Enhanced Antibody-Mediated Phagocytosis and Antibody-Mediated Cell Cytotoxicity Using Tetravalent, Bispecific Innate Cell Engagers (ICE®) in 3D Spheroids

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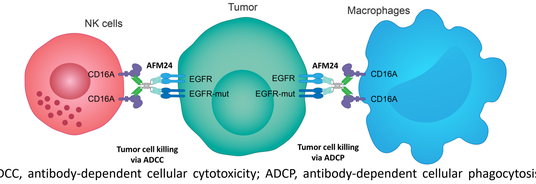
BACKGROUND

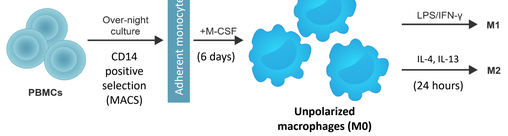
- **I**nnate **C**ell **E**ngager (ICE®) molecules bispecifically engage CD16A+ natural killer (NK) cells and macrophages and tumor antigens, resulting in enhanced anti-tumor activity of NK cells and macrophages
- AFM24 is an ICE® specific to epidermal growth factor receptor (EGFR), a protein which is overexpressed in many solid cancers, and which may indicate poor prognosis[1,2]
- Currently used anti-EGFR agents, such as tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAbs) have various limitations including: an inability to address broad patient populations due to primary mechanism of signal inhibition; activation of alternate pathways or downstream mutations can give rise to resistance in the EGFR signaling cascade; dose limiting toxicities may lead to treatment discontinuation or suboptimal dosing[3–6]
- AFM24 engages CD16A on NK cells and macrophages with a higher affinity than therapeutic monoclonal antibodies; once engaged, AFM24 can trigger responses against EGFR-expressing cancer cells including[7]:
  - NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC)
  - Macrophage-mediated antibody-dependent cellular phagocytosis (ADCP)
- The mode of action of AFM24 can overcome key limitations of current EGFR-targeted therapies by being independent of EGFR activity; signaling pathway resistance development cannot arise[7]
- Preclinical and clinical data suggest that ICE® molecules demonstrate promising safety and efficacy as monotherapies as well as in combination with other immunotherapeutic approaches[7,8]

AFM24 CAN BIND AND KILL EGFR EXPRESSING TUMOR CELLS REGARDLESS OF MUTATIONS IN EGFR SIGNALLING



ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; NK, natural killer cells.

MONOCYTE DIFFERENTIATION AND MACROPHAGE POLARIZATION



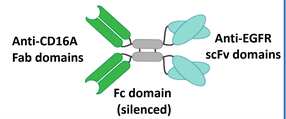
Sketch of PBMC-derived monocyte differentiation into M0 macrophages with M-CSF for 6 days. Polarization of M0 macrophages into M1 (with LPS and IFN-γ) and M2 (with IL-4 and IL-13) macrophages over 24 h. IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell.

OBJECTIVES

- To use two-dimensional (2D) patient-derived xenograft (PDX) cell cultures to access ADCP in the presence of ICE®, such as AFM24
- To establish three-dimensional (3D) spheroid cultures as an improved model to mimic the physiological micro-environment to test ICE®
- To assess the ability of AFM24, to induce ADCP and ADCC in 3D cultures of PDX cell lines

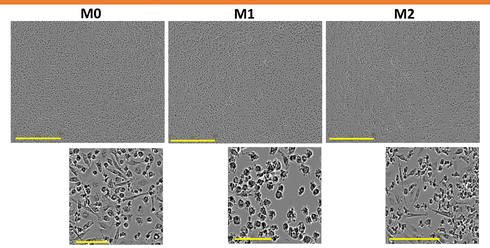
AFM24 STRUCTURE

- AFM24 is a prototypic ICE® derived from the **R**edirected **O**ptimized **C**ell **K**illing (ROCK®) antibody platform
- AFM24 is a bispecific, tetravalent EGFR/CD16A IgG1-scFv fusion antibody (scFv-IgAb) with a silenced IgG1 Fc



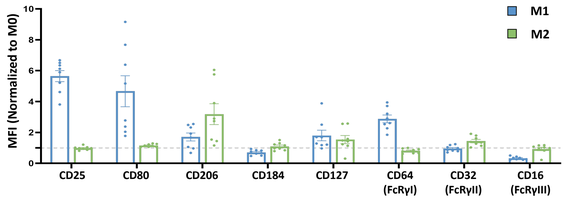
RESULTS

Morphology of different macrophage subsets – M0, M1 and M2, were consistent with previously published data[9,10]



Monocytes (CD14+)-derived from healthy donor PBMCs were isolated and differentiated into M0 macrophages and polarized to M1 or M2 macrophages. Cells were visualized using 10X magnification. Below is a zoom in view.

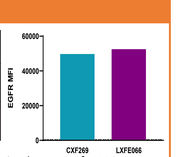
Macrophage subsets express characteristic surface proteins[9,10]



M0, M1 or M2 macrophages were stained for flow cytometry analysis, before acquisition on the BD FACS Fortessa (BD Biosciences). Graph shows M1 and M2 normalized MFI for each marker relative to M0 macrophages. Dotted line indicates relative M0 MFI equivalent to 1. MFI, mean fluorescence intensity.

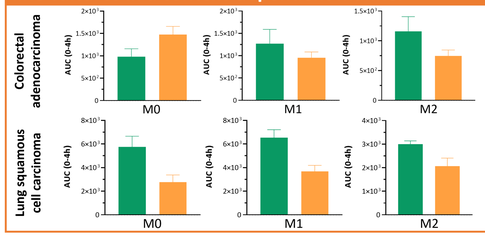
Consistent EGFR expression levels (RNA and protein) in selected PDX cell lines

Cell lines	EGFR gene expression (Log2 GCRMA)	KRAS mutation
Colorectal adenocarcinoma **(CXF269)**	Positive (8.8)	Unknown
Lung squamous cell carcinoma **(LXFE066)**	Positive (10)	No



Log2 GCRMA 8-10: reflects medium to high expression; PDX, patient-derived xenograft; MFI, mean fluorescence intensity

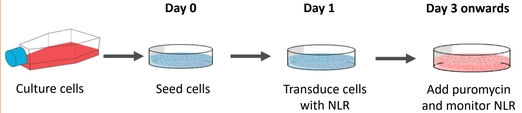
AFM24 can engage and induce phagocytosis in all macrophage subtypes in 2D PDX cell line cultures in a context-dependent manner



On day 7, target cells were stained with 250 ng/mL pHrodo Red before being co-cultured with M0, M1 or M2 macrophages, in the presence of AFM24 (10 µg/ml) for 8 hours. Wells were imaged every 20 mins for 8 hours with IncuCyte® S3. Target cell phagocytosis was then masked based on pHrodo fluorescence and the mean total area of the masked object for the first 4 hours of the assay was plotted. (E:T = 5:1). Higher AUC values depict increase in phagocytosis events.

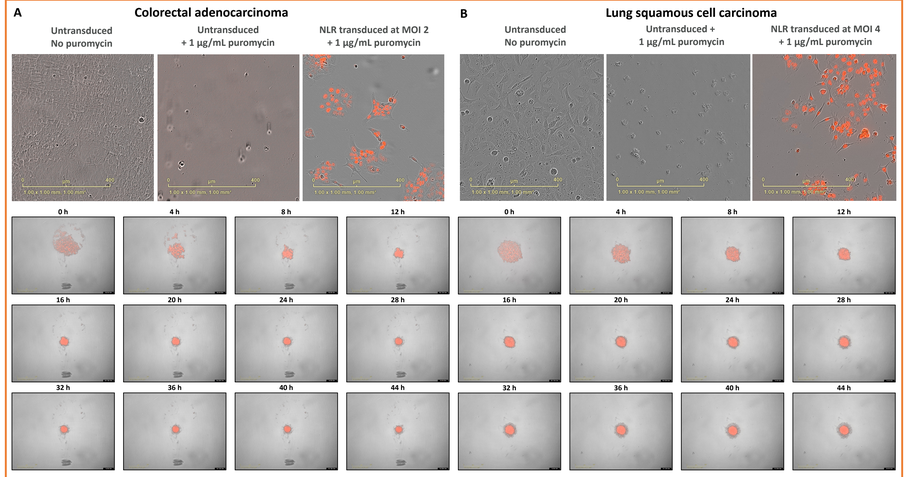
AFM24
Vehicle

Scheme for stable expression of NLR in selected PDX lines



NLR, Nuclight Lentivirus Reagents.

Successful establishment of a stable NLR+ PDX colorectal adenocarcinoma (A) and lung squamous carcinoma (B) cell lines and 3D spheroid cultures



CXF269 cells (panel A) and LXFE66 cells (panel B) were cultured for ~3 weeks prior to being plated and an antibiotic kill curve was used to determine the optimal concentration of puromycin, which in this case was 1 µg/mL or 2.5 µg/mL. The cells were transduced with NucLight Red Lentiviral particles (NLR, Essen Bio) and the transduction media replaced with media containing 1 µg/mL or 2.5 µg/mL puromycin 3 days later, respectively. The cells were then monitored for NLR expression using an IncuCyte®. Images shown were taken 7 days or 14 days post-puromycin selection, respectively, scale 0–400 µm. MOI, multiplicity of infection; NLR, Nuclight Lentivirus Reagents.

Timelapse of spheroid cells captured every 4 hours, scale 0–800 µm (below). CXF269-NLR and LXFE66-NLR cells were plated in ultra-low attachment (ULA) plates at 2,500 cells/well centrifuged at 200 x g for 10 min. Cultures were imaged every 2 h for 48 hours using IncuCyte® S3 to assess spheroid formation. These spheroids can then be used to test ICE® in the prescence of NK cells or macrophages over a 4-day period using the IncuCyte® S3.

CONCLUSIONS

- Here we successfully establish 2D and 3D assay conditions to investigate the effect of AFM24 in PDX cell lines, with the 3D model designed to replicate intrinsic physiological conditions
- Preliminary results show AFM24 can induce ADCP of tumor cells in 2D PDX cell cultures, engaging M0, M1 and M2 macrophage subsets in a context-dependent fashion
- We have successfully established a 3D spheroid platform using EGFR-expressing PDX cell lines

OUTLOOK STATEMENT

- Based on the successful generation of macrophage subsets and establishment of a 3D assay setup, we can use this platform to test Affimed's ICE® with various PDX cell lines to investigate the tumor response and better predict *in vivo* outcomes

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