Lactoferrin-Containing Immunocomplexes Drive the Conversion of Human Macrophages from M2- into M1-like Phenotype

Chen-Hui Gao1, Hong-Liang Dong*†‡, Li Tai†‡ and Xiao-Ming Gao*

Institute of Biology and Medical Sciences, Soochow University, Suzhou, China

Macrophages are multifunctional cells that perform diverse roles in health and disease and considered the main source of inflammatory cytokines in affected joints of patients with rheumatoid arthritis (RA). M2 macrophages are well known as anti-inflammatory and wound-healing cells; however, recent evidence suggests that they can also promote inflammation in RA, although the underlying mechanism remains to be clarified. Based upon our recent finding that lactoferrin (LTF)-containing IgG immunocomplex (LTF-IC), found elevated in RA sera, potent activators of human monocytes/macrophages, we herein demonstrate that LTF-IC was able to elicit immediate proinflammatory cytokine production by M2-polarized human macrophages through coligation with CD14/toll-like receptor (TLR) 4 and FcγRIIa (CD32a). The LTF-IC-treated M2 cells adopted surface marker expression profile similar to that of M1 phenotype and became functionally hyperactive to subsequent stimuli such as lipopolysaccharide, zymosan and IL-1β, which could provide a positive feedback signal to promote excessive inflammation in RA. They also acquired the ability to facilitate activation of Th17 cells that are known to play critical roles in RA pathology. We propose that IgG ICs containing TLR agonizing autoantigens are able to directly switch human macrophages from M2 into M1-like phenotype, thereby promoting excessive inflammation in autoimmune diseases such as RA.

Keywords: immune complex, lactoferrin, M2 macrophages, hyperactivity, rheumatoid arthritis

INTRODUCTION

Macrophages exhibit phenotypical and functional plasticity and can acquire a continuum of polarization states depending on the environmental cues (1–5). Classically activated macrophages (M1) are characterized by elevated expression of MHC class II, release of proinflammatory cytokines, generation of reactive oxygen species, and tumoricidal activity. Alternatively activated macrophages (M2), on the other hand, express high levels of anti-inflammatory cytokine IL-10, vascular endothelial growth factor, cyclooxygenase-2-derived PGE2 and possess tumor-promoting activity (6). In general, M1 macrophages predominate at the initial stages of inflammatory responses, whereas M2 macrophages drive the resolution of inflammation and tissue repair after injury and maintain tissue homeostasis (1, 2). It has been reported that transformation of tissue macrophages from M2 to M1 phenotype can occur in vivo which may hold the key to the break of self-tolerance and immune-pathogenic damage in autoimmune diseases (7–10).
Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic progressive joint inflammation that affects approximately 1% of the population worldwide (11–15). Production of autoantibodies is a hallmark of systemic autoimmune diseases such as RA and accumulating evidence suggests that immunocomplexes (ICs) between autoantibodies and self-antigens are pivotal pathogenic players in vivo, particularly through triggering Fc receptors (FcγRs) on infiltrating monocytes or tissue-resident macrophages. Macrophages/macrophages are the main source of proinflammatory cytokines (IL-1β, IL-6, and TNFα), which act both locally and systemically and are currently the main targets for RA immunotherapy (7–9). Both M1 and M2 macrophages could be identified in inflamed synovia of RA patients (9, 16), where the M1 cells were considered as the “bad guys,” while M2 the opposite. However, it has recently been shown that, after exposure to plate-coated IgG (representing insoluble IgG ICs deposited in local tissues) plus bacterial lipopolysaccharides (LPSs), a prototype Toll-like receptor (TLR)-4 agonist, M2 macrophages strongly produced inflammatory cytokines, indicating that costimulation through FcγRs and TLRs could drive M2-M1 macrophages conversion (10). Since some of the major autoantigens, such as lactoferrin (LTF) and citrulinated fibrinogen, in RA patients are also TLR agonists (17, 18), we propose that RA-related ICs might be able to drive the conversion of M2 macrophages into M1-like phenotype via synergistic signaling through TLRs and FcγRs.

Lactoferrin is an ~80 kDa multifunctional iron-binding glycoprotein of the transferrin family found in most mammalian exocrine secretions as well as secondary granules of neutrophils (19, 20). LTF-specific IgG autoantibodies are found in patients with various autoimmune diseases as RA, systemic lupus erythematosus (SLE), and antineutrophil cytoplasmic Ab-positive autoimmune vasculitis (21–23). We recently reported that LTF-containing ICs (LTF-ICs) are potent activators of human monocytes/macrophages (17). In the present study, LTF-IC was taken as a representative RA-related IC for investigation of their ability to drive the conversion of M2 into M1-like phenotype of human macrophages. Results from this study will have important implications for our understanding on the role of ICs in RA.

MATERIALS AND METHODS

In Vitro Macrophage Differentiation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood from healthy donors (HDs) by density gradient centrifugation at 500 g for 30 min on Ficoll lymphocyte separating solution (Dakewe Biotech) at room temperature. All donors gave written informed consent to participate in the study. CD14+ blood monocytes were purified from PBMCs by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Germany) and used for generation of M1 and M2 macrophages following the protocol of Vogelpoel et al. (10). Monocytes were cultured 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) for M2, or 500 U/ml recombinant human GM-CSF (Peprotech) for M1 macrophages. For M2, 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 IL-4 (Peprotech), respectively. Since the new guideline for in vitro polarization of M1 and M2 macrophages (1) was not followed in the present study, the differentiated M1 and M2 cells herein prepared are specified (GM-CSF)-M1 and (M-CSF)-M2 macrophages, respectively.

Preparation of LTF-ICs

Anti-huLTF antibodies from RA patients (LTF-Abs) and LTF-ICs were prepared as previously described (17). Briefly, LTF-Abs were sequentially purified by affinity chromatography on LTF-S4B (prepared in our laboratory) and Protein A-S4B columns (Pierce) from six pooled plasma samples shown by ELISA to contain high levels of anti-LTF antibodies. The eluted IgG fractions were concentrated by centrifugation with buffer exchange to phosphate-buffered saline (PBS) (Amicon Ultra, Millipore) and were depleted of endotoxin by filtration through a polymyxin B column (Detoxygel). IgG concentrations were determined by optical density at 280 nm; IgG was aliquoted, and stored at −80°C. Preparation and characterization of a mouse mAbs against huLTF (M860), bovine serum albumin (BSA) (J1) or chicken ovalbumin (M562) in this laboratory have also been documented (24). For preparation of LTF-IC, human LTF (2 µg/ml, Sigma-Aldrich) and M860 (2 µg/ml, mAbs of LTF, purified with protein G antibody affinity chromatography, GE Healthcare Life Sciences) or LTF-Abs were mixed in a sterile tube with gentle rotation at 37°C for an hour. IC between LTF and M860 were separated from the uncoupled Ab and antigen using Sephadex Superfine G-75 column. The elutions of IC were pooled, desalted and concentrated. Endotoxin was removed by polymyxin B coupled beads repeatedly and the level of endotoxin in IC was below 1 EU/mg which was detected by Chromogenic LAL Endotoxin Assay Kit (Genscript). ICs between BSA (Sigma-Aldrich) and J1 were used as control and prepared similarly.

Flow Cytometry

For phenotyping, cells were collected, washed with PBS, and the pellets were incubated for 30 min at 4°C with 50 µl APC-conjugated mouse anti-human CD14, CD163, CD16, or CD32, or PE-conjugated mouse anti-human CD86 or CD206, or FITC-conjugated isotype control Abs (Biolegend). After washes, the cells were subjected to analysis by flow cytometry Attune NxT (Life Technology).

Stimulations and ELISAs

In vitro differentiated macrophages were harvested by gentle pipetting and stimulated (3–5 × 10^4 cells/well) with 30 µg/ml LTF, M860 (LTF-Abs), M860-IC (LTF-IC), or 100 ng/ml LPS (from Escherichia coli 0111:B4; Sigma-Aldrich) in 96-well plates (Nunc) for 18 h in a 5% CO2 incubator at 37°C, then the supernatants were collected and stored at 4°C, for analysis by ELISA.

For PAMPs and inflammatory cytokine restimulation assays, LTF-IC-pretreated macrophages (5 × 10^4 cells/well) were restimulated with different stimuli, including 10 ng/ml LPS, 1 µg/
ml zymosan and 1 µg/ml curdlan, or with cytokines including 10 ng/ml IL-1β, 1,000 U/ml IFN-α/β, 2,000 U/ml IFN-γ, 100 ng/ml IL-6, 5 ng/ml IL-12, and the cytokines were collected. Cytokine levels were determined by TNFα ELISA kit.

The levels of TNFα, IL-1β, IL-6, IL-10, IL-17, IL-23, and IFN-γ in human macrophage culture supernatants were measured by using ELISA kits (from eBioscience) according to the manufacturer’s instructions. Standard curves were established using human recombinant cytokines provided in the kits.

**T Cell Activation**

Memory T cells were isolated from PBMCs of HDs using flow sorting (Aria III, BD Biosciences) stained with anti-CD45RO-PE (Biolegend) and anti-CD4-APC (MiltenyiBiotec). For *in vitro* activation of T cells, 2 × 10^5 (M-CSF)-M2 macrophages were stimulated with 30 µg/ml LTF, M860, M860-IC or 100 ng/ml LPS, and then cocultured with 2×10^5 allogeneic memory CD45RO+CD4+ T cells in presence of 2 µg/ml anti-IL-4 (R&D) and 10 µg/ml anti-IFN-γ (BD Pharmingen), costimulated with 100 ng/ml anti-CD3 antibodies (eBioscience) coated in the plates. Every 2 days, half of the medium was replaced by RPMI 1640 (Hyclone) containing 10% FBS and 20 U/ml recombinant human IL-2 (Peprotech). After 4 days, cells were transferred to 96-well flat-bottomed culture plates (Nunc).

For intracellular cytokine staining, T cells were restimulated by cell stimulation cocktail (including PMA, ionomycin, brefeldin A, and monensin, eBioscience) for 6 h. Cells were harvested and washed, fixed with fixation buffer (Biolegend) for 20 min at room temperature, washed again, permeabilized with Intracellular staining perm wash buffer (Biolegend) for 30 min. Cells were incubated with anti-IL-17- FITC (1:50, MiltenyiBiotec) and anti-IFN-γ-APC (1:50, MiltenyiBiotec) for 60 min at room temperature and analyzed by flow cytometry (Canto II, BD Biosciences). For cytokine analysis, resting T cells were restimulated with 1 µg/ml anti-CD3 and 1 µg/ml anti-CD28 (eBioscience). Supernatants were harvested after 24 h and stored at 4°C until analysis by ELISA.

**Quantitative RT-PCR**

For mRNA-level analysis, cells were lysed at the indicated time points, after which mRNA extraction was performed using Omega RNA Isolation Kit and cDNA synthesis using First Strand cDNA Synthesis Kit (Takara). Quantitative RT-PCR (StepOnePlus real-time PCR system; Applied Biosystems, Life Technology), was performed using SYBR green (Takara) and primer pairs as listed in Table 1. The mRNA levels were normalized to housekeeping gene expression [2^(-ΔΔCT)(housekeeping) – Ct(target)]^, and folds were calculated compared with an unstimulated control sample.

**Antibody and Inhibitor Blockade**

Blocking antibodies were preincubated with (M-CSF)-M2 macrophages for 2 h in a 5% CO2 incubator at 37°C, after which stimuli and culture medium were added resulting in a final antibody concentration of 5 µg/ml anti-CD16/32/64 and 2 µg/ml anti-CD14. Syk or TLR4 was inhibited by incubating M2 macrophages with titration of R406, Belnacasan VX-765 (both from Selleckchem) or CLI-095 (Invitrogen) for 2 h at 37°C before stimulation.

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**Statistical Analysis**

All experiments were repeated at least three times and the results are expressed as mean ± SD. Comparison of the data was performed using the Student’s *t*-test. Significance was defined as a *P* value of <0.05.

**Ethics Statement**

This study was approved by the Ethics Committees of Soochow University Medical School, Suzhou, China. Written informed consent was obtained from all participants prior to inclusion in the study.

**RESULTS**

**Proinflammatory Cytokine Production by Human (M-CSF)-M2 Cells following LTF-IC Stimulation**

Human (GM-CSF)-M1 and (M-CSF)-M2 macrophages were generated *in vitro* by treating freshly purified human CD14+ monocytes (Figure S1A in Supplementary Material) for 7 days with GM-CSF or M-CSF plus IL-4, respectively. The resultant cells displayed expected surface marker expression profiles of M1 (CD86 and CD64high) or M2 (CD14, CD163, and CD16 high) phenotypes (Figure S1B in Supplementary Material). Q-PCR results confirmed mRNA transcription for the genes of pro-inflammatory cytokine TNFα, while (M-CSF)-M2 produced anti-inflammatory cytokine IL-10 in response to subsequent LPS stimulation (Figure S1D in Supplementary Material).

Based on our recent finding that LTF-IC was able to coligate TLR4 and FcγRIIa on human monocytes/macrophages and induce a strong TNFα response (17), we wondered whether LTF-IC could also elicit inflammatory cytokine production by
human (M-CSF)-M2 cells \textit{in vitro}. In the experiment shown in \textbf{Figure 1}, (M-CSF)-M2 cells were stimulated with a mixture (1:1) of human LTF (hLTF) and LTF-specific IgG autoantibodies (affinity purified from RA sera) for 18 h followed by quantitation of TNF\(\alpha\), IL-1\(\beta\), IL-6, and IL-10 in the culture supernatant. Clearly LTF-IC, but not anti-LTF IgG or hLTF alone, or huLTF plus control hIgG, was able to elicit production of TNF\(\alpha\), IL-1\(\beta\), IL-6, and IL-10 in the culture supernatant. Like autoantibody-containing LTF-ICs, complex between hLTF and mouse anti-hLTF mAb M860 (M860-IC) is also capable of eliciting proinflammatory cytokine, but not IL-10, production by human monocytes (17), which can be explained by the fact that mouse IgG1 binds human Fc\(\gamma\)Rs with relatively high affinity (26, 27). In the present study, M860-IC strongly triggered
FigUre 2 | M860-IC enhances proinflammatory cytokines transcription in M2. (M-CSF)-M2 macrophages were stimulated with, or without (M), 30 µg/ml human lactoferrin, M860, M860-IC, or 100 ng/ml lipopolysaccharide (LPS) for 4 or 6 h and then analyzed for mRNA expression of (a–g) indicated genes (normalized to GAPDH expression, fold increase compared with unstimulated controls) by quantitative RT-PCR at indicated time points. The results are representative of three experiments with different donors.
heparin, CLI095, R406 or blocking mAbs against human CD14 or CD32. Taken together, both the CD14-TLR4 pathway and CD32-Syk axis play pivotal roles in LTF-IC-mediated (M-CSF)-M2 activation and subsequent conversion to an M1-like phenotype. Next we asked whether costimulation by unconjugated TLR agonist(s) and deposited IgG could also achieve similar M2 to M1 switch. As summarized in Table 2, combination of plate-coated IgG and soluble LTF was as effective as LTF-IC in eliciting IL-1β, IL-6, and TNFα secretion by (M-CSF)-M2 cells in vitro.

**LTF-IC-Primed M2 Macrophages Promote Th17 Activation**

Macrophages are regarded as antigen-presenting cells capable of inducing CD4+ T helper (Th) cell activation and polarization through cytokines (e.g., IL-1β, IL-6, and TNFα) they produce (28–30). It has also been reported that IgG-opsonized bacteria were able to promote human Th17 response via synergy between TLRs (TLR2, 4, 5) and FcγRIIa in dendritic cells (31). It is therefore reasonable to question whether LTF-IC-primed M2 macrophages could facilitate Th17 cell activation and/or polarization in a similar fashion. In the experiment shown in Figure 6, CD4+ T cells, freshly purified from PBMC of HDs, were cultured together with LTF-IC-stimulated (M-CSF)-M2 macrophages for 96 h, followed by quantitation of IL-17 and IFNγ in the culture supernatant and intracellular staining for the same cytokines in CD4+ cells. Percentage of IL-17+Th cells in the LTF-IC-primed group was significantly higher than that of the controls as evidenced by intracellular staining (Figures 6A,B). Apparently CD4+ T cells strongly responded to LTF-IC-primed (M-CSF)-M2 cells, but...
Figure 4 | Hyper-responsiveness of M860-IC-treated M2 cells. (A) Diagram showing the course of the experiment examining hyper-responsiveness of the lactoferrin (LTF)-containing IgG immunocomplex (LTF-IC)-primed M2 cells. (B,C) (M-CSF)-M2 macrophages were primed for 18 h with, or without (M), 30 µg/ml human LTF (hLTF), M860, M860-IC, or 100 ng/ml lipopolysaccharide (LPS), followed by washout and resting and then a 24 h restimulation with suboptimal concentration (100 ng/ml) LPS. Cytokines in culture supernatants were quantitated using ELISAs. Data from different donors (n = 5) are further compared in (C), each pair of dots represents one donor. ***P < 0.001. (D) Dose curve and (E) time course of M860-IC priming of (M-CSF)-M2 macrophages. The results are expressed as TNFα secretion by the LTF-IC-primed M2 cells in response to a 18 h stimulation with, or without (M), 100 ng/ml LPS. (F) (M-CSF)-M2 macrophages pretreated with, or without (M), M860-IC were restimulated with a panel of stimuli, including LPS (100 ng/ml), zymosan (1 µg/ml), curdlan (1 µg/ml), IL-1β (10 ng/ml), IFN-α/β (1,000 U/ml), IFN-γ (2,000 U/ml), IL-6 (100 ng/ml), or IL-12 (5 ng/ml), for 24 h. (G) (M-CSF)-M2 macrophages pretreated with, or without (M), M860-IC were allowed to rest 1, 3, 5 or 7 days in fresh medium, and then challenged with LPS (100 ng/ml) for TNFα production. The results, expressed as mean ± SD, are representative of three experiments with different donors.

not the controls, by producing large amounts of IL-17 and IFN-γ (Figures 6C,D).

DISCUSSION

An important finding of this study is that LTF-IC is able to not only elicit immediate proinflammatory cytokine production by human (M-CSF)-M2 macrophages but also drive their transformation into M1-like phenotype with sustainable hyperactivity. Interestingly, LTF-IC-primed (M-CSF)-M2 cells are also able to facilitate the activation of memory Th17 cells, a cell type highly activated both systemically and locally in inflamed synovium of RA patients (28). Note that LTC-ICs-treated (M-CSF)-M2 was unable to drive naive CD4 Th into IL17-producing cells in similar experiments (data not shown). Our results provide additional clues for the pathological roles of ICs between autoantibodies and biologically active autoantigens. Given that LTF concentration can be elevated significantly in circulation or synovial fluid of RA patients, the concentration range of LTF-ICs (10–30 µg/ml) used in this study is pathophysiologically relevant. We propose that LTF-ICs can be considered as novel proinflammatory mediators contributing to the pathogenesis of autoimmune diseases such as RA by steering macrophage polarization toward proinflammatory M1-like phenotype.

Some of the TLR agonist-containing IgG ICs from patients with systemic autoimmune disease such as SLE and RA possess potent stimulatory activities on myeloid cells, mostly through synergistic signaling of FcγRIIa and TLRs (10, 17, 18). In the “dual signal activation model,” simultaneous ligation of FcγRIIa (Signal 1) and TLR (Signal 2) results in immediate production of proinflammatory cytokines. For instance, complexes between...
FigURe 5 | M860-IC induced phenotype switching is dependent on CD14 and CD32. (A) (M-CSF)-M2 macrophages were exposed to M860-IC (30 ng/ml) for 18 h in the presence of heparin or R406 (0, 1, 3 µM), mAbs against human CD14, CD16, CD32, CD64, or CLI-095 (5 µM). Isotype-matched irrelevant Abs as well as untreated (M-CSF)-M2 cells (M) were included as controls. TNFα in the culture supernatant was quantitated using ELISAs. (B) (M-CSF)-M2 macrophages were exposed to M860-IC (30 µg/ml) for 18 h in the presence, or absence (None), of anti-CD14 (2 µg/ml), anti-CD32 (5 µg/ml), R406 (3 µM), CLI-095 (5 µM), or heparin (10 µM). Isotype-matched mAbs and DMSO were included as controls. The cells were then stained with fluorescence-labeled mAbs against human CD14, CD163 or CD206 for flow cytometric analysis. The results are shown as mean fluorescence intensity (MFI). (C) The above cells were also harvested and analyzed for mRNA expression of M2-specific markers including ALDH1A2, CLEC5A, INHBA (normalized to GAPDH expression, fold increase compared with un-stimulated control) by quantitative RT-PCR. The results are expressed as mean ± SD performed in parallel and representative of three experiments with different donors.

TABLE 2 | Cross-talk between FcγR and LTF-R in human M2 macrophages.a

| Cytokine quantitationb | Stimulators          |        |        |        |
|------------------------|----------------------|--------|--------|--------|
|                        | Medium               | clgG   | hLTF   | clgG + hLTF |
| IL-1β (pg/ml)          | 26.25 ± 3.64         | 4.95 ± 2.75 | 29.47 ± 0.91 | 558.73 ± 115.30 |
| IL-6(pg/ml)            | 23.27 ± 12.30        | 29.60 ± 1.12 | 39.90 ± 11.21 | 636.88 ± 45.66 |
| TNFα (pg/ml)           | 67.65 ± 7.55         | 84.00 ± 76.77 | 62.31 ± 3.78 | 5,180.38 ± 167.87 |

Data are shown as mean ± SD and representative of three experiments using blood samples from different donors.

aFreshly differentiated human (M-CSF)-M2 macrophages were stimulated with clgG (10 µg/ml), or hLTF(30 µg/ml), or clgG + hLTF for 18 h followed by quantitation of cytokines in the supernatant.

bCytokine levels were determined by TNFα, IL-1β, and IL-6 ELISA kit. Data are shown as mean ± SD and representative of 3 experiments using blood samples from different donors.

RA-related ACPA (anticitrulnated protein autoantibodies) and citrullinated fibrinogen or vimentin, could induce macrophage secretion of proinflammatory cytokines through FcγRIIa and TLR4 engagement (18). DNA-ICs found in SLE patients trigger activation cascade through cooperation of CD32 and TLR9 in monocytes/macrophages (32). It is reasonable to suggest that
all such ICs between autoantibodies and biologically active autoantigens might also be able to endorse M2-M1 polarization and act in a concerted manner to play pivotal roles in initiating overt inflammatory tissue damage in disease conditions (8, 32). Dominguez-Soto and colleagues recently reported that IVIG, a preparation of polyclonal and poly-specific Igs derived from the plasma of thousands of HDs, impaired the effect and function of M1 macrophages, but on the other hand caused a M2-to-M1 polarization switch (33). In their study, however, very high concentration (10 mg/ml) of IgG was used. It is quite unlikely that a significant amount of TLR agonists-containing ICs were present in IVIG preparations derived from HDs rather than patients with autoimmune disorders.

It has been demonstrated by previous investigators that human monocytes/macrophages can be trained, by exposure to C. albicans or β-glucans, to exhibit enhanced proinflammatory responsiveness and glycolysis (34–36). This "trained immunity" of monocytes/macrophages is mediated through Dectin-1/Raf-1 signaling pathway (34), which is different from that (FcγRIIa/Syk) triggered by LTF-IC. While functional polarization of macrophages induced by TLR agonist autoantigen-containing ICs are potentially pathological players in autoimmune disorders, β-glucan-trained macrophages displayed stronger ability in phagocytosis, indicative of more active roles in immunological defense and scavenging.

Implications for characterization of macromolecules capable of driving M2-M1 conversion go beyond the field of autoimmune diseases and infection immunity. Macrophages are the major tumor-infiltrating leukocytes and play a critical role in cancer-related inflammation (1, 2, 6), and depending on their polarization status, tumor-associated macrophages (TAMS) can either promote antitumor immune responses or contribute to tumor progression (6, 37, 38). The M2/M1 phenotype switch of TAM is especially relevant in tumor initiation, progression, and dissemination (37, 39). Currently, strategies to target M2 include depletion or blocking of recruitment (40, 41) and decreasing M2-like TAM via reeducation (42, 25). These
approaches employ chemotherapeutic drugs, Abs, or small-molecule inhibitors, which may cause unwanted adverse effects. In this aspect, targeting TAM with TLR agonist-containing ICs (e.g., LTF-IC) might provide a new potential therapy for cancers.

AUTHOR CONTRIBUTIONS

H-LD and X-MG designed the research. C-HG, H-LD, and LT carried out the experiment. H-LD analyzed the data. H-LD and X-MG prepared the manuscript. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00037/full#supplementary-material.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.