly as an indirect effect of AFM24. Overall, correlative science results show the activation of CD16$^+$ CD56$^{dim}$ NK cells and CD8 T cells in the peripheral blood and indicate a migration of cytotoxic cells into the tumor microenvironment. Taken together, these data support the activation of the innate immune system and the adaptive immune system by AFM24, with migration of cytotoxic NK and CD8 T cells into the tumor microenvironment.

These results not only provide proof-of-concept for AFM24 but also a rationale for combining AFM24 with other immunotherapies, in particular with those facilitating the crosstalk of innate and adaptive immunity to complement a multifaceted antitumoral immune response. In this context, AFM24 might be a key to render the tumor microenvironment to a "hotter" phenotype, where tumors are infiltrated with mostly T cells, which, in turn, could provide an environment primed for treatments aimed to stimulate T-cell responses such as checkpoint inhibitors (CPI). Despite the potential for synergy of innate and adaptive immunity, innate immunity has previously been under-explored as a therapeutic target (21). Immune checkpoint inhibitors, including mAbs against PD-1 and PD-L1 provided an alternative avenue for patients with EGFR-expressing tumors (22). However, primary resistance (immediately after treatment initiation), secondary resistance (after initially showing clinical benefit),

and progression after treatment discontinuation have been an issue in patients treated with these therapies (2, 23–25).

Collectively, based on the available data, the combination of AFM24 and CPIs may hold promise to cooperatively boost adaptive T-cell immune responses. AFM24 is considered to promote recruitment and activation of T cells, whereas CPIs, such as PD-1/PD-L1 inhibitors, release T cells from the immunosuppressive effects of the PD-1–PD-L1 axis (26).

Furthermore, studies on the potential of NK cells to promote favorable clinical outcomes are currently in development. An NKp30-engager molecule, based on the Fab variant of cetuximab, has shown targeted killing of EGFR-expressing tumor cells via efficient NK cell–mediated cytotoxicity in preclinical models (27). Co-engagement of several activating receptors potentiates NK-cell activation, and similarly, precomplexing ICE with NK cells has been shown to enhance the NK cell–mediated cytotoxicity in patients with relapsed or refractory $CD30^+$ lymphomas (28), thus demonstrating a potential avenue for future development (5).

AFM24 single-agent activity was modest in this unselected and heavily pretreated patient population. The phase IIa (dose expansion) study in EGFR-expressing tumor-specific cohorts is ongoing and includes those with a confirmed diagnosis of clear-cell renal cell carcinoma, MSS colorectal cancer, or EGFR-mutant NSCLC (29). The tolerable safety profile and promising correlative biomarker data, whereby AFM24 demonstrated activation of the innate and adaptive immune systems in paired tumor biopsies, highlighting a unique mechanism of action, could provide strong support for AFM24 treatment to be used in conjunction with other immunotherapies to enhance the innate and adaptive immune systems. The AFM24 clinical development program is currently exploring the potential of combination strategy to target tumors known to express EGFR by investigating AFM24 in combination with the PD-L1 inhibitor, atezolizumab (NCT05109442). Preliminary findings from this study indicate that AFM24 with atezolizumab shows promising signs of clinical efficacy, even in patients with resistance to prior CPI, and a well-tolerated and manageable safety profile (30); recruitment to this study is ongoing. Exploring whether immunotherapies should be introduced at earlier stages of disease warrants further investigation.

## Authors' Disclosures

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## Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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