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Research article

Activation of TLR-pathway to induce host Th1 immune response against visceral leishmaniasis: Involvement of galactosylated-flavonoids

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HIGHLIGHTS

- Galactosilated flavonoids treatment clears in-vivo drug-resistant *Leishmania donovani* infection.
- Quercetin 3-D-galactoside (Q-gal) & Kaempferol 3-O-D-galactoside (K-gal) induce host TLR4 pathway.
- These flavonoids up-regulate Th1 cytokines and suppress the disease-promoting IL-10.
- TLR4 deficient C3H/HeJ mice are unresponsive towards Q-gal and K-gal treatment.

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ABSTRACT

Immunotherapeutic strategies against visceral leishmaniasis (VL) are pertinent because of the emergence of resistance against existing chemotherapy, coupled with their toxicity and high costs. Various bioactive components with potential immunomodulatory activity, such as alkaloids, terpenes, saponins, flavonoids obtained primarily from medicinal plants, have been screened against different disease models. Reports suggested that glycans containing terminal β-galactose can skew host immune response towards Th1 by engaging TLRs. In this study, two synthesized terminal galactose-containing flavones, Quercetin 3-D-galactoside (Q-gal) and Kaempferol 3-O-D-galactoside (K-gal), are profiled in terms of inducing host protective Th1 response in both *in vitro & in vivo* animal models of experimental VL individually against antimony-resistant & antimony-susceptible *Leishmania donovani*. Further, we explored that both Q-gal and K-gal induce TLR4 mediated Th1 response to encounter VL. Molecular docking analysis also suggested strong interaction with TLR4 for both the galactosides, with a slightly better binding potential towards Q-gal. Treatment with both Q-gal and K-gal showed significant antileishmanial efficacy. Each considerably diminished the liver and splenic parasite burden 60 days after post-infection (>90% in AG83 infected mice and >87% in GE1F8R infected mice) when administered at a 5 mg/kg/day body-weight dose for ten consecutive days. However, the treatments failed to clear the parasites in the TLR4 deficient C3H/HeJ mice. Treatment with these compounds favors the elevation of TLR4 dependent host protective Th1 cytokines and suppression of disease-promoting IL-10. Q-gal and K-gal also triggered sufficient ROS generation in macrophages to kill intracellular parasites directly.

1. Introduction

Visceral leishmaniasis (VL) is triggered by the *Leishmania donovani* (LD), a protozoan parasite that lives and increases within the phagolysosomes of the host macrophages, where they transform into intracellular amastigotes. VL, characterized by the absence of a cell-mediated immune response caused specifically by *Leishmania* parasites (Murray et al., 2005), elicited clinical symptoms like

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hepatosplenomegaly, fever, blood cytopenia cachexia, and anemia (Mann et al., 2021). In the absence of effective vaccines, chemotherapy remains the mainstay for treating VL. Treatment of VL depends on a limited variety of drugs, including pentavalent antimonials (SbV), amphotericin B (AmB), and miltefosine (MIL). However, the present chemotherapeutic strategies are restricted by high effective dose with alarming cytotoxicity, drug resistance in parasites, and various side effects, including nephrotoxicity, nausea, vomiting (Murray, 2001; Mukherjee et al., 2014; Ponte-Sucre et al., 2017; Mann et al., 2021) emphasizing the need to develop an alternative strategy to combat VL. In this scenario development of immunotherapy has emerged as a viable alternative. Immunotherapeutic strategies against several pathogens highlighted the importance of toll-like receptors (TLRs) in the host defense (Aderem and Ulevitch, 2000; Ashour, 2015), particularly in pathogen-associated molecular patterns (PAMPs) modulation (Aderem and Ulevitch, 2000). As already reported, various drugs can induce cell activation through TLRs, ultimately leading to the secretion of subsets of cytokines, which could mediate immunomodulation (Sau et al., 2003; Hennessy et al., 2010).

Natural compounds, especially galactose-containing compounds, have been shown to possess different biological and therapeutic actions, such as antiinflammatory, antimicrobial and anticancer properties (Shukla et al., 2014). It has also been studied that glycans with terminal β(1–4) galactose can regulate host immune responses and mount Th1 mediated immunity through macrophage TLRs (Dissanayake and Shahin, 2007; Karmakar et al., 2012; Ricci-Azevedo et al., 2017). Flavone glycosides have earlier been shown to possess antitrypanosomal and antileishmanial activity (Ercil et al., 2005; Tasdemir et al., 2006; Schmidt et al., 2012). Exploiting established laboratory mouse model for VL, this study explored the potential of two flavone galactosides, quercetin 3-D-galactoside (Q-gal) & kaempferol 3-O-D-galactoside (K-gal), in activating host TLR pathway to confer protection against the parasite. Both Q-gal and K-gal are demonstrated to be associated with TLR4 mediated Th1 cytokine and ROS production, leading to parasite clearance.

2. Materials and methods

2.1. Animals, parasites and animal infection

Four to six weeks aged BALB/c mice (irrespective of sex, bought initially from Jackson Laboratory, Bar Harbour, ME, USA), and cultivated in the animal facility of the Indian Institute of Technology, Kharagpur, were used, with the Accreditation Number 147/1999/CPCSEA, approved by the animal ethics committee of the Institute. TLR4 defective C3H/HeJ mice were gifted by Dr. A. Surolia of the National Institute of Immunology, New Delhi. AG83 (MHOM/IN/83/AG83; antimony-responsive) & GE1F8R (MHOM/IN/89/GE1F8R; antimony-resistant) *L. donovani* parasite strains were used for experimental infection (Bhaumik et al., 2009). Parasites maintained in golden hamsters. Transformed promastigotes were used from infested spleen and sustained in M199 (Invitrogen Life Technologies) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (FCS) at 22 °C. CSA or Complete soluble antigen was prepared from NP-40-solubilized promastigote extracts, as described by De and Roy (1999). RAW 264.7, a murine macrophage cell line, was sustained at 37 °C, and 5% CO_2 in RPMI medium (SIGMA) complemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml of streptomycin (GIBCO), i.e., RPMI complete medium. Experiments were carried out in RAW 264.7 macrophages, *in vitro*, using 2nd passage promastigotes at a ratio of 20:1 for parasite: macrophage described earlier (Karmakar et al., 2011). Quercetin 3-D-galactoside and Kaempferol 3-O-D-galactoside were bought from Sigma Chemicals (St Louis, MO, USA). Q-gal and K-gal were solubilized in DMSO every time before use at the selected concentrations for *in vitro* experiments such that the final concentration of DMSO in culture was less than or equal to 1%. Q-gal and K-gal and other reagents were verified for endotoxin impurity by Limulus amoebocyte lysate (LAL) endpoint assay (QCL-1000; Bio-Whittaker, MD, USA) using manufacturer's protocol, and the data was found not as much of 0.1 EU/mL. Animals were infected through intra-cardiac route by inoculating 10^7 second-passage LD promastigotes (Bhaumik et al., 2009). Three different doses of Q-gal and K-gal ranging between 1 to 10 mg/kg/day (sonicated to make saline suspensions) were administered orally for ten successive days starting at 30 days post-infection. 20 days after the final drug treatment, animals were forfeited and the splenic & hepatic parasite burden were determined as described (Bhaumik et al., 2009) and the schedule has been described pictorially in Fig S4. Results were represented in LDU (Leishman Donovan units) of Stauber on Giemsa-stained imprints (LDU = number of amastigotes/1000 cells nuclei × mg organ weight) (Stauber, 1958).

2.2. Transfection of siRNA to RAW264.7 cells

1 μg of appropriate siRNA or control siRNA were added to the RAW264.7 cells to achieve transfection of the desired level, following manufacturer's instructions (Santa Cruz Biotechnology; sc-40261, sc-40257 & sc-45987 for TLR4 siRNA, TLR2 siRNA & MyD88 siRNA, respectively). Briefly, 100 μl siRNA Transfection Medium (prepared as per manufacturer's protocol) has been added to RAW264.7 (10^6) cells, for 6 h. Then the cells are washed to remove the medium and been infected with *Leishmania* parasites followed by treatment with Q-gal or K-gal. Pepinh-TRIF (TRIF Peptide Inhibitory; Cat Code: tlrl-pitrif) brought from InvivoGen. Briefly, the assay with Pepinh-TRIF involves platting of RAW264.7 cells (10^6) in presence or absence of Pepinh-TRIF (100 μM), for 6 h, followed by infection and treatment with *Leishmania* parasites and Q-gal/K-gal respectively.

2.3. Cytokine and iNOS analysis by semi-quantitative RT-PCR

Whole RNA was extracted from BALB/c mice spleen by the RNeasy minikit (QIAGEN) and analyzed separately (5 animals/group) using RT-PCR. Briefly, cDNA was prepared from 1 μg of RNA, isolated from different groups of mice (infected and treated) by random hexamers (Invitrogen) using Superscript II (Invitrogen). Expression levels of IL-10, IL-12 were analyzed in the synthesized cDNA using RT-PCR with gene-specific primers in a thermocycler (PerkinElmer model 9700) (Basu et al., 2005; Mukherjee et al., 2020a). Each gene was standardized against β-actin gene & the fold increase/decrease was measured against infected samples. The forward and reverse primers used are listed below: for IL-10, forward primer 5′TCCTTAATGCAGGACTTTAAGGGTT ACTTG3′ and reverse primer 5′ GACACCTTGGTCTTGGAGCTTAT-TAAAATC3' (238bp); for IL-12, forward primer 5′CTTAGGAGTTGG GGGTGGCT3′ and reverse primer 5′CTGGTGCAAAGAAACATGGA3' (276bp); for β-actin amplification, forward primer 5′GATGACGATATC GCTGCGCTG3′ and reverse primer 5′GTACGACCAGAGGCATACAGG3' (440bp) were used. ImageJ software (v1.41°) was used for densitometry analyses. DNA bands were visualized by staining with ethidium bromide under UV transilluminator. To determine band intensity, identical areas were used from the bands after normalizing against β-actin.

2.4. Cytokine analysis by ELISA

1 × 10^6 spleen cells/mL in a final volume of 0.2 mL from individual mice (five animals per group) were plated in 96-well plates and incubated for 72 h at 37 °C along with 50 μg/mL CSA. The release of cytokines in the supernatants was calculated by ELISA kits (Quantikine M; R&D Systems, Minneapolis, MN, USA; TGF-β, e-Bioscience), keeping in mind the detection limit of these assays. These were <2.0, <2.5, <5.1, <1.5, and <4.6 pg/mL for IFN-γ, IL-12p70, TNF-α, IL-10, and TGF-β, respectively. The data demonstrated as mean ± SD of all 5 animals of the group under consideration.

2.5. Measurement of ROS and NO

The amount of ROS was determined fluorometrically by using the cell-permeable H2DCFDA, as defined earlier (Bhaumik et al., 2009). ROS inhibitor, N-Acetyl-L-cysteine or NAC (Cat No: A7250) brought from Sigma-Aldrich. Splenocytes (10^6/mL) from infected and treated groups of animals were suspended in phenol-red free RPMI medium and incubated in the presence/absence of CSA (50 μg/mL) for 72 h in 5% CO2 incubator at 37 °C. The cells were further plated in RPMI medium and incubated either in presence or absence of NAC (1 mm NAC for 6h) with H2DCFDA (2, 7 Dichlorodihydrofluorescein diacetate; Cat No: D6883, Sigma; 2 μg/mL) at dark in room temperature for 20 min. Relative fluorescence was calculated in a PerkinElmer LS50B Spectrofluorometer with an excitation wavelength of 510 nm and an emission wavelength of 525 nm. Fluorometric measurements were done in triplicate and stated as fluorescence intensity unit for each experiment. The data are demonstrated as mean ± SD of all 5 animals/group under consideration. The culture supernatant was evaluated for its nitrite (NO^{2-}) content using the Griess reagent as defined earlier (Bhaumik et al., 2009). Nitrite measurement was an indication of NO produced by these cells. The data are demonstrated as mean ± SD of all 5 animals/group under consideration.

2.6. Homology model and molecular docking

The ligand-free structure of the mouse TLR4/MD-2 complex was acquired from PDB (PDB ID: 5IJB). PyMOL v1.3 was used to draw the structural models (Peters et al., 2006). Structural models of TLR4/MD-2 with Q-gal (CID 5281643) and K-gal (CID 5488283) were docked *in silico* using molecular docking simulation PyRX (Dallakyan and Olson, 2015) consisting of AutoDock Vina (Trott and Olson, 2010). The binding site residues were identified using BIOVIA Discovery Studio Visualizer (Dassault Systèmes) to demonstrate hydrophobic and hydrogen-bond interfaces.

2.7. Statistical analysis

Paired two-tailed Student's t-test was performed for all statistical analyses. Variances among means were calculated to show statistical importance, and P values of less than or equal to 0.01 were contemplated statistically significant.

3. Results

3.1. Flavone galactosides elicit a protective response in infected macrophages

In order to verify the ability of Q-gal and K-gal to induce host protective response, antileishmanial efficacy of these galactosides were checked in SbV-sensitive AG83 and SbV-resistant GE1F8R infected RAW264.7 cells in a dose (6.25–50 mg/L or 13.45–107.67 μM) and time (24–48 h) dependent manner. A significant, concentration-dependent decrease in infection was observed, with maximum of 87.15% AG83 amastigote multiplication and 78.52% GE1F8R amastigote multiplication, for 25 μg/ml (53.83 μM) Q-gal after 24 h (Figure 1). K-gal clears 89.8% of AG83 parasites and 78.28% of GE1F8R parasites, respectively, at 25 μg/ml (53.83 μM) concentration after 24 h (Figure 1). No obvious cytotoxicity of both K-gal and Q-gal were noted against macrophages at concentrations up to 100 μg/mL (Figure S5A & S5B).Flavone galactosides induced protection in infected macrophages through TLR-MYD88 pathway:

Various glycoconjugates with galactose residue show potent antileishmanial activity by inducing the TLR pathway (Dissanayake and Shahin, 2007; Karmakar et al., 2012). With this rationale, we wanted to find out whether Q-gal and K-gal could stimulate the TLR pathway to kill parasites in infected cells. To decipher the possible interaction of Q-gal and K-gal with TLR4, molecular docking analyses were performed for the

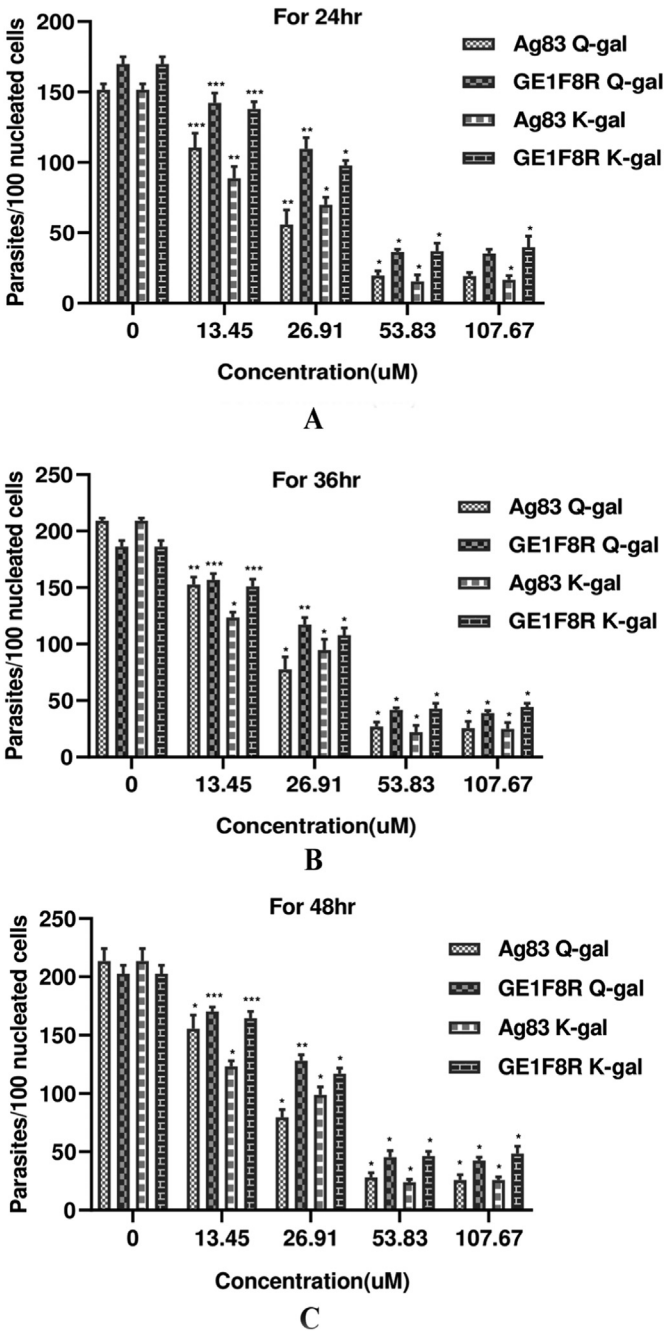

Figure 1. Comparison of the antileishmanial activity of different doses of Quercetin 3-D-galactoside (Q-gal) & Kaempferol 3-O-D-galactoside (K-gal) against intra-cellular amastigote forms of *L. donovani* Sb^S strain AG83 and Sb^R strain GE1F8R *in vitro*. RAW264.7 cells (10^6) were adhered for 24 h and subsequently infected with 20 times AG83 and GE1F8R promastigotes for 24 h. They were then treated with different doses of Q-gal and K-gal (as mentioned in the figure) for the next 24 h (A), 36 h (B) and 48 h (C), respectively. Intracellular parasite number was measured by Giemsa staining and expressed as parasites/100 mΦ. Results are demonstrative of three independent experiments and data shown are mean ± SD; n = 3. *p values versus corresponding infected control; paired two-tailed Student's t-test. *$p < 0.0001$, **$p < 0.001$ and the other values are not significant compared to $P < 0.01$.

two galactosides independently with the TLR4/MD2 complex. As depicted in Figure S1a and S1b, respectively, both Q-gal and K-gal displayed potential interaction with the formation of 11 H-bonds with TLR4/MD2. However, Q-gal displayed a higher affinity for the receptor complex with a binding energy of -9.1 kcal/mol compared to −8.8 kcal/mol for K-gal.

For TLR4 and TLR2 knockdown, RAW264.7 cells were transfected with TLR4 and TLR2 siRNA, respectively. Knockdown of TLR4 & TLR2 expressions in transfected cells were confirmed by western blotting (Figure 2A). Expression of GAPDH in the transfected cells was considered as the internal control. The number of parasites had increased in the Q-gal/K-gal treated TLR4 knocked-down cells in contrast with Q-gal/K-gal treated I-RAW (*Leishmania* infected RAW) cells. Along with that, there were not much difference between the parasite load of untreated parasite-infected cells and the Q-gal/K-gal treated TLR4 knocked-down RAW cells, suggesting the role of TLR-4 in galactose conjugated

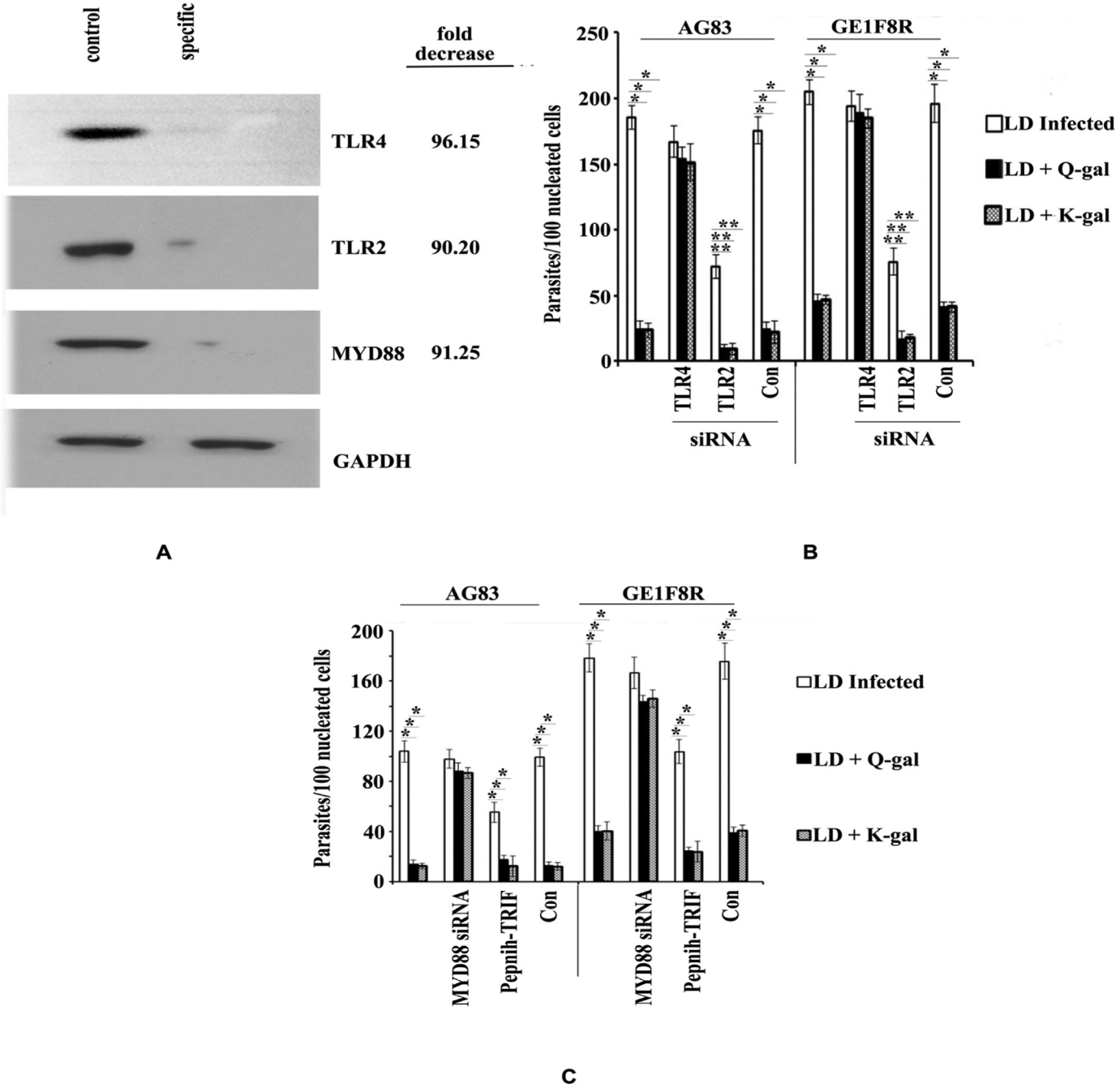

Figure 2. TLR4 and MyD88 are required for Q-gal and K-gal mediated antileishmanial response. A. RAW264.7 cells transfected with siRNAs specific to TLR4, TLR2 and MyD88. Control group of mice was transfected with control siRNA (control). 24 h post-transfection, cells were recuperated and TLR4, TLR2 and MyD88 levels measured by western blots. GAPDH was used as loading controls. Blots are representative of three separate experiments. For the original blots of TLR4, TLR2, MyD88 and GAPDH, refer to supplementary figure number S7A, S7B, S7C & S7D respectively. B. TLR4 transfected, untransfected and TLR2 transfected RAW264.7 cells were infected with LD (APC/parasite 1:20) for 24 h and then treated with 25 μg/mL of Q-gal and K-gal for the next 24 h. Intracellular parasite number was determined by Giemsa staining and expressed as parasites/100 mΦ. The results are demonstrative of three independent experiments and data presented are mean ± SD; n = 3. *p values versus corresponding infected control; paired two-tailed Student's t-test. *$p < 0.0001$, **$p < 0.001$ and the other values are not significant compared to $P < 0.01$. C. RAW264.7 cells transfected with MyD88 and control siRNA (control) and were infected with LD (APC/parasite 1:20) for 6h. RAW264.7 cells were also infected with LD (APC/parasite 1:20) in presence or absence of a TRIF inhibitory peptide, Pepinh-TRIF (100 μM), for six hours. Non-ingested promastigotes were removed by washing, and cells were cultured for another 18 h. Infected APCs were treated with Q-gal and K-gal (25 μg/mL) for twenty-four hours. Intracellular parasites were counted by Giemsa staining. Experiments were performed in triplicate and repeated three times each and one set of demonstrative data is shown. Error bars signify mean ± SD, *$p < 0.0001$ and the other values are not significant compared to $p < 0.01$; paired two-tailed Student's t-test.

flavonoid treatment. But in the case of Q-gal/K-gal treated TLR2 knocked-down cells it was observed that the parasite burden markedly decreased as compared to Q-gal/K-gal treated I-RAW cells. This suggested that the Q-gal and K-gal could still protect the TLR2 knocked down cells (Figure 2B).

Further, to investigate the role of downstream effector myeloid differentiation primary response 88 (MyD88), siRNA was used to produce MyD88 knocked down cells. Additionally, for TIR-domain-containing adapter-inducing interferon-β (TRIF) inhibition the MyD88 knocked down RAW cells were treated with 100 μM of TRIF inhibitory peptide or Pepinh-TRIF for 30 min at 37 °C (Invivogen, catalog code: tlrl-pitrif). MyD88 knocked down RAW cells were infected with AG83 parasites for 24 h, following treatment with Q-gal for another 24 h. The parasite burden in Q-gal treated MyD88 knocked down RAW cells was increased by 6.33 fold in AG83 parasite-infected MΦ, and 3.60 fold in GE1F8R parasite-infected MΦ, compared to Q-gal treated I-RAW cells respectively (Figure 2C). A similar trend was observed for K-gal also, with increase of parasite burden by 6.86 fold in AG83 parasite-infected MΦ and 3.60 fold in GE1F8R parasite-infected MΦ compared to K-gal treated I-RAW cells respectively. But in Pepinh-TRIF treated macrophage cells, no significant effect had been observed with Q-gal mediated therapy (Figure 2C). With K-gal, the same trend of results were observed as it also failed to clear the parasites in MyD88 knocked down macrophages (Figure 2C).

3.2. Flavone galactosides mediated Th1 cytokines production and ROS and NO up-regulation in infected macrophages

Taenia glycans and Lewis X pentasaccharide having terminal β-(1-4)-galactose residue have been shown to stimulate Th-1 biased response (Dissanayake and Shahin, 2007). TLR4 stimulation also initiates DC maturation and Th1- type responses through the release of IL-12 (Netea et al., 2005; Li et al., 2015). So, we have investigated proinflammatory cytokine profile generated by Q-gal and K-gal. A high amount of Th1 cytokines was produced in the Q-gal and K-gal treated AG83/GE1F8R infected cells. Maximum IL-12 generation, 3 fold increase in AG83 and 2.49 fold increase in GE1F8R infected cells, respectively (Figure 3C) were observed at 24h post-treatment with 25μg/mL of Q-gal. 2.89 fold increase in AG83 and 2.21 fold increase in GE1F8R infected cells were observed in 24h post-K-gal treatment. Very high amounts of IL-10 (1074.04 ± 90.31 pg/mL and 1178.73 ± 87.54 pg/mL respectively, Figure 3A) and TGF-β (1115.18 ± 91.11pg/mL and 1218.76 ± 110.56 pg/mL respectively, Figure 3B), were evident in AG83 and GE1F8R, respectively, as determined by ELISA. In Q-gal treated AG83 and GE1F8R infected TLR4 knocked down macrophages, IL-12 cytokine level (273.53 ± 35.46 pg/mL & 287.54 ± 40.65 pg/mL respectively, Figure 3C) and IL-10 level (1048.67 ± 69.95 pg/mL & 976.45 ± 81.76 pg/mL respectively Figure 3A) were almost similar to the cytokine levels in the infected macrophages. It has been found out that in Q-gal and K-gal treated LD infected macrophages, TLR2 expression is inhibited (Fig S6, middle panel), probably resulting in decrease in the expression of IL-10 and TGF-β in treated macrophages (Figure 3A & B). In AG83 and GE1F8R infected Q-gal and K-gal treated MYD88-knocked down macrophages, the IL-12 level was decreased by 2.57 fold & 2.63 fold for Q-gal and 2.72 fold & 2.31 fold for K-gal (Figure 3C), IL-10 level was increased by 5.14 fold & 4.19 fold for Q-gal and 4.44 fold & 4.31 fold for K-gal (Figure 3A), respectively, compared to Q-gal treated infected macrophages. But TRIF inhibition failed to produce any effect in Q-gal or K-gal mediated cytokine productions (data not shown). Semi-quantitative RT-PCR data also speculate similar trends of the cytokines by mRNA expression level (Fig S2). Interestingly, production of TNF-α, a major Th1 cytokine, was significantly altered by Q-gal or K-gal as determined by both ELISA & semi-quantitative RT-PCR data (Figure 3D).

Oxidative burst and generation of free radicals are important for killing pathogens to restrain the progression of infection. Unconjugated flavones can also induce the production of superoxide anion, hydrogen peroxide, and other reactive oxygen species (Cao et al., 1997; De Marchi et al., 2009; Procházková et al., 2011; Kumar and Pandey, 2013) in macrophages. Hence, we assessed the ROS generation following treatment with the cell-permeable dye H_2DCFDA. The levels of ROS in AG83 and GE1F8R infected Q-gal and K-gal treated cells were 5.25 fold and 6.4 fold higher, respectively, when compared to control AG83 infected cells (Figure 3E). The ROS levels were diminished in treated TLR4 knocked down and MYD88 knocked down cells (Figure 3E). Conversely, in the presence of N-acetyl cysteine (NAC, 50 μM; commonly used to inhibit ROS), Q-gal and K-gal treatment in both AG83 and GE1F8R infected macrophages displayed parasite burden, which was comparable with the untreated cells (Figure 3F). When examined for induction of nitric oxide generation, Q-gal and K-gal treatment did not elevate NO generation from infected macrophages (Figure 3G).

3.3. Antileishmanial efficacy of flavone galactosides in infected animals

Q-gal and K-gal kill the intracellular amastigotes by producing substantial amounts of Th1 cytokines and ROS. So, to evaluate the role of these galactosides in the modulating disease progression, BALB/c mice as animal model for VL was implemented. BALB/c mice were infected with AG83 for 60 days. Two months post-infection, Q-gal and K-gal (5 mg/kg/day) were given orally for 10 consecutive days, and the BALB/c animals were sacrificed 20 days after the last treatment, following which parasite burdens were scored. Q-gal and K-gal at almost reduced the parasite load by 92.58% and 92.97% in AG83 infected BALB/c mice spleen & 88.03% and 88.25% in GE1F8R infected BALB/c mice spleen, respectively (Figure 4A). Q-gal treatment clears more than 95% of liver parasites and 92% of bone marrow parasites from both AG83 and GE1F8R infected mice (Figure 4B & C). In contrast, K-gal treatment clears 88% of liver parasites and almost 92% of bone marrow parasites (Figure 4B & Figure 4C) in the AG83 infected BALB/c mice (on an average), which was evident from GIEMSA stained stamp smears when observed under light microscopy.

Since these galactosides did not confer protection in TLR4 deficient cells, we also evaluated the therapeutic efficacy of Q-gal and K-gal in AG83 and GE1F8R infected C3H/HeJ mice which are TLR4 defective. Two months old infected C3H/HeJ mice were given 5 mg/kg/day Q-gal and K-gal orally for 10 consecutive days and these animals were sacrificed 20 days after the last treatment. But Q-gal and K-gal produced no or little protection in C3H/HeJ mice, similar to DMSO treated mice, as shown in the spleens, livers and bone marrows of treated mice with high parasite load (Figure 4D & E). All these results indicate that flavone galactosides mediate their protective response against VL through TLR4.

3.4. Effect of flavone galactosides on Th1 immune response and ROS up-regulation in in vivo mice model

A detailed splenic cytokine analysis was performed in LD-infected mice after Q-gal and K-gal treatment by ELISA. Th1 cytokine profiles had markedly increased in these galactosides-treated BALB/c mice, whereas high IL-10 and TGF-β cytokines were detected in the AG83 and GE1F8R infected BALB/c and C3H/HeJ mice groups. Expression of IFN-γ and IL-12 cytokines were up-regulated with respect to 60 days infected mice in both Q-gal-treated AG83 (76.45% and 61.62% respectively) and GE1F8R (74.97% and 71.44% respectively) infected BALB/c mice (Figure 5A & B). In K-gal-treated mice, the same trends of IFN-γ and IL-12 were also seen (Figure 5A & B). Levels of the Th1 cytokines were diminished in both treated and untreated AG83 and GE1F8R infected C3H/HeJ mice (Figure 5A & B), suggesting a role of TLR4 in Q-gal and K-gal treated Th1 cytokine production. Levels of Th2 cytokines IL-10 and TGF-β also attained maximum (Figure 5C & D) at 60 days in both the AG83 and GE1F8R infected BALB/C and in both treated and untreated C3H/HeJ mice groups. Similar results for the IL-12 (Fig S3 A, B & G, H) and IL10 (Fig S3 C, D & I, J) transcripts were obtained at mRNA levels as shown by RT-PCR data in both Q-gal and K-gal treatment against AG83 and GE1F8R infection.

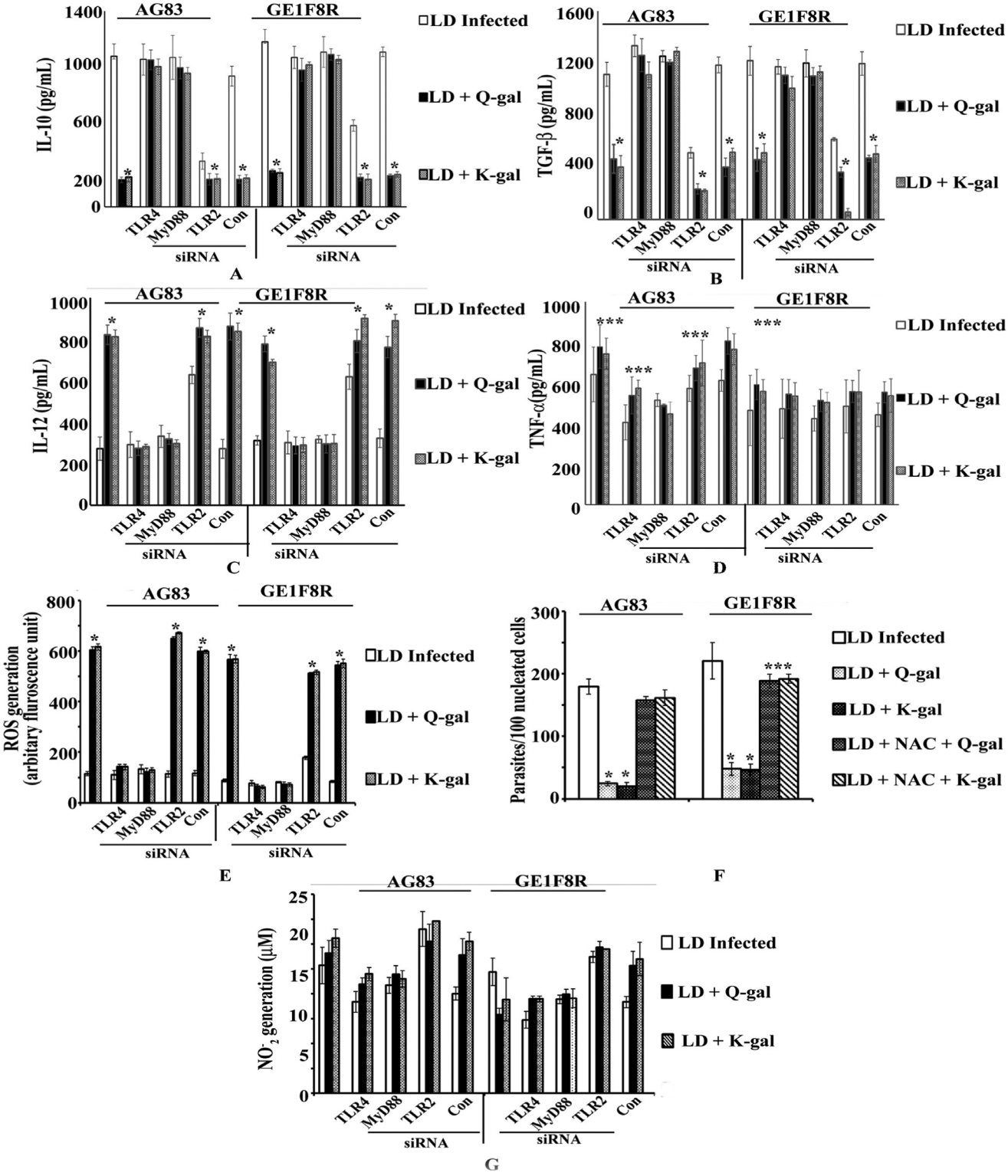

Figure 3. Effect of Q-gal and K-gal on cytokine response and free radical generation. Cells infected with LD promastigotes (24 h) at 20:1 ratio of parasite-to-cell trailed by treatment with 25 μg/mL of Q-gal and K-gal for 24 h. IL-10 (A), TGF-β (B), IL-12 (C) and TNF-α (D) cytokines levels in culture supernatants were also assessed by ELISA. ROS production was determined by H2DCFDA (E). The results are demonstrative of three independent experiments and data presented mean ± SD; n = 5. *p values vs corresponding infected control; paired two-tailed Student's t-test. *$p < 0.0001$, **$p < 0.001$ and the other values are not significant compared to $p < 0.01$; paired two-tailed Student's t-test. F. RAW264.7 cells (10^6) were adhered for 24 h and subsequently infected with 20 times AG83 promastigotes for another 24 h. Infection was then followed by treatment with 25 μg/mL of Q-gal and K-gal for 24 h in the presence or absence NAC (50 μM). Intracellular parasite number was counted by Giemsa staining and expressed as parasites/100 MΦ. The results are demonstrative of three independent experiments and data presented mean ± SD; n = 5. *p values vs corresponding infected control; paired two-tailed Student's t-test. *$p < 0.0001$, **$p < 0.001$ and the other values are not significant compared to $P < 0.01$; paired two-tailed Student's t-test. G. Cells were infected with LD promastigotes (24 h) at 20:1 parasite-to-cell ratio followed by treatment with 25 μg/mL of Q-gal and K-gal for 24 h. Endogenous NO production was determined by Griess reagent in the supernatants.

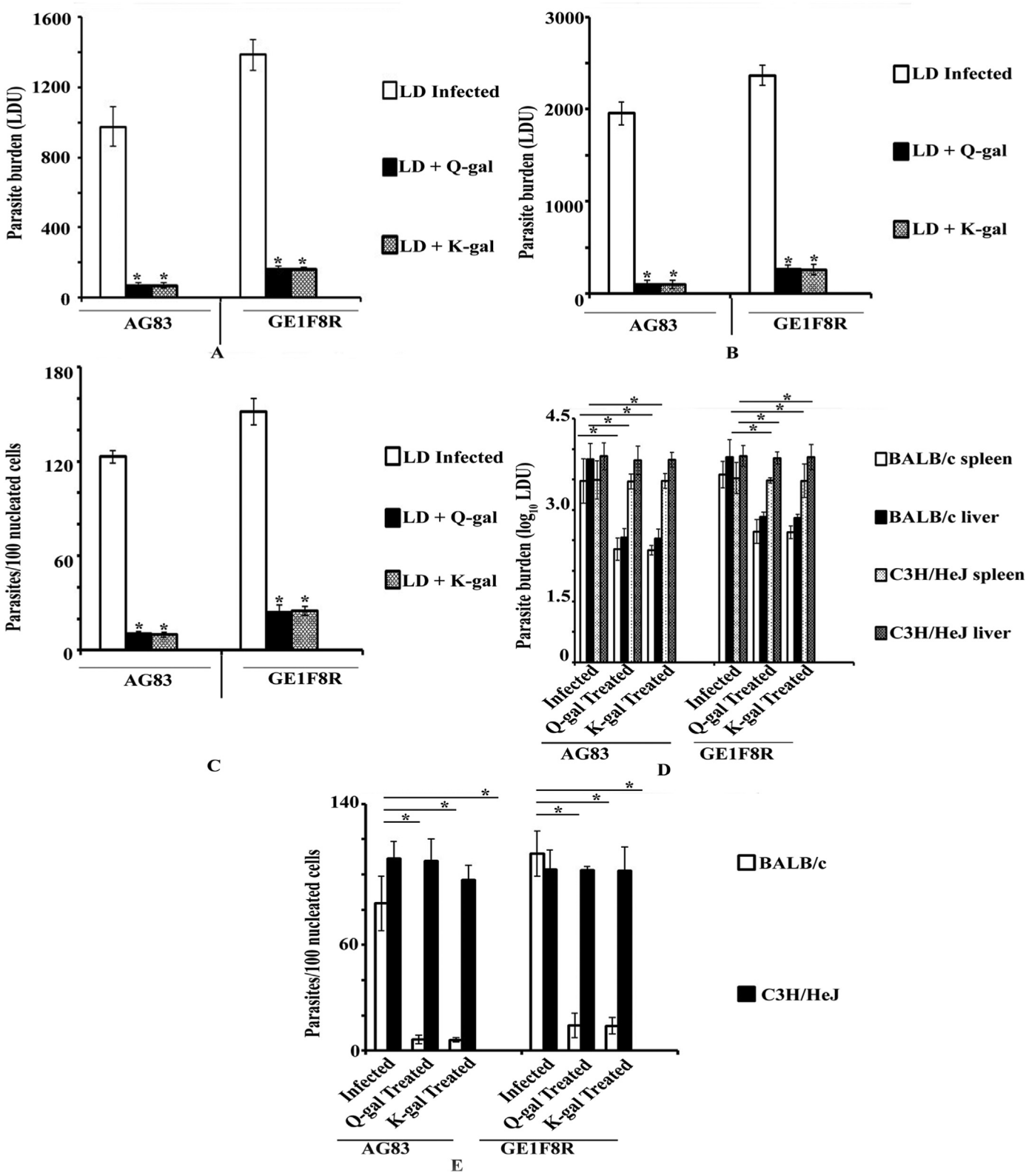

Figure 4. *In vivo* efficacy of Q-gal and K-gal against *L. donovani* infection through TLR4. Q-gal and K-gal were administered at an amount of 5 mg/kg/day orally for 10 successive days starting at 60th day post-infection in BALB/c mice. Animals were sacrificed 20 days after treatment and splenic (A) and hepatic parasite loads (B) were determined for all groups in LDU as described in the materials and method section previously. Parasite load in bone marrow was determined as parasites/100 nucleated cells (C). Data shown as mean ± SD of ten animals/group, and are demonstrative of three independent experiments. *$p < 0.0001$ and the other values are not significant compared to $P < 0.01$; related with infected control groups at all time points; paired two-tailed Student's t-test. 5 mg/kg/day of Q-gal and K-gal orally were given to sixty days AG83 and GE1F8R infected TLR4 deficient C3H/HeJ mice for 10 consecutive days. The parasite burdens in liver and spleen (log_{10} LDU, D) and in bone marrow (parasites/100 nucleated cells, E) of each animals were determined at 20 days post-treatment. The results are demonstrative of three independent experiments and data represent as mean ± SD; $n = 5$. *$p < 0.0001$ vs corresponding infected group; paired two-tailed Student's t-test.

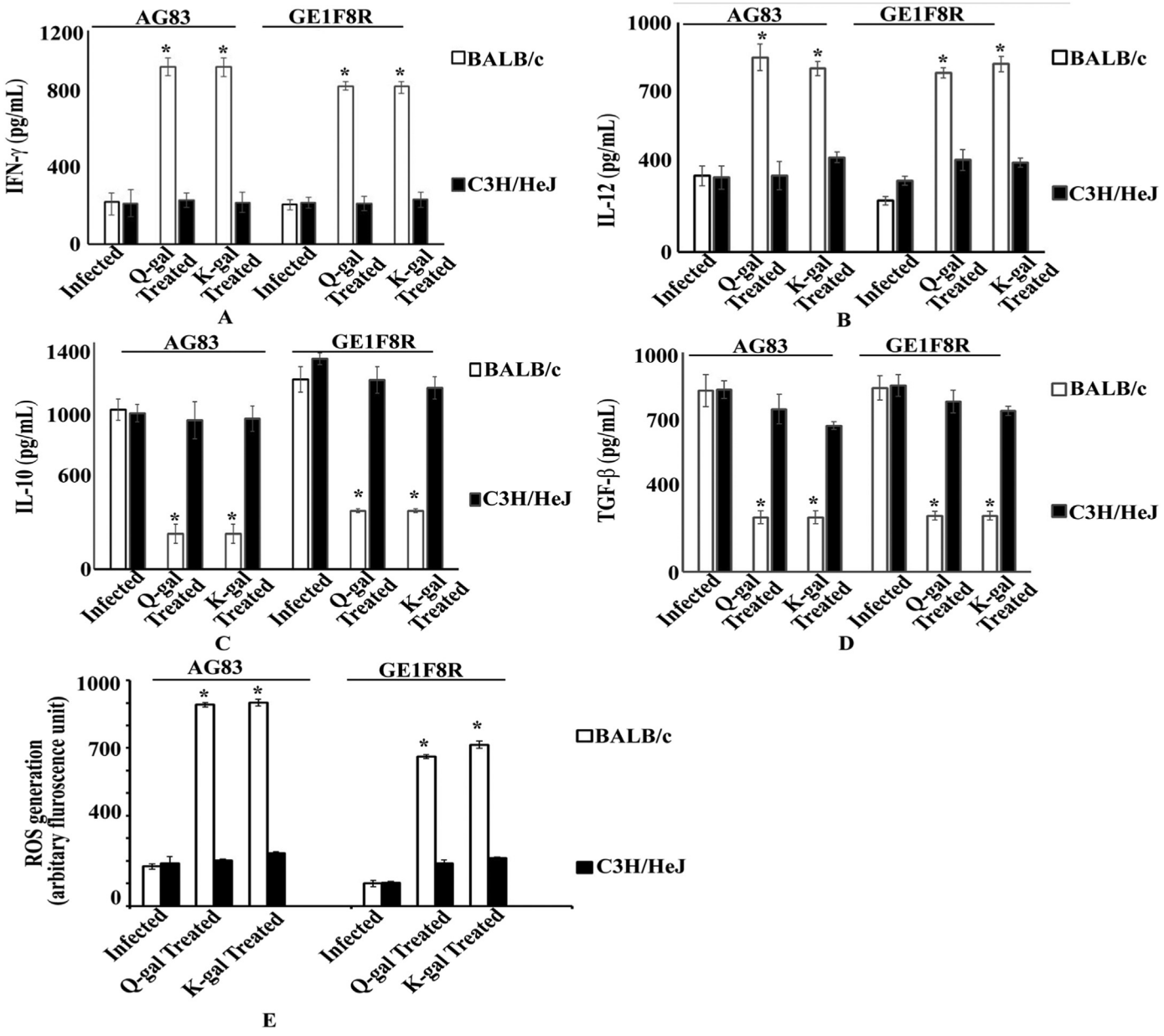

Figure 5. Effect of Q-gal and K-gal on Th1 cytokine and ROS production *in vivo*. 5 mg/kg/day of Q-gal and K-gal were given orally (10 days consecutively) staring on the 60th day after infection in both BALB/c and C3H/HeJ mice. Animals were sacrificed 20 days after the treatment. Culture supernatants from different groups of experimental mice used to see the expressions of Th1 (A, IFN- γ; B, IL-12) and Th2 (C, IL-10; D, TGF-β) cytokines at protein level were detected by ELISA. The results are demonstrative of three independent experiments and data represents as mean ± SD; *$p < 0.0001$ and the other values are not significant compared to $p < 0.01$; paired two-tailed Student's t-test. BALB/c and C3H/HeJ mice were infected with *L. donovani* AG83 and GE1F8R promastigotes and treated with Q-gal and K-gal as described above. Splenocytes (10^6 cells) from different infected and treated groups were isolated and incubated with 50 μg/mL CSA in a 5% CO_2 incubator at 37 °C for 72 h. *In vivo* ROS production was determined by H2DCFDA at 525nm (E). The data shown are representative of three independent experiments.

As the ROS level was elevated in Q-gal and K-gal treatment *in vitro*, we also observed the level of ROS in the Q-gal and K-gal treated cells in AG83 and GE1F8R infected animals. Neutrophils and macrophages produce ROS in response to different ligands of pattern recognition receptors (PRRs). Moreover, impaired ROS generation was observed in MyD88 deficient macrophages. Q-gal and K-gal treated mice produced substantial amount of ROS with H2DCFDA by fluorometric analysis compared with AG83 and GE1F8R infected animals (Figure 5E). The high amount of ROS produced in Q-gal and K-gal treated mice almost came down to a negligible amount in treated C3H/HeJ groups, comparable with infected BALB/c mice (Figure 5E). Overall, these data suggested that Q-gal and K-gal have the ability to induce protection against VL by switching the cytokine balance towards host protective Th1 mode.

4. Discussion

Manifestation of the infection in visceral leishmaniasis depends on the IL-10 dependent suppression of T cell responses in the host (Ghalib et al., 1993; Kane and Mosser, 2001; Kupani et al., 2020) and amelioration is mediated through the induction of IL-12 driven IFN-γ production (Mukherjee et al., 2020b; Parmar et al., 2020), generation of reactive nitrogen and oxygen species through activated macrophages (Mukherjee et al., 2020b) and a decrease in IL-10 production. Treatment of leishmaniasis is based on pentavalent antimonial drugs that are toxic and prone to drug resistance (Croft and Coombs, 2003). However, with the emergence of resistance (Mukhopadhyay et al., 2011) new drugs like paromomycin, miltefosine and amphotericin B came into picture (Jha

et al., 2013). But resistance to these new generation drugs are also emerging at an alarming rate (Hefnawy et al., 2017). Several new strategies have been applied to discover a successful alternate approach (Mukherjee et al., 2014). With this rationale, importance of TLR mediated therapy against VL has been widely investigated (Paul et al., 2014a, 2014b). *Leishmania* infection suppresses TLR4 mediated Th1 cytokine productions by inducing TLR2 mediated IL-10 production (Re and Strominger, 2004). It has already been reported that terminal galactose-containing glycans can induce a strong Th1 response against VL by engaging the TLR4 pathway (Dissanayake and Shahin, 2007; Karmakar et al., 2012). Here we have shown that two Flavone galactosides, quercetin 3-D-galactoside (Q-gal) & kaempferol 3-O-D-galactoside (K-gal), both having significant binding affinity towards TLR4 (Fig S1a & Fig S1b), offers significant protection against both antimony sensitive AG83 and antimony resistant GE1F8R infection in both *in vitro* and *in vivo* conditions by engaging TLR4 pathway. Q-gal and K-gal treatment positively and negatively regulate the expression of TLR4 and TLR2 respectively in macrophages (Fig S6, upper panel& S6 middle panel). Independent treatment with either Q-gal or K-gal removes LD parasites in a time and dose-dependent manner, killing most of them. Q-gal or K-gal treatment results in significant parasite clearance within 24 h treatment, with Q-gal showing slightly better efficacy than K-gal which might be due to their higher binding affinity towards TLR4 (Fig S1a & Fig S1b). Infection of macrophage cells with LD promastigotes failed to stimulate a proinflammatory response due to defective antigen presentation (Pradhan et al., 2021), ultimately leading to negligible IL-12 and IFN-γ production. TLR stimulates the production of an impressive level of IL-12 from proinflammatory mature DC cells (Caielli et al., 2010). Our results suggest both these galactosides induced the TLR4 pathway, which is associated with the up-regulation of IL-12 and other Th1 cytokines, as along with the generation of ROS and suppression of IL-10 within the LD infected host (Figure 3). Also it has been observed that, Q-gal and K-gal inhibited TLR2 expression (Fig S6, middle panel), which could be the probable cause of decreased expression of the level of IL-10 and TGF-β in treated cells as shown in Figure 3A & B. Interestingly, this galactosidase-mediated activation of TLR4 is independent of the nature of input infection, as it is observed in case of both antimony-susceptible and antimony-resistant LD infected hosts. Involvement of TLR4 in offering galactosidase mediated protection is clearly evident as treatment with Q-gal and K-gal failed to offer any protection. Clinically validated TLR4-increasing compounds, such as BCG, have already proved to reduce parasite burden in LD infected hosts. In LD infected macrophage cells, the parasite load became almost negligible 24 h post-treatment (Figure 2B). In infected cells, there was a suppression of IL-12 and IFN-γ production accompanied by simultaneous stimulation of IL-10 production, which was reversed by treating the cells with Q-gal and K-gal as suggested by RT-PCR (Fig S2 & Fig S3) and ELISA data (Figure 3).

MyD88, one of the most important molecules in the TLR4 pathway, plays a significant role against resistance to leishmaniasis (Tuon et al., 2008; Mukherjee et al., 2015). Inhibition of MyD88 by siRNA also negates Q-gal and K-gal mediated protection in the AG83 infected macrophages, whereas no role of TRIF had been seen in these galactoside treatments (Figure 2C). High concentrations of Th-2 cytokines with low amounts of Th1 cytokines in Q-gal and K-gal treated MYyD88 knocked down cells suggest that these galactosides might exert protection through MYD88 dependent pathway.

In vitro protection results of these galactosides-treated AG83 and GE1F8R infected macrophages with no significant cytotoxicity in host macrophage cells led to the therapy-based studies *in vivo*. In coherence to the macrophage infection model, *in vivo* studies with AG83 and GE1F8R infected BALB/c mice revealed that the parasite burden became almost negligible in the spleen (Figure 4A), liver (Figure 4B) and bone marrow (Figure 4C) 20 days post-treatment. Levels of Th1 cytokines were suppressed in infected mice while were up-regulated in Q-gal and K-gal treated mice at a Q-gal/K-gal concentration of 5 mg/kg/day, as suggested by RT-PCR (Figures S2 & S3) and ELISA data (Figure 5A, B). In TLR4 deficient C3H/HeJ mice, no apparent amelioration of pathophysiology in terms of parasitic load could be detected even after 20 days post-treatment (Figure 5D & E). Alongside, a high amount ofIL-10 (Figure 5C) and TGF-β (Figure 5D) with very low levels of Th-1 cytokines (Figure 5A, B) could be detected, suggesting the crucial role of TLR4 in the Th1 cytokine production for the killing of LD parasites in the cells.

The generation of ROS and NO in the macrophage is critical in controlling *Leishmania* infection. There was no significant change of NO in infected versus Q-gal/K-gal treatment as seen by the Griess reagent (Figure 3G). We also observed the ROS levels in the Q-gal and K-gal treated cells. Neutrophils and macrophages produced ROS in response to different ligands of pattern recognition receptors (PRRs). Moreover, impaired ROS generation was observed in MyD88 deficient macrophages (Ichikawa et al., 2012). Q-gal and K-gal treated macrophages produced a substantial amount of ROS with H2DCFDA by fluorometric analysis (Figure 3E). In Q-gal/K-gal treated mice, high amount of ROS was also observed, almost down to a negligible amount in treated C3H/HeJ groups, comparable with infected BALB/c mice (Figure 5E). Treatment with NAC completely abolished the protective effect of Q-gal and K-gal in the infected cells. Overall, these data suggested that Q-gal and K-gal have the ability to induce protection against VL by switching the cytokine balance towards host protective Th1 mode.

As a carbohydrate-binding protein, galectin-3 represents as a viable target for numerous therapeutic interventions as it influences several biological pathways including proinflammatory response through recognizing small β-galactosides. Though this particular study is restricted to evaluating Q-gal and K-gal as trigger for TLRs, the possibility of involvement of galectins like galectin-3 cannot be excluded and warrants intricate exploration in future (MacKinnon et al., 2008).

In conclusion, the naturally occurring or synthesized galactosides tested here have proved very promising in inducing host defensive mechanisms by stimulating the TLR4 pathway. These compounds show excellent *in vitro* antileishmanial potentials. Successful clearance of LD parasites from the BALB/c mice by these galactosides depends on the up-regulation of Th-1 cytokines and direct killing of the parasites through reactive oxygen intermediates, such as ROS, by engaging the TLR pathway. From this study, it can be inferred that different galactoterminal compounds have the potential to be used as antileishmanial drugs. The emergence of drug resistance is a severe problem of modern drug therapy. Thus, targeting the TLR pathway by different galactoterminal compounds exhibits a new, safe, and alternate therapy strategy against VL.

Declarations

Author contribution statement

Supratim pradhan: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Snehlata, Debolina Manna: Analyzed and interpreted the data; Wrote the paper.

Subir karmakar, Manoj Kumar singh: Performed the experiments.

Arijit bhattacharya: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Budhaditya mukherjee: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Joydeep paul: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

No data was used for the research described in the article.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

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