Supplementary Material

Lactoferrin-containing immunocomplex mediates antitumor effects by resetting tumor associated macrophages to M1 phenotype

Hongliang Dong[1*], Yueyao Yang[1], Chenhui Gao[2], Hehe Sun[1], Hongmin Wang[1], Chao Hong[1], Jun Wang[1], Fangyuan Gong[1], Xiaoming Gao[1]

*** Correspondence:** Corresponding author: Dr. HL Dong, hldong@suda.edu.cn; or Dr. XM Gao, xmgao@suda.edu.cn;

1 Supplementary Data

1.1 In vitro macrophage differentiation.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood from HD by density gradient centrifugation at 500g for 30 min on Ficoll lymphocyte separating solution (Dakewe Biotech) at room temperature. The PBMCs were collected and washed twice with PBS. All donors gave written informed consent to participate in the study. Monocytes were purified from PBMCs by magnetic cell sorting using CD14 microbeads (MiltenyiBiotec, Germany). Macrophages were generated by culturing freshly separated monocytes for 6 days in RPMI 1640 (Hyclone) containing 10% fetal bovine serum (FBS, Biological industries) supplemented with 20 ng/ml recombinant human M-CSF (Peprotech). At day 3, half of the medium was replaced by new medium containing cytokines. At day 7, the medium was totally replaced in the presence of 20 ng/ml recombinant human IFN-γ for M1, and IL-4 (Peprotech) for M2, respectively. TAMs derived human blood monocytes or mouse bone marrow cells were in vitro exposed to cancer cell (MDA-MB-231 or B16)-conditioned media plus the cytokine cocktail of IL-4, IL-10, and M-CSF.

Supplementary Material

1.2 Cell proliferation assay.

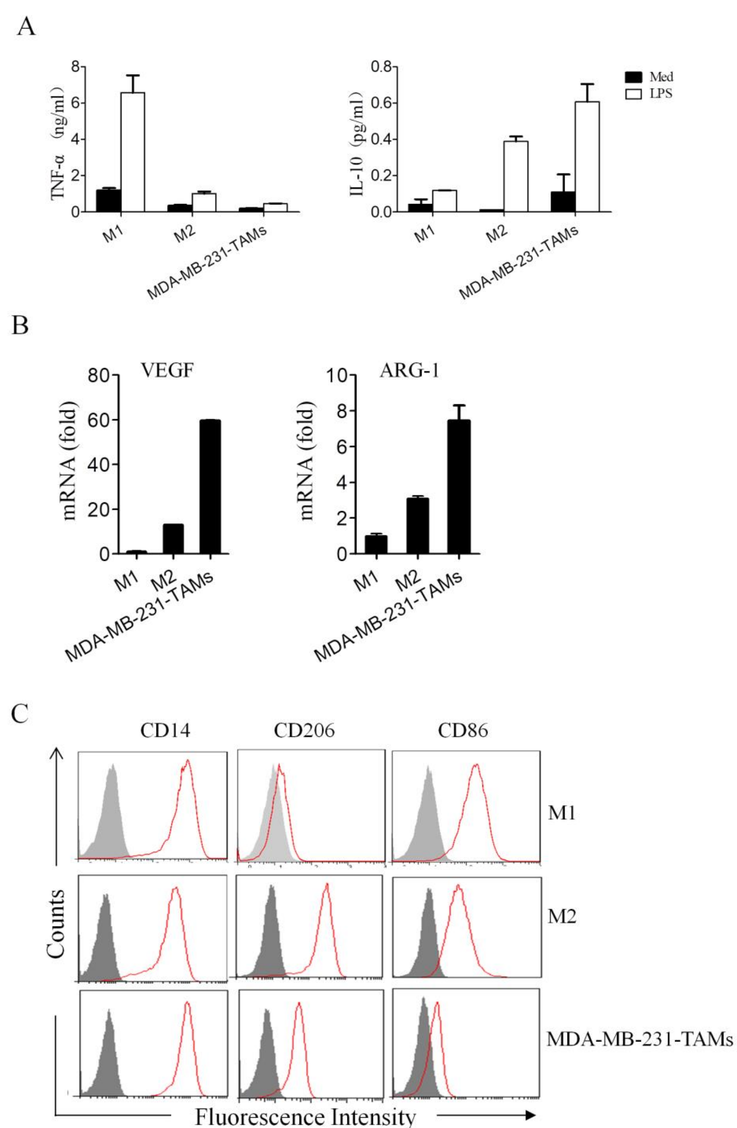
Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MDA-MB-231 cells were seeded in 96-well plates at a final density of 5×10^3 cells per well, treated with or without (Med) LTF, M860, LTF-IC (30μg/ml) for 48 hrs, and then 20μl of MTT was added to each well and incubated for 4 hrs at 37°C. Absorbance values were measured at 492nm with an automated plate reader (Synergy H4 Hybrid Reader, Biotech). The results of the cell viability assay in three independent experiments (3 wells per condition) were normalized to the medium control group and expressed as mean ± SD.

1.3 Stimulation.

MDA-MB-231-TAMs or freshly purified monocytes were harvested by gentle pipetting and stimulated ($3\sim5\times10^4$ cells/well) with 0-300 μg/ml LTF or LTF-IC in 96-well plates (Nunc) for 24h in a 5% CO_2 incubator at 37˚C, then the supernatants were collected and the levels of TNFα were measured by ELISA kits (eBioscience) according to the manufacturer's instructions.

2 Supplementary Figures and Tables

2.1 Supplementary Figure 1

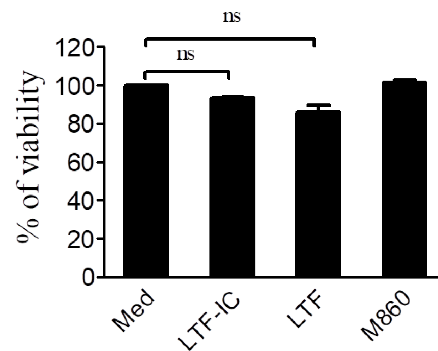


Supplementary Fig. 1

Supplementary Figure 1. M1, M2 macrophages and TAMs differentiation and identification. Freshly differentiated M1, M2 macrophages and MDA-MB-231-TAMs (5×10^4 cells/well) were incubated with 100 ng/ml LPS for 16 hours and the supernatants were collected. Concentration of TNFα and IL-10 was detected by using human TNFα and IL-10 ELISA kit **(A)**. M1, M2

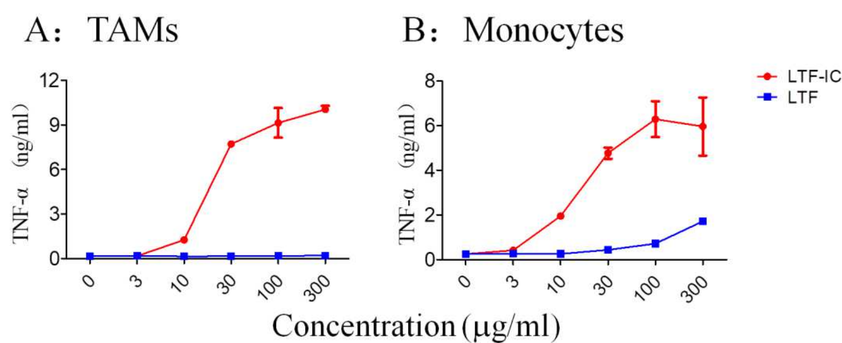
macrophages and MDA-MB-231-TAMs were collected and analyzed for mRNA expression of VEGF and ARG-1 (normalized to GAPDH expression, fold change compared with M2) that was determined by quantitative RT-PCR **(B)**. Human monocyte-derived M1/M2 macrophages and MDA-MB-231-TAMs were stained with APC-conjugated anti-human CD14, APC-conjugated anti-human CD86, and FITC-conjugated anti-human CD206 for 30 minutes, and subjected to analysis by flow cytometry **(C)**. The results are representative of three experiments from different donors.

2.2 Supplementary Figure 2



Supplementary Figure 2. LTF-IC didn't inhibit proliferation of tumor cells. MDA-MB-231 cells (5×10^3 per well) were cultured in 96-well plates in the presence of LTF, M860, LTF-IC (30μg/ml) for 48 hrs. Cell viability following treatment was measured by MTT assays.

2.3 Supplementary Figure 3



Supplementary Figure 3. TAMs are unresponsive to lactoferrin. MDA-MB-231-TAMs (A) or freshly purified monocytes (B) were treated with indicated concentrations of LTF-IC or LTF for 18 hrs. Cytokine levels were determined by TNF-α ELISA kit. The results are expressed as mean±SD performed in parallel and representative of at least three experiments with different donors.

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