Abstract
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CD16A-Specific Tetravalent Bispecific Immuno-Engagers Potently Induce Antibody-Dependent Cellular Phagocytosis (ADCP) by Macrophages

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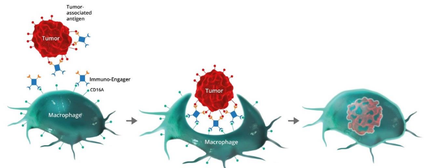
Introduction

Affimed has developed high affinity tetravalent bispecific immuno-engagers for redirected optimized cell killing (ROCK® platform). Using anti-CD16A and anti-tumor target-specific antibody domains, the engagers activate NK cells to efficiently kill target cells. The most advanced immuno-engager is AFM13 targeting CD30 on tumor cells and CD16A on innate immune effectors and is currently evaluated in several clinical trials to treat CD30-positive malignancies[1,2].

Based on the fact that CD16A is not exclusively expressed on NK cells, but also on macrophages, we hypothesized that CD16A-specific immuno-engagers would also be able to activate CD16A-expressing macrophages through antibody-dependent cellular phagocytosis (ADCP) contributing to anti-tumor response.

Macrophages can be broadly classified into different subtypes including M1 (classically activated, generally characterized as pro-inflammatory and immuno-supportive) and M2 (alternatively activated primarily of an anti-inflammatory profile) subtypes. Those subtypes greatly differ in their phenotype and function and appear to be highly plastic. While M1 macrophages are generally considered to be tumoricidal, M2 macrophages are mostly tumorigenic depending on their context within the tumor microenvironment.

Peripheral monocytes derived from primary human hematopoietic cells of healthy donors were used to generate macrophages in vitro. We were able to demonstrate binding to macrophages and robust tumor cell killing by ADCP for several CD16A-specific immuno-engagers which appeared to be at least as potent as that mediated by Fc-gamma receptors pan-specific classical monoclonal antibodies. Importantly, anti-tumoral response by ADCP was not restricted to a certain macrophage subtype offering the potential to boost tumoricidal function within the tumor microenvironment in solid tumors irrespective of the phenotype of high abundant macrophages.

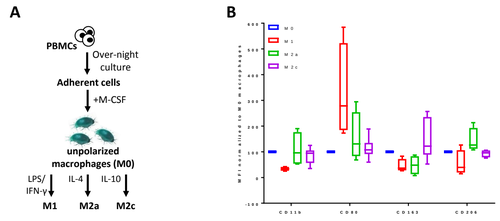


Antibody-dependent cellular phagocytosis (ADCP) induced by Affimed's ROCK® antibodies.

In vitro differentiation and characterization of human macrophages

- ***In vitro*** **polarized M1 macrophages upregulate the M1-specific marker CD80 on the cell surface**
- ***In vitro*** **polarized M2a and M2c macrophages upregulate the M2-specific markers CD163 and CD206 on the cell surface**

In vitro differentiation, polarization and characterization of human macrophages

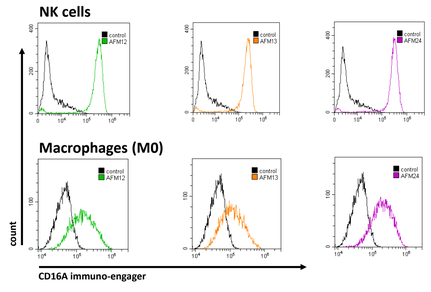


In vitro **differentiation and characterization of human macrophages from primary human monocytes.** A) Experimental scheme of *in vitro* differentiation and polarization of human macrophages. C) Flow cytometric assessment of cell surface markers expressed on *in vitro* differentiated and polarized macrophages (n=4). Mean fluorescence intensities (MFI) of the indicated surface markers were normalized to their expression on unpolarized (M0) macrophages.

Specific binding of ROCK® immuno-engagers to CD16A⁺ macrophages and NK cells

- **Binding of ROCK® antibodies AFM12, AFM13 and AFM24 to human NK cells and** ***in vitro*** **differentiated human macrophages**

Binding of ROCK® antibodies to human CD16A expressing cells

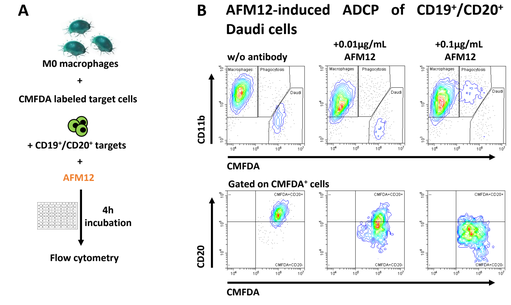


Binding of ROCK® antibodies to freshly isolated NK cells or ***in vitro*** **differentiated macrophages from primary human PBMCs.** Binding of CD19/CD16A AFM12, CD30/CD16A AFM13, or EGFR/CD16A AFM24 was performed on human NK cells or unpolarized human macrophages at 37°C and assessed by flow cytometry.

AFM12 induces ADCP by M0 macrophages

- **Flow cytometry-based** ***in vitro*** **phagocytosis allows quantification of ADCP**
- **Loss of the independent antigen CD20 during phagocytosis validates tumor cell engulfment**

Flow cytometric assessment of ADCP

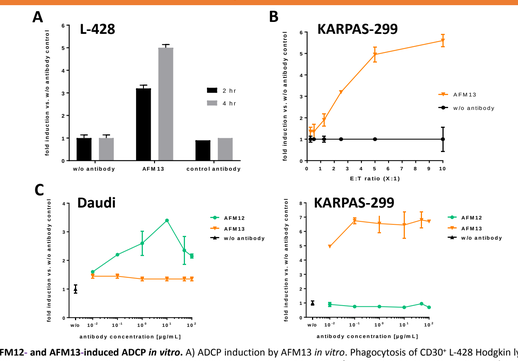


Flow cytometric assessment of ADCP ***in vitro*****.** A) Experimental setup for analyzing AFM12-induced ADCP by M0 macrophages *in vitro*. B) Exemplary FACS plots of AFM12-induced ADCP using CD19⁺/CD20⁺ Burkitt's lymphoma Daudi cells as targets and M0 macrophages as effectors. Cells were co-incubated for 4h at an E:T of 5:1. Engulfment of target cells was monitored by co-expression of macrophage-specific CD11b and intracellular CMFDA staining, as well as loss of CD20 on the surface of phagocytosed target cells.

AFM12 and AFM13 rapidly and specifically induce ADCP by macrophages

- **Fast onset of CD16A-engager-mediated ADCP within 2h**
- **ADCP by CD16A-engagers is an antibody dose- and E:T ratio-dependent process**
- **CD16A-engagers induce specific ADCP towards target antigen-positive cells but not towards target antigen-negative cells**

Characterization of ADCP induced by AFM12 or AFM13

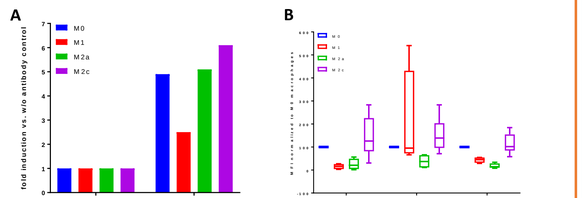


AFM12- and AFM13-induced ADCP ***in vitro*****.** A) ADCP induction by AFM13 *in vitro*. Phagocytosis of CD30⁺ L-428 Hodgkin lymphoma cells upon co-culture with *in vitro* M2c macrophages with or without 10µg/mL AFM13 or control antibody after 2 and 4h respectively. EGFR/CD16A antibody was used as negative control. Phagocytosis was determined by flow cytometry quantifying CMFDA^{high}CD11b⁺ cells as fold induction of control w/o antibody. B) Titration of the ratio between M0 macrophages as effector and CD30⁺ KARPAS-299 as target cells in presence or absence of 30µg/mL AFM13. Phagocytosis was determined by flow cytometry quantifying CMFDA⁺CD11b⁺ cells as fold induction of control w/o antibody. C) AFM12- and AFM13-induced target cell phagocytosis at an E:T of 5:1 after 4h co-culture of M0 macrophages with CD19⁺CD30⁺ KARPAS-299 or CD19⁺CD30⁻ Daudi cells. Phagocytosis was determined by flow cytometry quantifying CMFDA⁺CD11b⁺ cells in % as fold induction of control w/o antibody.

Strong AFM13-induced ADCP mediated by different macrophage subtypes

- **All CD16⁺ macrophage subtypes, but especially M2c macrophages mediate AFM13-induced ADCP**
- **Efficacy of ADCP correlates with CD16 expression level on macrophage subtypes**

ADCP mediated by differentially polarized macrophages



AFM13-induced ADCP ***in vitro*** **mediated by different macrophage subtypes.** A) Phagocytosis of CD30⁺ anaplastic large cell lymphoma KARPAS-299 cells upon co-culture with *in vitro* differentiated human macrophages of the indicated subtypes in presence or absence of 10µg/mL AFM13. Cells were co-cultured for 4h at an E:T of 5:1. Phagocytosis was determined by flow cytometry quantifying CMFDA⁺CD11b⁺ cells as fold induction of control w/o antibody. B) Flow cytometric assessment of CD16, CD32 and CD64 expressed on *in vitro* differentiated and polarized macrophages (n=4). MFIs of the indicated Fcγ receptors were normalized to its expression on M0 macrophages.

AFM24 induces robust ADCP by M0 macrophages

- **AFM24 (EGFR/CD16A) is able to induce robust ADCP on EGFR+ target cells**
- **ADCP is specific and dependent on both EGFR- and CD16A-binding of AFM24**

In vitro ADCP induced by AFM24

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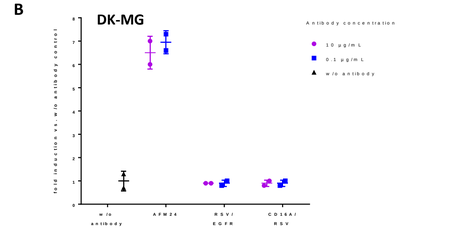
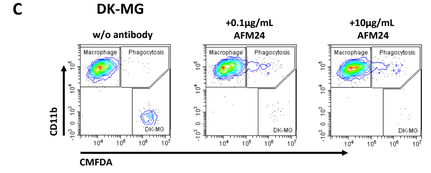
Cell line	Origin	EGFR surface expression (SABC)
DK-MG	Glioma	222,648





AFM24-induced ADCP ***in vitro*****.** A) Table summarizing the expression level of EGFR as determined by specific antibody binding capacity (SABC) using QIFIKIT and anti-EGFR mAb H11. B) AFM24-induced phagocytosis of EGFR-expressing DK-MG after 4h co-culture with M0 macrophages at an E:T of 5:1. Phagocytosis was assessed by flow cytometry quantifying CMFDA⁺CD11b⁺ cells as fold induction of control w/o antibody. C) AFM24-induced phagocytosis of EGFR-expressing DK-MG cells mediated by M0 macrophages. Exemplary FACS plots showing phagocytosis of DK-MG cells upon 4h co-culture with or without AFM24 at the indicated concentrations. Cells were co-cultured at an E:T of 5:1.

Key Conclusions

- All ROCK® based CD16A immuno-engagers binding different tumor targets tested in this study were able to induce ADCP
- ADCP induced by CD16A immuno-engagers:
  - Is tumor antigen-specific and depends on binding to both CD16A on macrophages and target antigen on tumor cells
  - Can be induced in all CD16+ macrophage subsets
- AFM24 induces ADCP of EGFR+ target cells, offering the possibility to target macrophage-prevalent solid tumors
- Macrophages contribute to the ROCK® CD16A immuno-engager mechanism of action

#Corresponding author; Presenting author; Disclosures: J.P. and A.C. received research funding from Affimed; S.W., U.R., A.B., J.K. and M.T. are employees of Affimed.

References: [1]) Reusch et al., MAbs. 2014 May-Jun;6(3):728-39. [2]) Rothe et al., Blood. 2015 Jun 25;125(26):4024-31.