A novel immunocytokine fusion protein combining tumor-targeting anti-CD47 antibody with GM-CSF cytokine for enhanced antitumor efficacy

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AACR 2018 #5622

ABSTRACT

CD47 blockade has emerged as a promising cancer immunotherapy by promoting phagocytosis of tumor cells. However, this treatment strategy may be limited by the predominant accumulation of macrophages with pro-tumor M2 phenotype. Therefore, reprogramming of M2-type macrophages into M1-type macrophages has been considered as a better potential therapy to boost the antitumoral effects by macrophages. The key myeloid cytokine GM-CSF is approved as an adjunctive agent in the treatment of cancer by virtue of its ability to stimulate hematopoiesis and particularly induce the differentiation of pro-inflammatory M1 macrophages. Here we report the development of a novel immunocytokine composed of a newly discovered CD47 IgG1 fused with GMCSF at the C-terminus (1F8-GMCSF). In macrophage-tumor cell co-culture, treatment with 1F8-GMCSF fusion molecule led to significantly higher levels of tumor cell phagocytosis and M1 characteristic cytokines production than that with anti-CD47 alone. 1F8-GMCSF demonstrated superior efficacy in suppression of tumor growth as well as shift of tumor associated macrophages from M2 to M1 phenotype compared to 1F8 or GM-CSF treatment alone or in combination. In vivo tracking of injected anti-CD47 antibodies by live imaging confirmed the enriched localization of antibodies in the CD47$^+$ tumor site. Taken together, our data showed that 1F8-GMCSF fusion exhibited enhanced phagocytosis and M1 macrophage activation, leading to the superior antitumor efficacy.

METHODS

Generation of anti-CD47-GMCSF fusion molecule: A lead anti-CD47 mAb (1F8) was identified from panning of a naïve human library for binding to human CD47 extracellular domain (ECD). All binders with unique VH and VL were converted to full IgG and screening through a series of functional assays. The human GMCSF sequence was then fused via various flexible linkers to the C-terminus of 1F8's Fc to generate different formats of anti-CD47-GMCSF fusion molecules.

In vitro phagocytosis assay: Human PBMC-derived macrophages were seeded in 24-well plates (1 x 10^5 cells/well) overnight. On the next day, 5 x 10^5 CFSE-labeled Raji cells were added to the macrophages followed by addition of IgG control or anti-CD47-GMCSF fusion molecules. Phagocytosis activity was measured by analyzing the percentage of CFSE and CD14 double-positive cell population by flow cytometry.

TF-1 proliferation assay: Human erythroleukemic TF-1 cells were seeded at 1 x 10^4 cells/well of a 96 well plate. Recombinant human GMCSF control or anti-CD47-GMCSF fusion was added to the cells and incubated for 72 hours at 37°C. Cell proliferation was measured by adding CellTiter-Glo® according to the manufacturer's recommendation.

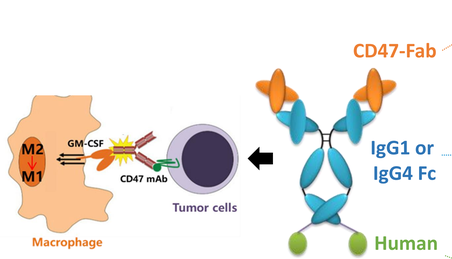
Human M1 macrophage activation: Primary human macrophages were co-cultured with tumor cells in the presence of IgG control, GMCSF alone, anti-CD47 mAb alone or anti-CD47-GMCSF fusion for 24 hours. The culture supernatant was collected for the production of TNF-a, IL-6 and IL-12 and the cells were analyzed for the expression of CD80 by flow cytometry.

In vivo xenograft model and M1/M2 staining: NSG mice were subcutaneously engrafted with Raji cells and dosed twice a week with IgG control, 1F8 mAb, mouse GMCSF, 1F8 mAb + mouse GMCSF combo or 1F8-mouse GMCSF fusion (70 nmol per mouse) when the average tumor volume reached 100-150 mm^3. Tumors were excised and fixed in 4% paraformaldehyde for immunofluorescence (IF). Sections were stained with anti-iNOS antibody for M1 macrophages or anti-CD163 antibody for M2 macrophages and counterstained with DAPI for nuclei.

In vivo biodistribution: The biodistribution of anti-CD47 antibody was analyzed by in vivo live imaging. 1F8 was labeled with indocyanine green (ICG) according to the manufacturer's protocol (Dojindo Molecular Technologies) and intravenously injected into Raji cell-bearing mice (10 mg/kg). At the time of injection and at 6, 24 and 72 hours after administration, mice were imaged with IVIS Lumina III system to monitor the distribution of the ICG-1F8 antibody.

RESULTS

Figure 1. Design of anti-CD47-GMCSF Fusion (C4GM)



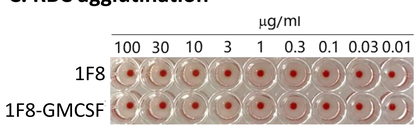
- Promote tumor phagocytosis
- Enhance anti-tumor immune responses
- Retain the RBC-sparing properties
- Maintain the potency of fusion cytokine with the use of flexible linker
- Trigger the shift of M2 to M1 phenotype (potentiate phagocytosis)
- Modulate the cytokine milieu of the tumor microenvironment

Figure 2. C4GM demonstrated comparable potency in CD47 activity to the parental mAb.

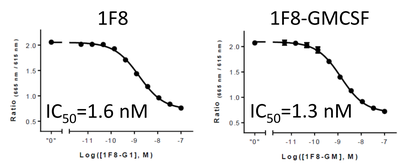
A. CD47 binding

	ka (1/Ms)	kd (1/s)	KD (M)
1F8	3.61E+05	2.82E-03	7.81E-09
1F8-GMCSF	7.21E+05	4.43E-03	6.14E-09

C. RBC agglutination



B. SIRPα competition



D. Phagocytosis by macrophage

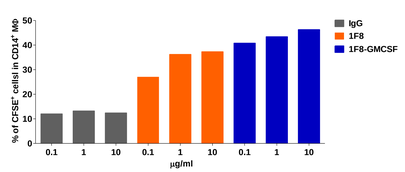
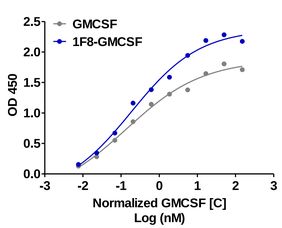
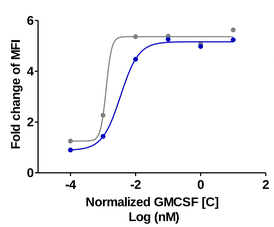


Figure 3. C4GM demonstrated comparable potency in GM-CSF activity to the recombinant protein.

A. GMCSF receptor binding



B. pSTAT5 signaling



C. TF-1 proliferation

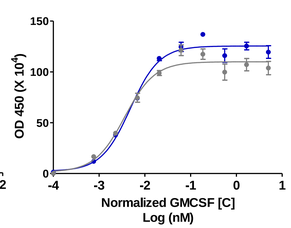
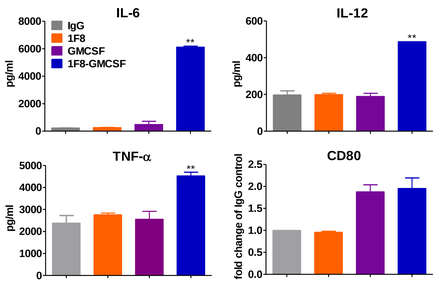
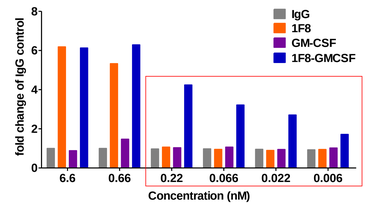


Figure 4. C4GM induced a robust M1 activation and enhanced phagocytosis in macrophage-tumor co-culture assay.

A. M1 activation in co-culture



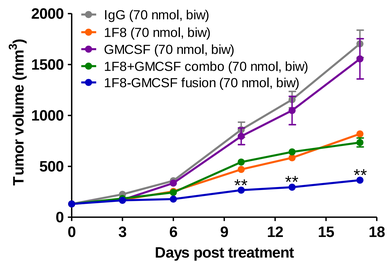
B. Enhanced phagocytosis in co-culture



1F8-GMCSF fusion induced higher levels of tumor phagocytosis **at lower concentrations** compared to 1F8 alone.

Figure 5. C4GM showed superior efficacy in Raji xenograft model with a shift of towards M1 polarization in TAMs.

A. In vivo efficacy of C4GM in Raji model



B. Shift of M1/M2 phenotype in TAMs

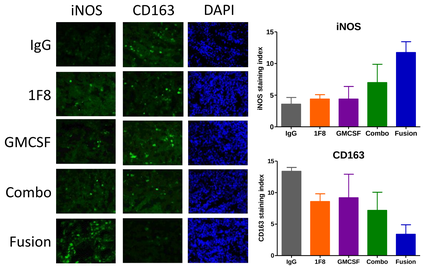
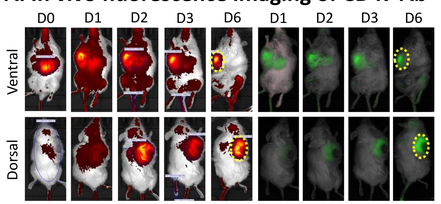
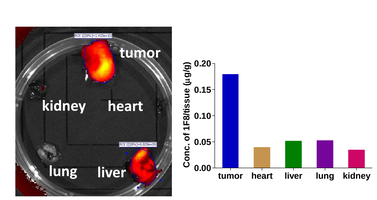


Figure 6. Anti-CD47 Ab was enriched in tumor site after in vivo injection.

A. In vivo fluorescence imaging of CD47 Ab



B. Enrichment of CD47 Ab in tumor



SUMMARY

- C4GM is specifically designed to treat solid tumor with Mϕ infiltration – as opposed to CD47 mAb for hematological malignancies.
- C4GM demonstrates synergistic effects in enhancing M1 macrophage activation and tumor phagocytosis in vitro.
- C4GM shows superior efficacy in tumor growth inhibition in vivo compared to anti-CD47 alone or in combination with GMCSF, partly through focused local polarization of TAMs towards the M1 phenotype.
- Anti-CD47 mAb is highly enriched in tumor compared to other organs after systemic injection.