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Toll-like receptor-induced inflammatory cytokines are suppressed by Gain of function or overexpression of $G\alpha_{i2}$ protein

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Abstract

Previous studies have implicated a role of $G\alpha_i$ proteins as co-regulators of Toll –like receptor (TLR) activation. These studies largely derived from examining the effect of $G\alpha_i$ protein inhibitors or genetic deletion of $G\alpha_i$ proteins. However the effect of increased $G\alpha_i$ protein function or $G\alpha_i$ protein expression on TLR activation has not been investigated. We hypothesized that gain of function or increased expression of $G\alpha_i$ proteins suppresses TLR2 and TLR4 -induced inflammatory cytokines. Novel transgenic mice with genomic “knock-in” of a Regulator of G-protein Signaling (RGS)-insensitive Gnai2 allele ($G\alpha_{i2}{}^{G184S/G184S}$; GS/GS) were employed. These mice express essentially normal levels of $G\alpha_{i2}$ protein, however the $G\alpha_{i2}$ is insensitive to its negative regulator RGS thus rendering more sustained $G\alpha_{i2}$ protein activation following ligand/receptor binding. In subsequent studies, we generated Raw 264.7 cells that stably overexpress $G\alpha_{i2}$ protein (Raw $G\alpha_{i2}$). Peritoneal macrophages, splenocytes and mouse embryonic fibroblasts (MEF) were isolated from WT and GS/GS mice and were stimulated with LPS, Pam3CSK4 or Poly (I:C). We also subjected WT and GS/GS mice to endotoxic shock (LPS 25mg/kg i.p.) and plasma TNFα and IL-6 production were determined. We found that *in vitro* LPS and Pam3CSK4 induced TNFα and IL-6 production are decreased in macrophages from GS/GS mice compared with WT mice ($p<0.05$). *In vitro* LPS and Pam3CSK4 induced IL-6 production in splenocytes and *in vivo* LPS induced IL-6 were suppressed in GS/GS mice. Poly (I:C) induced TNFα and IL-6 *in vitro* demonstrated no difference between GS/GS mice and WT mice. LPS induced IL-6 production was inhibited in MEFs from GS/GS mice similarly to macrophage and

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splenocytes. In parallel studies, Raw $G\alpha_{i2}$ cells also exhibit decreased TNFα and IL-6 production in response to LPS and Pam3CSK4. These studies support our hypothesis that $G\alpha_{i2}$ proteins are novel negative regulators of TLR activation.

Keywords

$G\alpha_i$ protein; TLR signaling; LPS; endotoxemia; inflammatory cytokines

INTRODUCTION

Sepsis is the clinical manifestation of a systemic maladaptive host response to invasive infection. Sepsis leading to septic shock and multiple organ failure is the leading cause of death in intensive care units and remains a major health problem in the United States and worldwide [1,2]. The interaction of bacterial components such as LPS, peptidoglycan, lipoteichoic acid, and exotoxins with macrophages, monocytes, endothelial cells or other host cells induces the release of inflammatory mediators that play a major role in the pathophysiology of septic shock [3,4].

The Toll-like receptor (TLR) family plays a critical role in mediating the innate and adaptive immune response [5]. The Gram-negative bacteria cell wall component LPS-induced signaling is mediated by TLR4 coupled with CD14 and MD-2 [6]. Lipoprotein from the Gram-positive bacteria -induced signaling pathways is mediated, in part, through TLR2 and other receptors [7]. Pam3CSK4 is a synthetic triacylated lipoprotein signaling through TLR1/2 [8]. TLR3 recognizes double-stranded RNA along with its synthetic analog, polyinosinedeoxycytidylic acid (Poly I:C) [5]. Stimulation of TLR2, TLR3 or TLR4 signaling pathways results in activation of a series of signaling proteins leading to expression of pro-inflammatory cytokine and chemokine genes [9]. These cytokines and chemokines contribute to the complex inflammatory milieu of sepsis.

Heterotrimeric guanine nucleotide binding regulatory (G) proteins of the G inhibitory class (G_i) are involved in signaling to microbial stimuli. Our recent studies demonstrated that Gαi proteins were directly activated by LPS [10]. Inhibition of $G\alpha_i$ protein with pertussis toxin (PTx) augment LPS induced inflammation *in vitro* and *in vivo* [11–14]. In addition to pharmacologic inhibition of $G\alpha_i$ protein, mice with genetic deletion of $G\alpha_{i2}$ protein exhibit an enhanced inflammatory response and reduced survival in response to cecal ligation and puncture-induced sepsis [10]. Collectively, these findings suggest an anti-inflammatory role of $G\alpha_i$ proteins in inflammatory response.

Since inhibition of $G\alpha_i$ protein augments the inflammatory response, we hypothesized that increased $G\alpha_i$ protein function or overexpression of $G\alpha_i$ protein will suppress TLR induced inflammation. WT and novel transgenic mice with genomic knock-in of a RGS-insensitive $G\alpha_{i2}$ (GS/GS) were employed [15]. The TLR4 ligand LPS, TLR2 ligand Pam3CSK4 and TLR3 ligand Poly (I:C) induced TNFα and IL-6 production in splenocytes, macrophage and MEFs were determined. LPS shock induced plasma TNFα and IL-6 production were assessed in GS/GS vs WT mice. In subsequent studies, Raw 264.7 cells stably expressing control vector and $G\alpha_{i2}$ proteins were generated. LPS and Pam3CSK4 induced TNFα and IL-6 production were determined. Collectively these demonstrate an anti-inflammatory function of $G\alpha_{i2}$ proteins in regulating TLR induced inflammatory response.

MATERIALS AND METHODS

Mice

$G\alpha_{i2}^{G184S/G184S}$ (GS/GS) mice and littermate WT mice with C57BL/6 background were generated by breeding heterozygous mice as described previously [15]. Studies employed 7 to 9 week old GS/GS and age matched WT mice. The gain of function $G\alpha_{i2}^{G184S/G184S}$ mice were obtained from Dr. Richard R. Neubig. (University of Michigan) The investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and operated under the approval of the institutional animal care and use committee.

Raw $G\alpha_{i2}$ cell line

WT human $G\alpha_{i2}$ gene was cloned into a ViraPower Lentiviral Expression System using pLenti6/V5 directional TOPO cloning kit. A pLenti6/V5-GW/*lacZ* plasmid was used as a control plasmid. Lentivirus containing $G\alpha_{i2}$ and control vector were generated following manufacture's instructions (Invitrogen). Raw 264.7 cells were transduced with lentivirus and selected by Blasticidin to generate the Raw cells lines stably expressing $G\alpha_{i2}$ and β-galactosidase. Since the pLenti6/V5 vector contains a V5 tag sequence, a V5 antibody was used to detect the overexpression of target protein. A representative Western blot shows the overexpression of $G\alpha_{i2}$ and β-galactosidase in Raw cell lines (Fig 1).

Cell culture and stimulation

RAW 264.7 cells stably expressing $G\alpha_{i2}$ and β-galactosidase were grown in Dulbecco's modified Eagle's medium (Gibco Invitrogen Corporation, Carlsbad, CA) supplemented with heat inactivated 10% fetal bovine serum (Cellgro Mediatech Inc., Herndon, VA), 2% Penicillin/streptomycin (BioWhittaker Inc., Walkersville, MD) and 2ug/ml Blasticidin (Invitrogen) in 150 cm^2 tissue culture flasks and maintained at 37°C in 5% CO_2, 95% air. The confluent cells were detached using 0.05% trypsin-EDTA (Gibco Invitrogen Corporation, Carlsbad, CA) and passaged every 2–3 days. RAW 264.7 cells within 20 passages were used for experiments.

RAW 264.7 cells expressing $G\alpha_{i2}$ and β-galactosidase were stimulated with LPS (10ng/ml, ultra-pure LPS from *Escherichia coli* O111:B4, List Laboratories, Campbell, CA), Pam3CSK4 (1μg/ml, Invivogen) or Poly (I:C) (10μg/ml Invivogen) for 24 hours. LPS-induced TNFα and IL-6 production were analyzed by enzyme-linked immunosorbant assay (ELISA).

Peritoneal macrophages, splenocytes and MEFs were isolated from GS/GS mice and littermate WT mice as previously described [10,16,17] and maintained in RPMI 1640 medium (Cellgro Mediatech Inc., Herndon, VA), supplemented with heat inactivated 1% fetal calf serum (FCS, Sigma, St. Louis, MO), 50U/ml penicillin, 50μg/ml streptomycin (Cellgro Mediatech Inc., VA). The macrophage (1×10^6 cells/well) were incubated at 37°C for 2 hours. Non-adherent cells were washed off. Typically, more than 95% of macrophages were obtained as examined by stain with fluorescein isothiocyanate-conjugated anti-mouse F4/80 antibody (Caltag) and analyzed by flow cytometry. Macrophages were stimulated with 10ng/ml of LPS, 1μg/ml of Pam3CSK4 and 10μg/ml Poly (I:C) for 24 hours. Splenocytes (5×10^6 cells/well) in 24-well plates were stimulated with 10ng/ml of LPS, 1μ/ml of Pam3CSK4 and 10μg/ml Poly (I:C) for 24 hours. MEFs were stimulated with LPS (10ng/ml) for 24 hours. The supernatants were collected for assay of mediator production.

Endotoxemia

Endotoxemia was induced by intraperitoneal injection of LPS (25mg/kg). Six hours later plasma was taken for TNFα and IL-6 measurements.

Western blot

The Raw cells expressing $G\alpha_{i2}$ and β-galactosidase were washed and lysed with ice-cold RIPA lysis buffer (10 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). The supernatant was collected and stored at −20°C until Western blot analysis.

For Western blotting, lysates were added to Laemmli sample buffer and boiled for 4 min. Subsequently, protein from each sample was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membranes were washed with Tris-buffered saline-Tween 20 (TBST; 20 mM Tris, 500 mM NaCl, and 0.15% Tween 20) and blocked with 5% milk in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20) for 1 h. After being washed twice with TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20), membranes were incubated with polyclonal anti-V5 antibody (Invitrogen) overnight at 4°C. The membranes were washed twice with TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20) and incubated with HRP conjugated secondary antibody in blocking buffer for 1 h. After being washed three times with TBST (TBST; 20 mM Tris, 500 mM NaCl, and 0.15% Tween 20), immunereactive bands were visualized by incubation with ECL plus detection reagents (GE Healthcare) for 5 min and exposure to ECL Hyperfilm (GE Healthcare). The densitometry of bands was quantified with NIH image software.

Assay for TNFα and IL-6 production

TNFα and IL-6 production were measured using an ELISA with mouse TNFα or IL-6 ELISA kits (eBioscience, San Diego, CA).

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined by analysis of variance (ANOVA) with Fisher's probable least-squares difference test or students' *t* Test using GraphPad Prism software. A $p<0.05$ value was considered statistically significant.

RESULTS

Gain of function of $G\alpha_{i2}$ suppresses LPS and Pam3CSK4-induced TNFα and IL-6 production in macrophage

Peritoneal macrophages were isolated from WT and GS/GS mice and stimulated with the TLR4 ligand LPS, TLR2 ligand Pam3CSK4 and TLR3 ligand Poly (I:C) for 24 hours. LPS and Pam3CSK4 induced TNFα (Fig. 2A) and IL-6 (Fig. 2B) production were decreased (33±3% and 46±4%; 69±7% and 58±16% respectively, $p<0.05$) in GS/GS mice compared with WT mice. Poly (I:C) induced TNFα and IL-6 production was not different between GS/GS mice with WT mice (Fig. 2A, 2B).

Gain of function of $G\alpha_{i2}$ decreases LPS and Pam3CSK4-induced IL-6 but not TNFα production in splenocytes

Splenocytes were isolated from WT and GS/GS mice and were stimulated with LPS, Pam3CSK4 and TLR3 ligand Poly (I:C) for 24 hours. There was no difference between WT

and GS/GS mice in LPS and Pam3CSK4 induced TNFα production in splenocytes (Fig. 2C). However, LPS and Pam3CSK4 induced IL-6 production was decreased (64±13% and 70±4% respectively, $p<0.05$) in GS/GS mice compared with WT mice (Fig. 2D). Poly (I:C) induced TNFα and IL-6 production was not different between GS/GS mice with WT mice (Fig. 2C, 2D).

Gain of function of $G\alpha_{i2}$ inhibits LPS-induced IL-6 production in MEFs

Mouse embryonic fibroblasts (MEF) were isolated from WT and GS/GS mice and were stimulated with LPS for 24 hours. LPS induced IL-6 production in MEFs was decreased (65±2%, $p<0.05$) in GS/GS mice compared with WT mice (Fig. 3).

Gain of function $G\alpha_{i2}$ mice exhibit decreased plasma IL-6 production compared to wild type mice in response to endotoxemia

WT and GS/GS mice were subjected to endotoxemia. In GS/GS mice, LPS-induced plasma TNFα was not significant different from WT mice (Fig. 4A). However LPS-induced IL-6 production was significantly decreased (60±10%, $p<0.05$, Fig. 4B) in GS/GS mice compared to WT mice.

Overexpression of $G\alpha_{i2}$ protein decreases LPS and Pam3CSK4-induced TNFα and IL-6 production in Raw cell lines

The lentiviral expression system was used to generate Raw 264.7 cells that stably overexpressed $G\alpha_{i2}$ protein (Raw $G\alpha_{i2}$) and β-galactosidase (Raw-control vector). LPS and Pam3CSK4 induced TNFα and IL-6 production were determined by ELISA. The Raw $G\alpha_{i2}$ cells exhibited decreased TNFα (27±5% and 25±1% respectively, $p<0.05$, Fig 5A) and IL-6 (39±0.4% and 52±3% respectively, $p<0.05$, Fig 5B) production in response to LPS and Pam3CSK4.

DISCUSSION

These studies provide the first evidence that gain of function of $G\alpha_{i2}$ protein suppresses LPS and Pam3CSK4 induced inflammatory cytokines. We found that TLR4 ligand LPS and TLR2 ligand Pam3CSK4 induced TNFα and IL-6 production were decreased in peritoneal macrophages and IL-6 in splenocytes from gain of function of $G\alpha_{i2}$ mice. Gain of function of $G\alpha_{i2}$ protein also inhibited LPS-induced IL-6 production in MEFs. LPS-induced plasma IL-6 production was decreased in gain of function of $G\alpha_{i2}$ mice compared to WT mice. The response of Raw 264.7 cell lines overexpressing $G\alpha_{i2}$ protein to TLR2 and TLR4 ligands also support the notion that $G\alpha_{i2}$ protein suppresses TLR2 and TLR4 ligand induced inflammatory cytokines.

Our previous studies and others' demonstrated that mastoparan (MP)-7, which activates $G\alpha_i$ proteins reduce LPS-induced inflammatory responses *in vitro* and *in vivo* [10,12,14]. Lentschat et al. demonstrated that MP-7 differentially regulate TLR2 and TLR4 activation [11]. In contrast to MP-7, pretreatment with PTx, which inhibits $G\alpha_i$ proteins significantly augments LPS induced inflammatory cytokines *in vivo* whereas PTx without LPS also did not induce an inflammatory response [10]. However a limitation of MP-7 or PTx is that it is not $G\alpha_i$ isoform specific and as with any pharmacologic agent may have other effects. Our current studies employing isoform specific targeting suggest that $G\alpha_{i2}$ protein regulates both TLR2 and TLR4 signaling. We also employed TLR3 ligand Poly (I:C) in our studies. Interestingly we found that Poly (I:C) induced only modest TNFα and IL-6 production in macrophage and splenocyte from WT mice. Gain of function of $G\alpha_{i2}$ proteins exhibited no effect on Poly(I:C) induced TNFα and IL-6 production. Similar results were observed when $G\alpha_{i2}$ protein were overexpressed in Raw 264.7 cells and stimulated the cells with TLR

ligands. Since both TLR2 and TLR4 activate MyD88 signaling and TLR3 activates Toll-IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF) signaling [5], these data suggest that $G\alpha_i$ protein may only regulate MyD88 dependent signaling and have no effect on TRIF dependent signaling in macrophage and splenocytes.

The mechanism by which $G\alpha_i$ proteins inhibits TLR-induced inflammation is being intensively investigated. Our previous studies demonstrated that a $G\alpha_{i2}$ minigene plasmid or dominant negative $G\alpha_{i3}$ blocked TLR4 induced ERK1/2 activation in HEK293 cells [13]. In endothelial cells, PTx suppressed ERK 1/2 and AKT induced by TLR activation independent of tumor necrosis factor receptor-associated factor 6 (TRAF6) [14]. Both ERK 1/2 and PI3 kinase induced signaling may have anti-inflammatory effects [18,19].

It has been demonstrated that TLR4 clusters with multiple receptors upon LPS stimulation [20]. For example the G protein coupled receptor (GPCR) CXCR4 which is coupled to Gi protein has been postulated as a co-regulator of TLR4 [20]. Blocking antibodies to CXCR4 increase TLR4 activation whereas a CXCR4 ligand reduces TLR4 activation [21,22]. Also in Raw264.7 cells we have demonstrated that LPS directly activates $G\alpha_i$ protein determined by an increase the active $G\alpha_i$-GTP complex [10]. Thus TLR receptors may directly activate $G\alpha_i$ proteins perhaps through a consensus motif for binding $G\alpha_i$ in the TLR intracellular domain [14,23]. Alternatively but not exclusively ligand binding to TLRs may indirectly activate $G\alpha_i$ proteins through transactivation of GPCRs or through autocrine pathways eg. CXCR4 ligands [14,22]. Thus activation of $G\alpha_i$ signaling may constitute a novel negative feedback to limit TLR induced inflammation. Our findings that gain of function $G\alpha_{i2}$ cells and mice and overexpression of $G\alpha_{i2}$ supress TLR2 and TLR4 activation are consistent with such as hypothesis.

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Figure 1. Raw 264.7 cell lines expressing $G\alpha_{i2}$ and β-galactosidase

Raw 264.7 cell line and Raw cells lines stably expressing $G\alpha_{i2}$ and β-galactosidase were subjected to Western blot analysis. An anti-V5 antibody was used to detect the overexpression of target protein. The band at 121 KDa is vector control (Raw *Laz*) corresponds to β-galactosidase. The 42 band corresponds to overexpressed $G\alpha_{i2}$ in Raw $G\alpha_{i2}$ cells

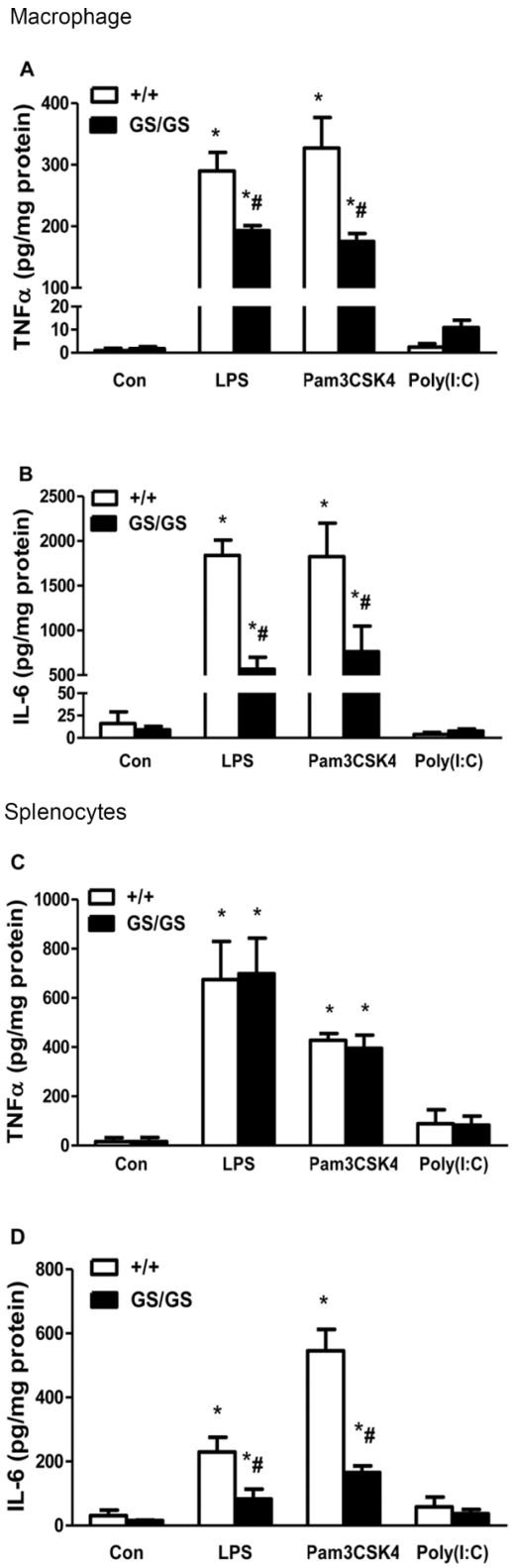

Figure 2. Effect of gain of function of $G\alpha_{i2}$ protein on LPS and Pam3CSK4-induced TNFα and IL-6 production in peritoneal macrophage and splenocytes

Peritoneal macrophage and splenocytes were isolated from WT and GS/GS mice and stimulated with LPS and Pam3CSK4 for 24 hours. LPS and Pam3CSK4 induced TNFα (A) and IL-6 (B) production in peritoneal macrophages and TNFα (C) and IL-6 (D) production in splenocytes were determined. *, $p<0.05$ compared to basal group; #, $p<0.05$ compared to WT group. N=3.

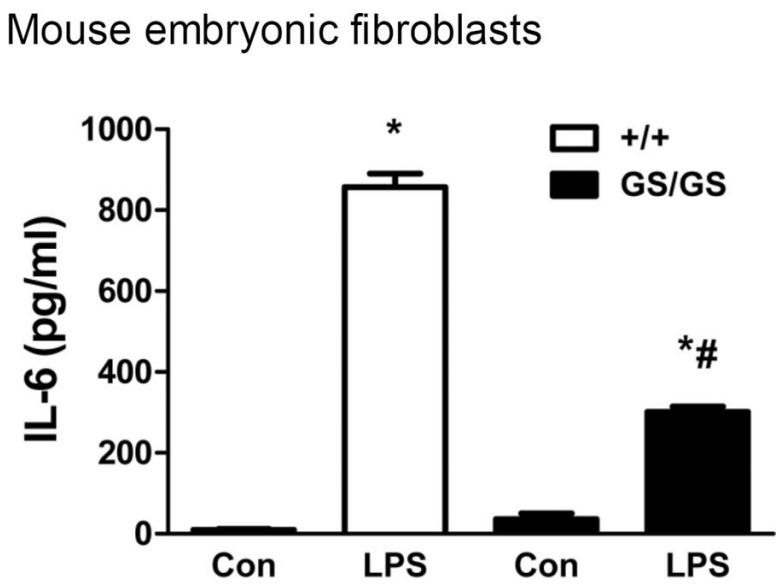

Figure 3. Effect of gain of function of $G\alpha_{i2}$ protein on LPS-induced IL-6 production in mouse embryonic fibroblast (MEF)

MEFs were isolated and cultured from WT and GS/GS mice and stimulated with LPS and Pam3CSK4 for 24 hours. LPS induced IL-6 production was determined by ELISA. *, $p<0.05$ compared to basal group; #, $p<0.05$ compared to WT group. N=6.

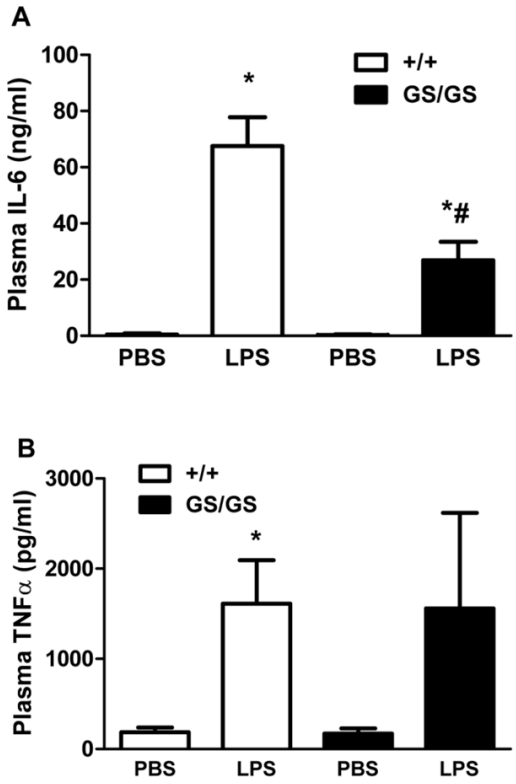

Figure 4. Effect of gain of function of $G\alpha_{i2}$ protein on LPS -induced plasma TNFα and IL-6 production in mice

WT and GS/GS mice were subjected to endotoxemia for 6 hours and LPS-induced plasma TNFα (A) and IL-6 (B) production were determined. *, $p<0.05$ compared to basal group; #, $p<0.05$ compared to WT group. N=4–6.

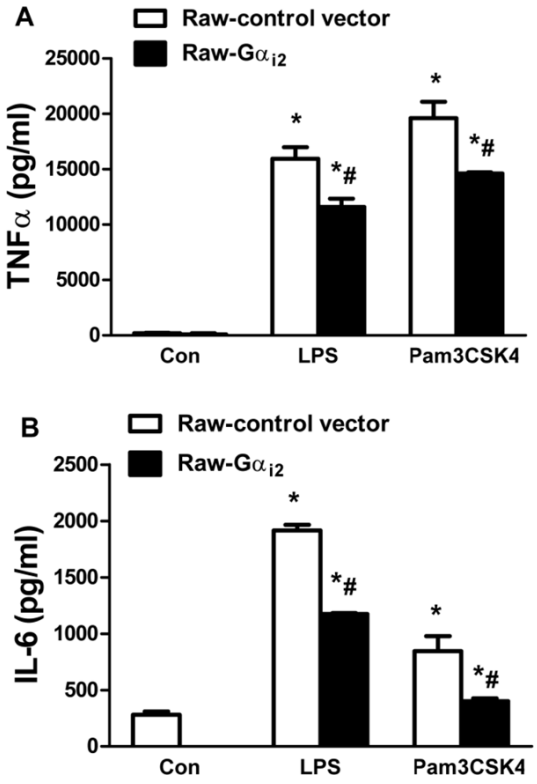

Figure 5. Effect of overexpression of Gα$_{i2}$ protein on LPS and Pam3CSK4-induced TNFα and IL-6 production in Raw 264.7 cells

Raw 264.7 cells that stably overexpressing Gα$_{i2}$ protein (Raw Gα$_{i2}$) and α-galactosidase (Raw-control vector) were employed. Cells were stimulated with LPS and Pam3CSK4 for 24 hours. LPS and Pam3CSK4 induced TNFα (A) and IL-6 (B) production were determined by ELISA. *, $p<0.05$ compared to basal group; #, $p<0.05$ compared to WT group. N=3.