ToxoGRA15II promote macrophages polarization against Hepatic carcinoma by targeting TRAF6

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Research

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Abstract

Background: There is increasing evidence that tumor-associated macrophages play an important role in the development of tumors. The more macrophages, the faster the tumors develop. On the one hand, studies have shown that the dense granule protein molecule of Toxoplasma gondii (ToxoGRA15II) tends to induce the differentiation of classically activated macrophages (M1). GRA15II can also induce M2 reversal polarized to M1. Promote the occurrence of Th1 immune response.

Methods: Here, we constructed LV-gra15, which was transferred to M1-like and/or M2-like macrophages. Furthermore, we injected LV-gra15 by vein into tumor bearing C57BL/6 mice.

Results: We found that M2-like macrophages could be polarized to M1-like macrophages, inducing NO, IL12, TNF-αexpression. Mechanistically, ToxoGRA15 activate NF-κB signaling pathway by binding to TRAF6. Additionally, LV-gra15 promoted p65 nuclear translocation to polarize TAM to M1-like macrophages and induce IFN-γ,TNF-α,IL12, NO expression, inhibiting tumor growth. Subsequently, ToxoGRA15 has a role in cancer pathway and metabolism.

Conclusions: Taken together, these findings could lead to the development of immunotherapy strategies to tumor, due to ToxoGRA15 as polypeptide effector molecule of T.gondii and non-toxicity to mammalians.

Author Summary

Immunotherapy is considered to be a crucial part of cancer treatment in recent years. Specially, Tumor-associated macrophages are deeply involved in hepatocellular carcinoma proliferation, invasion and growth. So we have been looking for ways to polarize macrophages. Toxoplasma gondii is a widespread parasite of the phylum apicomplexa can induce host's immunity response. We found a polypeptide effector of T.gondii, ToxoGRA15, could inhibit tumor development by restoring the immune killing ability of macrophage polarization and investigated the mechanism of its action in tumor envrionment. ToxoGRA15 as a protein capable of early activation of specific cell populations and is no-toxic to mammals, has potential value for a novel immunotherapeutic strategy against tumor.

Background

Hepatocellular Carcinoma (HCC) is one of the most common malignant tumors in the world, accounting for more than 90% of primary infections, with the second highest mortality rate and a 5-year survival rate of approximately 51%[1]. At present, surgery, radiotherapy, chemotherapy, biological therapy and traditional Chinese medicine treatment are the basic comprehensive treatment modes. Due to the early metastasis of tumors and the possibility of recurrence, toxicity and drug resistance, the treatment of hepatocellular carcinoma is still unresolved. In recent years, with the deeper understanding of many fields such as tumor epidemiology, etiology, immunology and genetics, the importance of biotherapy has been further emphasized.

Tumor immunotherapy can activate the body's immune system and rebuild normal immune warning function. It has the advantages of strong targeting, low toxic and side effects, and can produce immune memory. It is one of great significance for the treatment of tumors and prevention of tumor recurrence and metastasis. Tumor microenvironment is a complex ecological environment that contains important factors that determine the malignancy of tumor cells. In the tumor microenvironment, a variety of immune cells participate in the adjustment, the macrophages(Mφ) in large numbers and in each stage of the development of tumor expression of important role, is called tumor associated macrophage (TAMs). It is recruited into tumor tissue under the influence of numerous chemokines (such as CCL2, M-CSF, etc.)[2]. Because Mφ has obvious plasticity[3], it can differentiate into M1 and M2 under different stimulating factors. The M1 type is obtained by the classical activation route. It is induced by Th1 cytokine environment (such as IFN-γ, LPS and TNF), and the expression of MHCII, CD80 and CD86 on M1 surface is enhanced, and it has high antigen-presenting ability[4–5]. High levels of pro-inflammatory cytokines such as IL-12 TNF-α are secreted by triggering respiratory bursts and releasing nitric oxide (NO) to kill intracellular pathogens. The M2 type is obtained by an alternative activation route. It is highly expressed by Th2 type cytokines (such as IL-4, IL-10 and IL-13), scavenger receptor (scavenger receptor), mannose receptor (mannose receptor CD206) and galactose receptor (half Lactose receptor), and synthesize Arginase1 (Arg1). M2 mainly secretes IL-10 and TGF-β1, etc., and has weak antigen presentation ability and tumor killing ability, and participates in negative immunoregulation. Studies have clearly shown that biological concomitant infections can inhibit tumor growth by regulating the host immune system[6]. Among them, Toxoplasma gondii infection can inhibit the proliferation of LLC by inducing Th1 immune response. After inoculation of tumor cells in mice infected with Toxoplasma gondii type II (ME49), the survival rate was significantly higher than that of uninfected Toxoplasma gondii, and CD8 + T cells, IFN-γ, serum IgG2a titers increased significantly, tumors Angiogenesis is inhibited[7].

Toxoplasma gondii is an opportunistic pathogenic obligate intracellular parasitic protozoan[8], including type worm strains (RH strain, GT1 strains, etc.), type worm strains (such as PRU strains, ME49 strain, etc.), and type worm strains (CTG strains, etc). Fifty-one strains of Toxoplasma gondii were isolated from cats and humans. Genotyping results showed that 80% of isolates were genotype Chinese1 (ToxoDB#9). Combining with the genotyping results of other domestic scholars on the animal source strains, Chinese1 was identified as the dominant genotype of Toxoplasma gondii unique to China[9–10]. The tips of Toxoplasma tachyzoites have complex cellular structures and secretory organelles[11]. There are three main types of Toxoplasma tachyzoites, including the microneme, rhoptry (ROP) and dense-granule, which secrete different parasite proteins[12–13]. Genotyping of Toxoplasma gondii and immune regulation of polymorphic effector molecules are important advances in Toxoplasma gondii research in recent years. In the process of anti-toxoplasma infection, cell immunity plays a leading role, and Mφ is one of the main effector cells. The insect strains with different genotypes have induced the body to have different trends of bias and even polarization, with two polymorphism effector molecules playing an important role: ROP16, a member of ROPs family protein, and GRA15, a member of GRAs family [12]. There are not kinase activity with GRA15 and the GRA15 can directly activate the host cell NF-κB, drive Mφ to M1 polarization, induction of IL − 12 high expression of NK cells and T cells secrete IFN-γ, free play

Th1 type Immune response. If the polarization direction of TAMs is regulated to reverse its M2-type into M1-type with antigen-presenting ability, it is of great significance to inhibit tumor invasion and metastasis and improve the prognosis of tumor patients, and this method has been proved to be effective in treating tumors[14]. So we want to take advantage of toxoplasma explore GRA15$_{\square}$ can reverse polarization, the M2 can be reeducated M1 polarization trend, regulate the body's immune response, inhibit tumor proliferation. The preliminary study of our research group found that after injection of Mφ which was polarized by GRA15$_{\square}$ into tumor-bearing mice, tumor growth was significantly inhibited[15].Our aim was to further verify the mechanism of GRA15$_{\square}$ to reeducated M2 to M1 and inhibition of tumor growth in vivo and in vitro.

Methods

Reagents

In this study we use many reagents including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), which bought from Wisent (Montreal, QC, Canada). Puromycin (PM), penicillin and streptomycin were purchased from Sigma (St. Louis, Missouri, USA).

Parasites, Plasmid construction, and lentvirus infection

The acquisition of *Toxoplasma gondii* tachyzoites (type II) from the PRU strain was initially passaged through homogenate of the brain containing the encapsulated mouse. The open reading frame encoding TgGRA15$_{II}$ (missing signal peptide 15bp, http://ToxoDB.org) was amplified by real-time (RT)-PCR in the total RNA of the fast-growing spores, and the recombinant plasmid pEGFP-gra15$_{II}$ was obtained to obtain a recombinant lentivirus (LV) vector (LV-pEGFP-gra15$_{II}$)[16].

Cell culture

The murine HCC cells, Hepa1-6, were purchased from the Chinese Academy of Sciences Cell Bank in Shanghai. Mouse cell line RAW264.7 is kept by laboratory; Mouse peritoneal macrophage was injected into the mouse peritoneal cavity with 200 PRU tachyzoites, and the mice were anesthetically killed at 48, 72, 96 and 120h respectively. Peritoneal lavage solution (PEC) was collected under aseptic conditions, and then cultured in a 9cm petri dish with complete medium RPMI1640. After 3h, the non-adherent cells were washed away, and the adherent cells were counted as peritoneal macrophages, and the complete medium RPMI1640 was added. The medium used for all cells was 10%-15% serum and penicillin-streptomycin mixture in DMEM, and the culture conditions were 37 ° C and 5% CO_2.

Flow cytometry assay

Flow cytometry was used to detect GRA15$_{II}$-transfected macrophages and M1 and surface markers. Specifically, four groups of cells (M1 LV-vector, M1 LV-gra15, M2 LV-vector, M2 LV-gra15) were separately prepared as single cell suspensions washed with PBS containing 1% fetal bovine serum and adjusted in

concentration for to 1 × 10^6 cells per 100 μl PBS with 1% FBS, respectively. All cells were incubated at 4 ° C for 20 min in the dark, washed twice with PBS, and detected by flow cytometry, then use Flow Jo to analysis results.

Tumor Models

As for the subcutaneous tumor model, saline containing 3×10^6 hepa1-6 cells were injected subcutaneously into the right lower groin of mice. After three days, a lump was palpable and the tumor volume reached 50mm^3. Tumors were observed in all infected mice. Thirty mice were randomly divided into 10 mice per group, namely, LV-gra15- Mφ, LV - Mφ and saline (NS) control. On the third and fifth days after inoculation, mice in the first and second groups were injected with saline containing 2.5 × 10^6 corresponding macrophages per 100ul through the tail vein, while mice in the control group were injected with saline of equal volume. On days 3, 5, 7, and 10 of inoculation, tumor size was measured using vernier calipers and 0.52* long tumor diameter * short tumor diameter. The animals were euthanized 10 days later before the results were analyzed.

RNA Extraction and qRT-PCR

Total RNA of the LV-vector and LV-gra15 groups was extracted with Trizol reagent, and RNA was reverse-transcripted into cDNA according to the manual using the Prime Script first Stand cDNA Synthesis Kit. Relative expressions of TNF-α, IL-12, IL-10 and Arg1 were detected by qRT-PCR using SYBR Premix Ex Taq Kit. The instrument used in the experiment was ABI Prism 7500 sequence detection system. The internal reference gene was GAPDH, and each sample was repeated three times to increase the accuracy of the data. Finally, the relative expression quantity is calculated by $2^{-\Delta\Delta Ct}$ to calculate.

Western Blotting Analyse

Total protein was extracted from the five groups of LV-gra15_{II}, TNF-α positive, TNF-α negative, LV-vector, and TRAF6-KO co-cultured with Mφ, respectively. Separated with 12% SDS-polyacrylamide gel. Then, the membrane was transferred, 5% skim milk was blocked, the primary antibody was incubated overnight at 4 ° C, the secondary antibody was incubated for two hours, developed, and the band intensity was observed.

RNA Chip

Mouse ascites were taken and washed with PBS two to three times to collect toxoplasma tachyzoites. RNA was extracted with Trizol and sent to the company for related experiments.

ELISA

LV-gra15_{II}-M1, LV-vector-M1, LV-gra15_{II}-M2 and LV-vector-M2 were separately seeded in 6-well plates (2 × 10^6 cells per well), suspended in 2 ml common culture medium, and cultured at 37°C with 5% CO2 for

48 h. Afterwards, we collected four cell supernatants, LV-gra15II-M1, LV-vector-M1, LV-gra15$_{II}$-M2 and LV-vector-M2, and determined IL-10, IL-12, respectively, using an ELISA kit. The concentration of these three cytokines was statistically different.

Immunoprecipitation

We collected LV-gra15$_{II}$- Mφ and cell IP lysis buffer (containing protease inhibitor) added in, then lysed at 4 ° C for 30 minutes, and centrifuged at 12000 rpm for 30 minutes, last took the supernatant. A small amount of lysate was taken for WB analysis. For the remaining lysate, we added the corresponding 1 ul antibody and 10-50 ul protein A/G-beats to the corresponding lysate and incubate overnight at 4 °C. After co-immunoprecipitation, centrifuge at 3000r for 5 min at 4 °C, centrifuge protein A/G-beats to the bottom of the tube, discard the supernatant, rinse 3 times with 1 ml of lysis buffer, and finally add 15 ul of 2×SDS. Add sample buffer and boil for 10 minutes. Ultimately, the SDS-PACE, Western Blotting was to understand comprehend if GRA15 is connected with TRAF6, MyD88, RIP1I, and RAK1.

Animal care and ethical statements

C57BL/6 female mice (6 weeks old) were purchased from changzhou gavens laboratory animal company, China (production license no. Scxk2013-003).The relevant animal care and experimental programs are implemented strictly in accordance with the guidelines for the care and use of experimental animals of the national institutes of health of China (1998) and approved by the institutional review committee of the institute of biomedical sciences of anhui medical university (license no. Amu26-080610).Try to minimize animal suffering during the study.

Immunofluorescence technique

The five groups of cells (LV-gra15- Mφ, LV- gra15$_{II}$ -M1, LV-vector-M1, LV- gra15$_{II}$ -M2 and lv-vector-M2) were counted respectively. After culture for 24h, the culture solution was discarded and washed with PBS twice. Formaldehyde fixation, membrane penetration, sealing, overnight incubation with primary antibody, incubation with secondary antibody for one hour, and finally sealing tablet observation

Results

ToxoGRA15$_{II}$inhibits HCC growth is determined by skewing TAMs from M2-like, towards M1-like phenotype.

On the basis of the pronounced capacity of ToxoGRA15$_{II}$ to reprograme macrophages phenotype, we next tested the efficacy of ToxoGRA15$_{II}$ expression in mice with HCC. The experimental strategy is shown in Figure 1A. Consistent with the observations in the size of tumor in mouse, ToxoGRA15$_{II}$ was specifically expressed in the TAMs using LV- *gra15$_{II}$* (Figure 1B). We observed that 30 days after injection of the control or *gra15$_{II}$*–encoding virus, there was substantially inhibit solid tumor growth. The tumor size in macrophages-depleted mice was not as large as the in macrophages-presenting mice with LV- *gra15$_{II}$*

treatment revealed that macrophage is involved in mediating the anti-tumor effect of ToxoGRA15$_{Δ}$(Figure 1C).Immunohistochemistry staining with F4/80 showed successful removal of macrophages by liposomal clodronate treatment, and LV- *gra15$_{Δ}$* did not significantly increase the residence of macrophage in tumor(Figure 1D).All these indicated that the role of ToxoGRA15$_{Δ}$ in mediating anti-tumor effect is dependent to the macrophages. This was further evidenced by the observation there was no significant decrease in tumor cells after LV- *gra15$_{Δ}$* infection *in vitro* (data not shown).

To examine if ToxoGRA15$_{Δ}$ could re-programme macrophages in tumor towards M1-like phenotype, we measured the presence of M1/M2 macrophages in the tumor with LV- *gra15$_{Δ}$* treatment. LV- *gra15$_{Δ}$* injection increase the CD86+ M1-like macrophages population while significant reduction of CD206+ M2-like population in tumor of mice (Figure 2A).Consistent with the phenotypic analysis, we observed upregulation of the pro-inflammatroy genes, TNF-α and IL12, while down regulation of IL10 and Arg1, the anti-inflammatory genes in LV- *gra15$_{Δ}$*-treated F4/80+ TAMs (Figure 2B). TAMs secreted higher level of pro- inflammatory cytokines (IL6 and TNF-α) while lower level of IL10 in LV- *gra15$_{Δ}$* injection mice(Figure 2C).To further classify the inflammatory expression in tumor, we observation the IFN-γ,IL10,TNF-α,and VEGF by immuohistochemistry staining(Figure 2D).Overall, TAMs reprogramming by ToxoGRA15$_{Δ}$ is functional in regulating cytokine production. ToxoGRA15$_{Δ}$ could promote the TAM repolarisation towards M1-like phenotype.\

Exploring the anti-cancer mechanism of LV-*gra15$_{Δ}$-macrophages* in nude mouse

In order to investigate the rules of ToxoGRA15$_{Δ}$ in tumor, we explored the molecular mechanisms underlying the injection of LV- *gra15$_{Δ}$* using microarray assays and multivariate analyses. The microarray results showed that inflammatory factor expression was significantly up-regulated and was associated with the NF-κB pathway. This phenomenon was also found in confocal microscopy (Fig 3).

ToxoGRA15$_{Δ}$reprograme M2-like Mφ to M1-like phenotype *in vivo*.

According to the preview study, we reported that ToxoGRA15$_{Δ}$ could drive Mφ from M0 to M1 polarization. To further examine the action of ToxoGRA15$_{Δ}$ on reducation of differentiated Mφ, we polarized M0 to either M1-like or M2-like phenotype. Then the polarized Mφ were infected with LV- *gra15$_{Δ}$*. Interestingly, ToxoGRA15$_{Δ}$ particularly skewed M2-like macrophage towards M1-like Mφ without causing any significant phenotypic changes in M1-like Mφ(Figure 4A). This was further evidenced by the fact that expression of pro-inflammatory factors in LV- *gra15$_{Δ}$*-M2-like Mφ was increased while anti-inflammatory factors were reduced (Figure 4B), which was consist with expression of pro- and anti-inflammatory genes (Figure 4C)

ToxoGRA15$_{Δ}$binding of TRAF6 to activate NF-κB pathway and mediates repolarisation of Mφ.

ToxoGRA15□ could modulate NF-κB in MEFs, providing no clues to which components of NF-κB signaling pathway were necessary for ToxoGRA15□ activity[17]. We next sought to determine the precise mechanism by which ToxoGRA15□ regulates the activation of NF-κB. ToxoGRA15□ protein detected as a control for successful infection. Uninfected cells, either nonstimulated or stimulated with TNF-α, served as controls for NF-κB activation. We observed that the levels of phosphorylated IKKβ were higher in infected cells. The analysis of IκBα and p65 phosphorylation also revealed higher levels of phosphorylation in control compared to LV-*gra15□* compared to LV-vector or control Mφ. Additionally, ToxoGRA15□ could not induce their phosphorylation in TRAF6$^{-/-}$ Mφ (Figure 5A). The increased levels of IKKβ and IκBα phosphorylation in presence of ToxoGRA15□ suggested that the translocation of p65 into the nucleus might also be diminished by LV-*gra15□* infected cells. This assumption was investigated by immunofluorescence analysis. We found that p65 could localized in the nuclei of Mφ with LV-*gra15□* (Figure 5B).

NF-κB transcription factors play a crucial role in the activation of the innate immune system. They are activated by the binding of cytokines like interleukin-1β (IL-1R), TNF-α to tumor necrosis factor receptor 1 (TNF-R1) or Toll-like recpetors 4 (TLR4). To verify the interaction of ToxoGRA15□ with these receptor complexes, we investigate the protein in NF-κB signaling pathway. In MyD88$^{-/-}$, IRAK1$^{-/-}$ and RIP1$^{-/-}$ macrophages, ToxoGRA15□ induced a 3.2-flod, 6.4-flod and 6.2-flod increase in p65 phosphorylation compared with uninfected cells(data now shown). However, ToxoGRA15□ could not induce p65 phosphorylation in TRAF6$^{-/-}$ macrophages (Figure 5A). To further confirm ToxoGRA15□ acts in concert with TRAF6, coimmunoprecipitation assays were performed. Endogenous expression of MyD88, IRAK1, TRAF6, and RIP1 was similar in the cell lysates of all samples, independent of ToxoGRA15□ expression. Most importantly, MyD88, IRAK1, and RIP1 did not coimmunoprecipitate with the ToxoGRA15□ protein, while TRAF6 did (Figure 5C). These findings support the assumption that the ToxoGRA15□ activate NF-κB signaling pathway by directed interaction with the TRAF6.

Discussion

T.gondii as intracellular parasite could infect all of sort of nucleated cells, including macrophages and related mononuclear phagocytes. The ability of various infections to inhibit tumor growth has been well reported[18]. Generally, T.gondii increased IL-12 and IFN-γ, stimulating natural killer (NK) cells and macropahges(Mφ) that cause Th1 immune response[19]. One of important recently reports that T.gondii-derived genotype-associated effectors of ToxoGRA15□ polarized macrophage to M1-like phenotype[12].The tumor microenvironment is potently immunosuppressive which is enrolling abundant tumor-associated macrophages (TAMs) present as M2-like phenotype. Our previous study found that transfusion of M1-like macrophages which were induced by ToxoGRA15□, leading to inhibit HCC growth and migration[15].Here we identified ToxoGRA15□ could anti-tumor in mice is effected by macrophages' phenotype activated with NF-κB after transfer LV-gra15□ directly. M2-like macrophages were reprogrammed as M1-like phenotype, consistently presenting NO, TNF-α,iNOS and IL-12p40, subjected

with LV-gra15, as well as in nude mice. Interestingly, despite injection with LV-gra15, there was no significant reduction in tumor volume in the macrophages removal murine model.M1 phenotype successfully activated may play a significant role in restriction of tumor progression in tumor microenvironment[20].

In the present investigation, we explored the alternation in tumor environment and found the LV-gra15 remarkably effected on the pathways in cancer. Our microarray data defines the placement of ToxoGRA15 in the NF-κB singnaling pathway in tumor bearing mice, such as p65,IκBα,TRAF6, etc. Additionally, the number of macrophages labeled by F4/80 in the group treated with LV-gra15 was no significantly alternated. However, M1 phenotype was evoked by LV-gra15 was injected through veins. We also observed remarkably increased of IFN-γ and TNF-α production and decreased tumor growth-associated cytokines such as IL-10, VEGF in tumor bearing mice. However, it is not clear that how to provoke the surveillance mechanisms via effects on systemic immunity. Further approaches are needed to sort T cells, DCs cells and NK cells in tumor tissues and their ability to reduced tumor size.

On the basis of our findings, we propose that the targeting of TRAF6 for macrophage polarization is the primary cellular mechanism underlying the physiological function of ToxoGRA15 in mice transplanted with Hepa1-6. In the previous reports, ToxoGRA15 activated the p65 location is not dependent on either MyD88 or TRIF, which are essential for TLR signialing[17]. ToxoGRA15 induced p65 phosphorylation in MyD88-/-,IRAK-/-,RIP1-/- more than TRAF6-/- macrophages. Furthermore, we confirmed that ToxoGRA15 acted in concert with TRAF6, are not MyD88, RIP1 and IRAK by coimmunoprecipitation. The targeting of TRAF6-ToxoGRA15 represents distinct molecular mechanisms in macrophages' reprogramment and thus would produce different thearapeutic approaches and drug candidates.

This funding encourages future screening studies for apicomplexan parasite, such as T.gondii, as potential immunotherapy for cancer.According to our investigation, ToxoGRA15 as effective molecular derived form T.gondii, could reeducate M2-like to M1-like macrophages, but not effect original M1-like phenotype, may play a potential role in activating innate and/or adaptive immunity to antitumor by it's ability binding TRAF6 to induce NF-κB singnalling pathway. It is currently unclear what the other effecting of ToxoGRA15 in tumor microenvironment.ToxoGRA15 could alternate expression of any chemokines and adhesion moleculars(our microarray data) is associated with cancer pathway. Further studies are needed to address these issues.

Conclusions

ToxoGRA15 as polypeptide effector molecule of T.gondii could bind TRAF6 to up-regulated NF-κB pathway in macrophages. Furthmore, M1 –like is polarized into M2-like macrophage in vivo and in vitro, inhibiting the proliferation and migration of tumor cells.

Declarations

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Ethics declarations

Ethics approval and consent to participate

The study has been submitted to the local ethics committee (Anhui Medical University,LLSC20150030).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All datasets used and/or analyzed during the current study are included in this published article and its additional files.

Authors' contributions

YHC and JLS designed the work; YHC , YYX, YLW, and YZ collected the data; YHC , YYX, YLW, and YZ analyzed the data; YHC and YYX drafted the article; YHC , LY and JLS did critical revision of the article. All authors read and approved the final manuscript.

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Figures

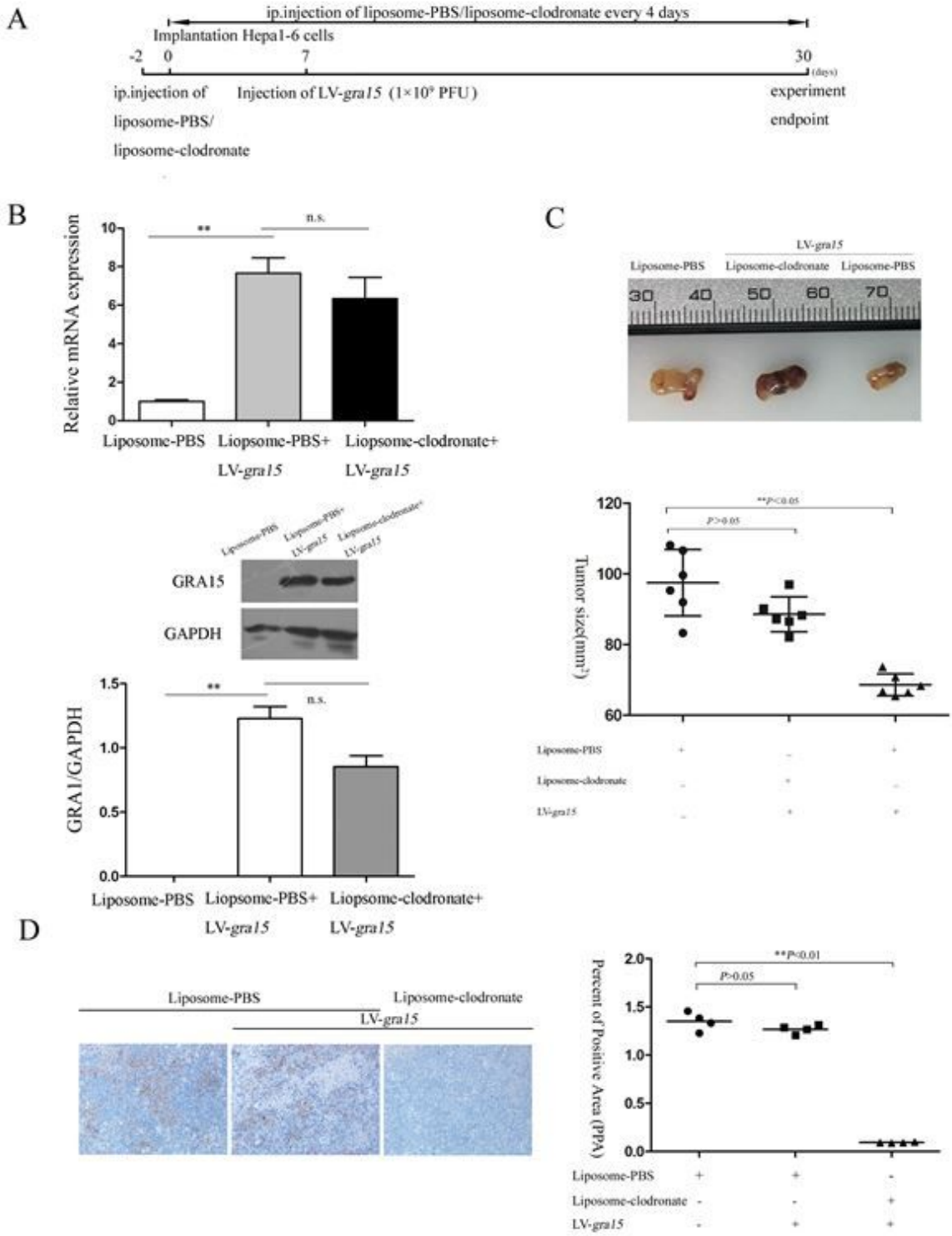

Figure 1

ToxoGRA15 inhibited HCC growth requires presence of macrophages in mice. (a) Schematic representation of the timing strategy used to evaluate the role of ToxoGRA15 in HCC in mice. (b) gra15 mRNA and GRA15 protein expression levels in tumor(n=6 mice per group for qPCR analysis; n=6 western blots in total).GAPDH was used as a loading control. (c) Removal of macrophage from mice completely blocked the anti-tumor effect of ToxoGRA15. The tumor growth in mice (n=6)with LV- gra15 injection

was not obvious, whereas removal of macrophage by intraperitoneally injecting clodronate liposome resulted in relapse of tumor. (d) Presence of clodronate liposome remarkably reduced hepatic macrophage in mice by Immunohistochemistry staining with F4/80. For all statistical results, the data are shown as the mean ± SD.*P□0.05, **P□0.01;n.s., not significant; by one-way ANOVA or two-tailed Student's t-test.

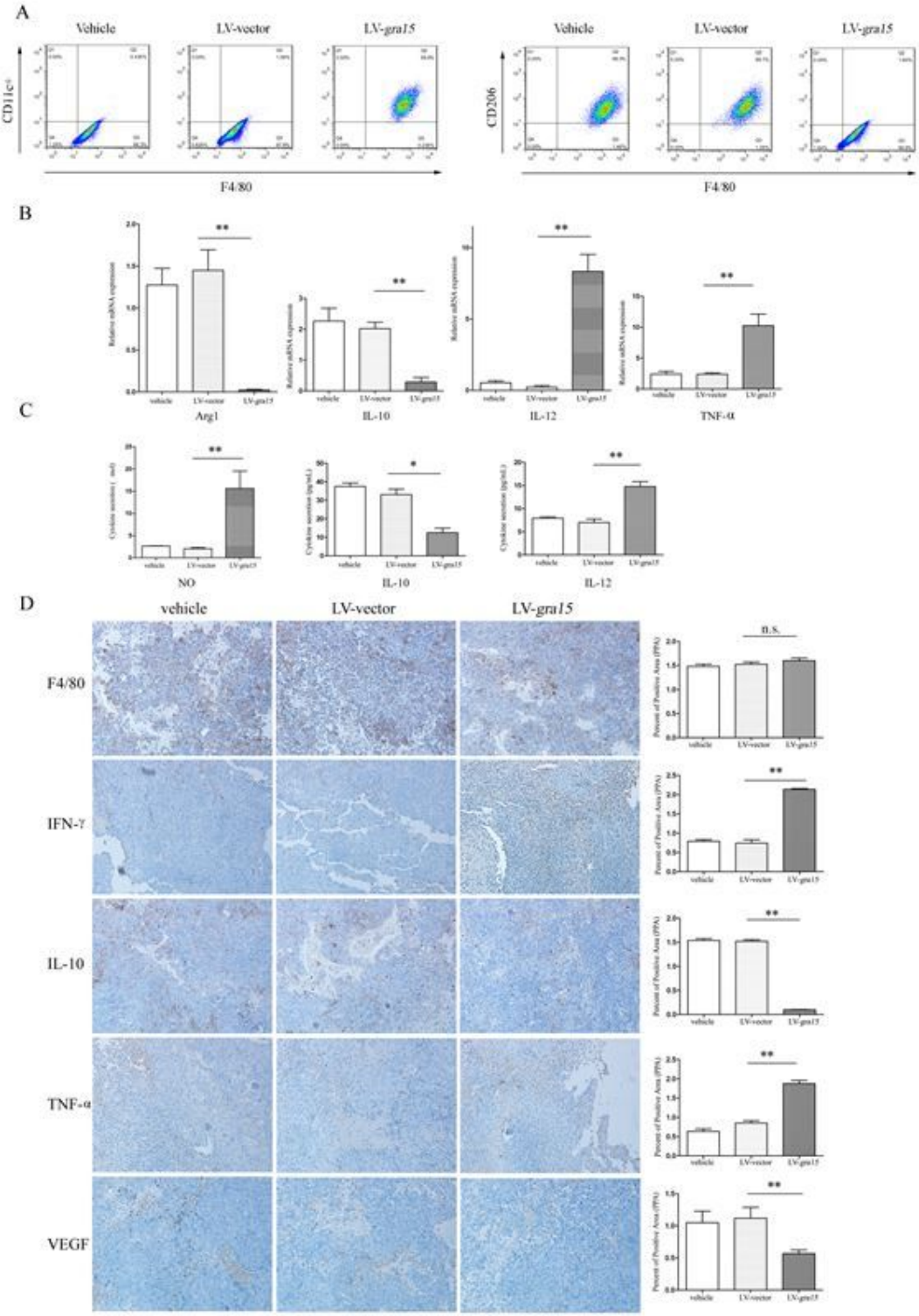

Figure 2

Treatment of ToxoGRA15 results in reprgramming of M2-like TAMs to M1-like phenotype. (a) LV- gra15 injection increase of hepatic M1-like TAMs with reduced M2-like populations. Antibodies against CD86 and CD206 were used to stain cell surface marker of TAMs. F4/80 was co-stained to identify differentiated macrophage population. M1-like macrophage had higher expression of CD86 with lower expression of CD206, while CD206 was induced and CD86 was relatively low in M2-like macrophage. (b) Cytokine profile in ToxoGRA15 TAMs favouring M1-like phenotype. RNA from tumor was collected and expression of TNF-α, IL12(M1-like macrophage markers) and IL10,Arg1(M2-like macrophage markers) was determined by qPCR In crease of M1 markers but decrease of M2 markers was observed in tumor with LV- gra15 injection. (c) LV- gra15 injection induced elevated secretion of pro-inflammatory cytokine. Cytokine concentration in tumor was determined with ELISA kit. NO, IL-12(pro-inflammatroy cytokines) levels were increased, while IL-10 (anti-inflammatroy cytokines) levels were decreased. For all statistical results, the data are shown as the mean ± SD.*P0.05, **P0.01;n.s., not significant; by one-way ANOVA or two-tailed Student's t-test. (d) Immuohistochemistry staining of tumor tissues after treatment with LV- gra15 injection. LV- gra15 injection did not increase the number of macrophages with F4/80 staining. While the expression of IL10, TNF-… was increased in tumor. For all statistical results, the data are shown as the mean ± SD.*P0.05, **P0.01;n.s., not significant; by one-way ANOVA or two-tailed Student's t-test.

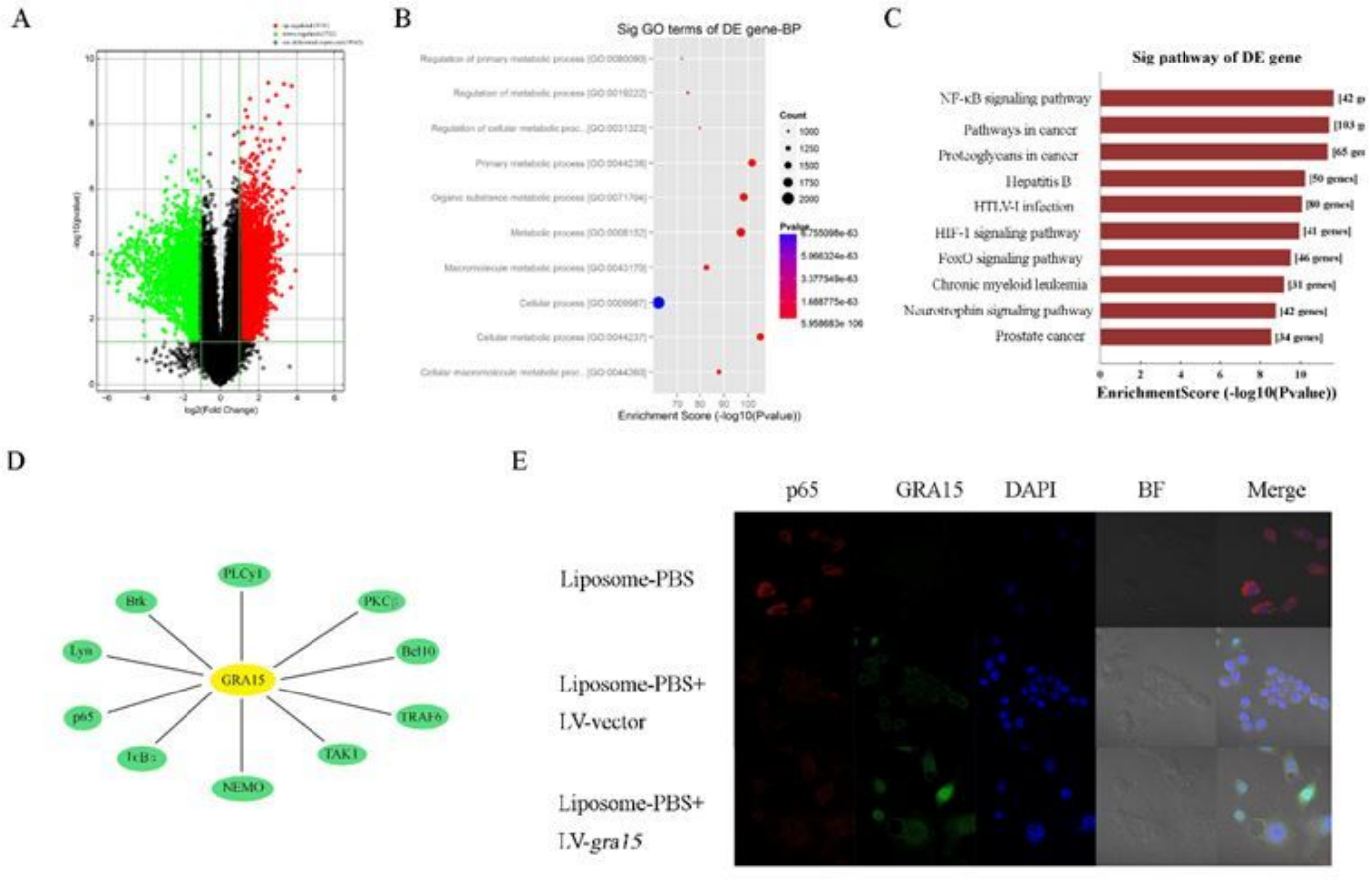

Figure 3

NF-κB signaling is required for ToxoGRA15 function. (a) volcano plot indicating the genes that were differentially expressed (red, upregulated genes; green, downregulated genes in ToxoGRA15 injection group relative to vector group) in a microarray analysis of tumor samples from ToxoGRA15 group and vector group mice. (b) Gene Ontology (GO) pathway enrichment analysis of the identified differentially expressed genes (DEGs). (c) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis data showing the potential upstream regulators of the DEGs (d) Ingenuity pathway analysis (IPA) data showing the potential upstream regulators of the DEGs that are involved in NF-kB signaling. (e) Representative confocal microscopy images showing the distribution of p65 translocation in nuclear of TAMs that have expressed ToxoGRA15 protein in ToxoGRA15 injection.

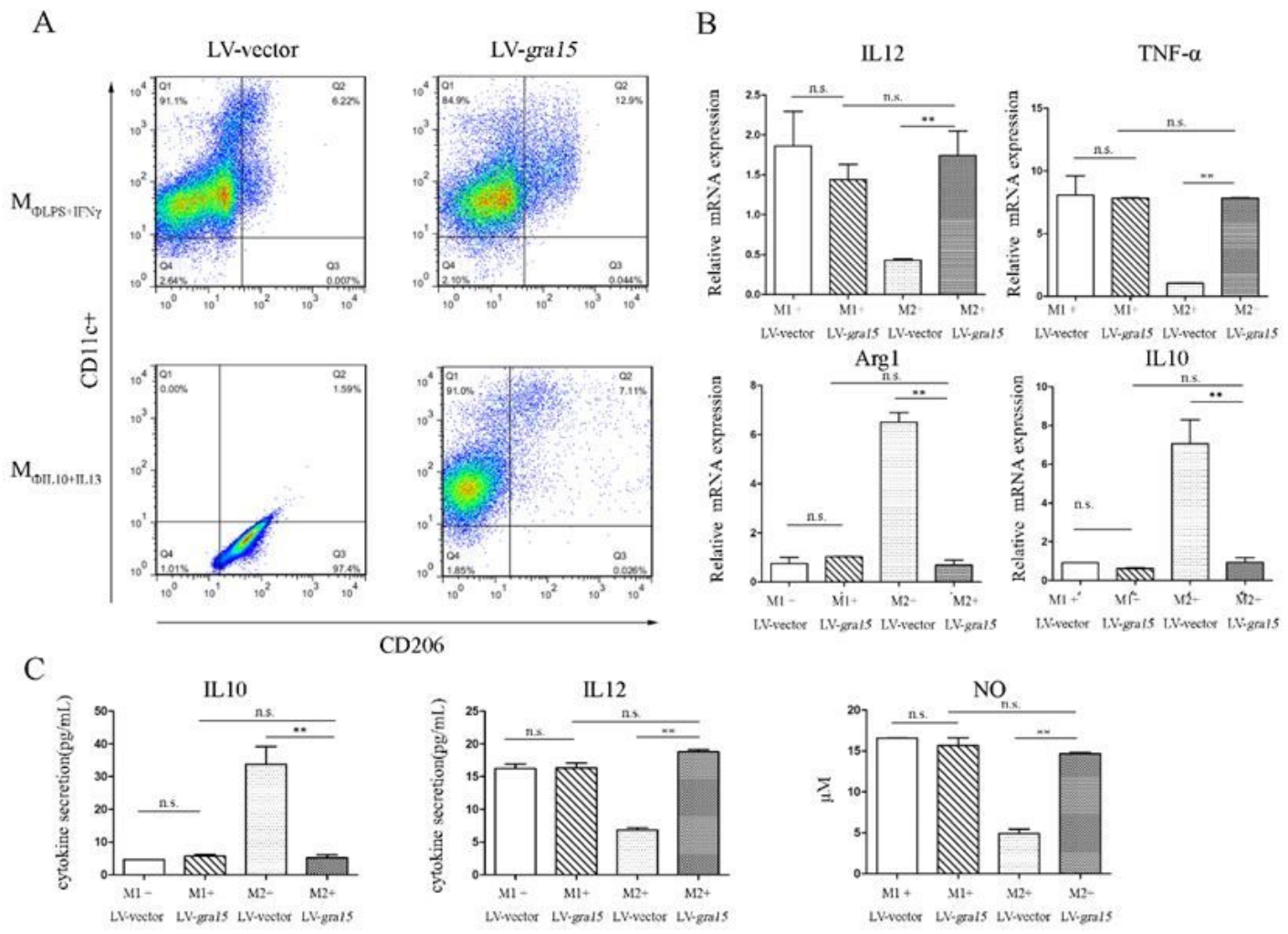

Figure 4

ToxoGRA15 induces repolarization of M2-like Mφ without affecting M1 phenotype. (a) LV- gra15 infection resulted in skewing of M2-like Mφ to M1-like phenotype without affecting M1 phenotype. ARAW264.7 was induced to M1-like and M2-like phenotype as described in Materials and Methods. (b) Cytokine profile in LV- gra15-M2-like Mφ favoring M1-like phenotype. RNA from cells was collected and expression of TNF-α, IL-12(M1-like Mφ markers) and IL-10, Arginase 1(Arg1) (M2-like Mφ markers) was

determined by quantitative real-time PCR. Increase of M1 markers but decrease of M2 markers was observed in LV- gra15-M2-like Mφ. (c) LV- gra15-M2-like Mφ secreted pro-inflammatory cytokine. Cytokine concentration in cultured cells supernatant was determined with ELISA kit. NO, IL-12(pro-inflammatroy cytokines) levels were increased while IL-10 (anti-inflammatroy cytokines) levels were decreased. For all statistical results, the data are shown as the mean ± SD.*P0.05, **P0.01; n.s., not significant; by one-way ANOVA or two-tailed Student's t-test.

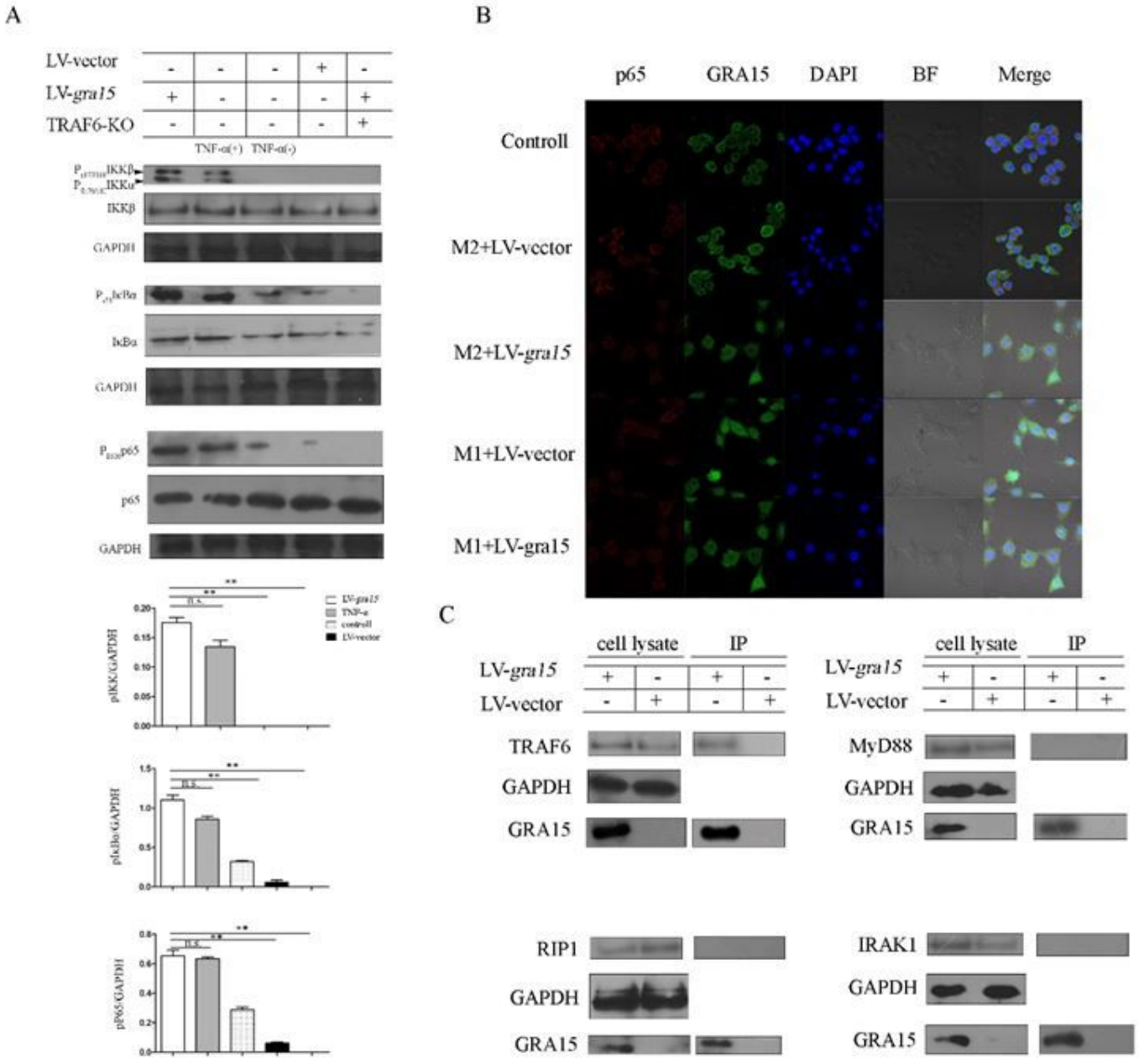

Figure 5

(a) ToxoGRA15 could not induce p65 phosphorylation in TRAF6-/- macrophages .(b) p65 translocation in ToxoGRA15 Mφ. (c) Co-immunoprecipitation of LV- gra15- Mφ with proteins of the TNFR1, IL-1R, and TLR4 receptor complexes. Bound proteins were eluted and separated by SDS-PAGE and analyzed via

Western blotting using an anti-FLAG antibody and specific monoclonal antibodies against MyD88, IRAK1, TRAF6, and RIP1. GAPDH served as a control. For all statistical results, the data are shown as the mean ± SD.*P□0.05, **P□0.01;n.s., not significant; by one-way ANOVA or two-tailed Student’s t-test.