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SHORT COMMUNICATION

Use of TLR9 and TLR7/8 Agonists in Combination With D-Galactosamine in Exploring Models for Distinct Severities of Systemic Inflammation Relative To Liver Injury

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Summary

Challenges with various TLR ligands (TLRLs) in combination with D-galactosamine (GalN) in rodents may mimic diverse conditions of acute inflammation and organ failure. Here, we report that CpG (ODN1826, TLR9 agonist)/GalN induced a liver-specific injury with modest systemic effects, whereas R848 (resiquimod, TLR7/8 agonist)/GalN exhibited systemic and liver toxicity. We also observed the protective effect of Gr-1$^+$ cells (the population containing neutrophils) against liver injury in both the R848/GalN and CpG/GalN models. In cytokine measurements, the intraperitoneal administration of antibodies showed a non-specific tolerance induction effect, which was more pronounced in the CpG/GalN than in the R848/GalN model. Cytokine analyses also suggested that the TLR9 agonist/GalN induced a limited degree of systemic inflammation compared to TLR7/8 agonist/GalN models. The relevance of this finding to the TLR9-mediated induction of stress tolerance (protective effect) in non-immune cells is discussed.

Key words

IL-6/IL-10 • Liver injury • Endotoxin tolerance • LPS • Intravenous immunoglobulin therapy

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Introduction

Administration of lipopolysaccharide (LPS) in combination with GalN is a widely studied model for acute liver failure and inflammation-mediated organ injury (e.g., Kemelo *et al.* 2017). GalN can impede uridine metabolism, possibly inducing cell stress. Various TLR ligands (TLRLs) in combination with GalN can induce liver injury (Seki 2017). Both TLR7/8 and TLR9 are endosomal receptors, but in our experiments, R848/GalN induced more severe signs of illness as compared to CpG/GalN, despite the use of the doses inducing similar levels of liver injury (Seki 2017). In this study we further compared these models.

Methods

The serum biomarker analyses were performed as previously described (Seki 2017). Briefly, 18 six-week-old C57BL/6 mice divided into three groups (n=6) were injected intraperitoneally with 100 µl PBS, 200 µg LPS in PBS, 5 µg R848+5 mg/mouse GalN in PBS, 20 µg CpG DNA+15 mg/mouse GalN in PBS.

For the antibody (Ab)-blocking analysis, 72 mice were divided into 12 groups (n=6) and the following Abs were intraperitoneally administered: 150 µg anti-Gr-1 (Ly6G, Ly6C) Ab (RB6-8C5) or isotype-matched control (IgG2bκ, BioLegend) 24 h before TLRL/GalN challenge; 40 µg rat anti-mouse P-selectin Ab (RB40.34) or isotype-matched control (rat IgG1λ Control A110.1, BD Pharmingen) 4 h before TLRL/GalN; and 100 µl rabbit anti-thrombocyte serum (CLA31440, Cedarlane) or the control serum (CL1000-10) 24 h before TLRL/GalN. We confirmed that these

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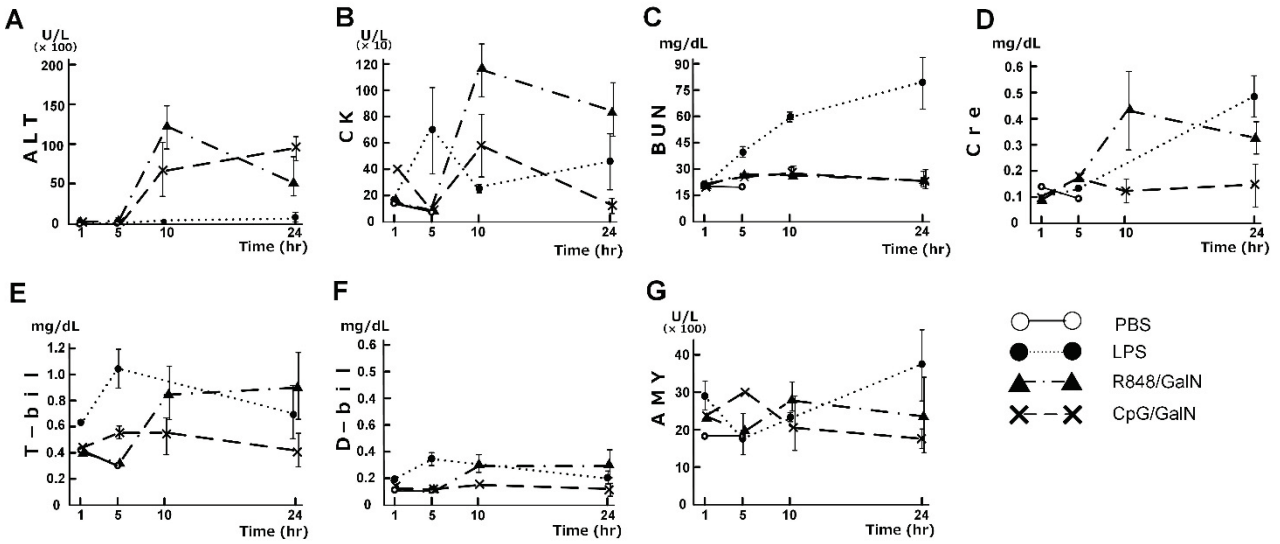


Fig. 1. Serum clinical biomarker analysis. (**A-G**) ALT, creatine kinase (CK), blood urea nitrogen (BUN), creatinine (Cre), total bilirubin (T-bil), direct-bilirubin (D-bil) and amylase (AMY) in serum samples of PBS, LPS, CpG/GalN, and R848/GalN-injected mice were analyzed using DRI-CHEM4000 (Fujifilm) at the indicated time points. The samples of the PBS-injected mice were analyzed only for 1 and 5 h time points. Some error bars (SD) are hidden for clarity.

pretreatments depleted >89 % neutrophils and >94 % of platelets in blood. All experiments were performed in accordance with protocols approved by the experimental animal committee of Teikyo University.

Severe extrahepatic effects of TLR7/8 but not TLR9 agonist in galactosamine mice model

To further compare the models, we measured several serum biomarkers for the mice treated with PBS, LPS, R848/GalN, and CpG/GalN. The serum alanine aminotransferase (ALT) level showed similar increases in the R848/GalN and CpG/GalN models (Fig. 1A). For most biomarkers other than ALT, the CpG/GalN model did not show notable increases except for CK, and showed lower values relative to the R848/GalN model (Fig. 1B-G). Thus, at the doses causing a similar level of liver injury, R848/GalN induced systemic responses, whereas CpG/GalN exerted more liver-specific effects. LPS mice showed a pattern of multiple organ injury with modest liver injury.

Blocking analysis supports protective roles of neutrophils

For further comparison, we conducted Ab-blocking/depletion analyses focusing on neutrophils and platelets. The depletion of Gr-1$^+$ cells exacerbated liver injury compared to the pretreatment with the isotype-matched control in the R848/GalN model (Fig. 2B). Consistent with this, IL-6 levels were higher in the Gr-1$^+$ cell-depleted mice compared to those in the isotype-matched control (Fig. 2E). Following the IL-6 rise, IL-10 increased in Gr-1$^+$-depleted R848/GalN mice (7 h of Fig. 2F). Thus, the depletion of Gr-1$^+$ cells caused aggravation of inflammation and liver injury in the R848/GalN model. Interestingly, previous studies have demonstrated the protective/anti-inflammatory effect of Gr-1$^+$ cells (or neutrophils) (Steinshamn *et al.* 1995. Omert *et al.* 1998. Daley *et al.* 2005.).

Non-specific tolerance induction by antibody injection: proneness of TLR9 pathway to tolerance induction

Depletion of Gr-1$^+$ cells led to a modest aggravation of liver injury in the CpG/GalN model (Fig. 2B) and a marked decrease in IL-6 level (Fig. 2E), lowering the IL-6/IL-10 ratio (to <1.0 at 7 h), suggestive of the tolerance (anti-inflammatory) state at 7 h (Fig. 2E, F). Notably, this tolerance induction was observed even with the control Ab, suggesting a non-specific anti-inflammatory effect of Ab injection. From our experience, IL-6 levels typically reached ~5 ng/ml at 7 h in this CpG/GalN model without pretreatment, but it remained <0.5 ng/ml in the Ab-pretreated CpG/GalN mice (either anti-Gr-1$^+$ or isotype-matched control) (Fig. 2E, F). This non-specific tolerance-inducing effect of Ab was not clear in the R848/GalN model, but significant in the CpG/GalN model based on IL-6 levels at 1 and 7 h.

P-selectin is considered important for leukocyte recruitment and hepatocellular injury in endotoxemic anti-P-selectin Ab tended to aggravate liver injury relative to the isotype-matched control for both models

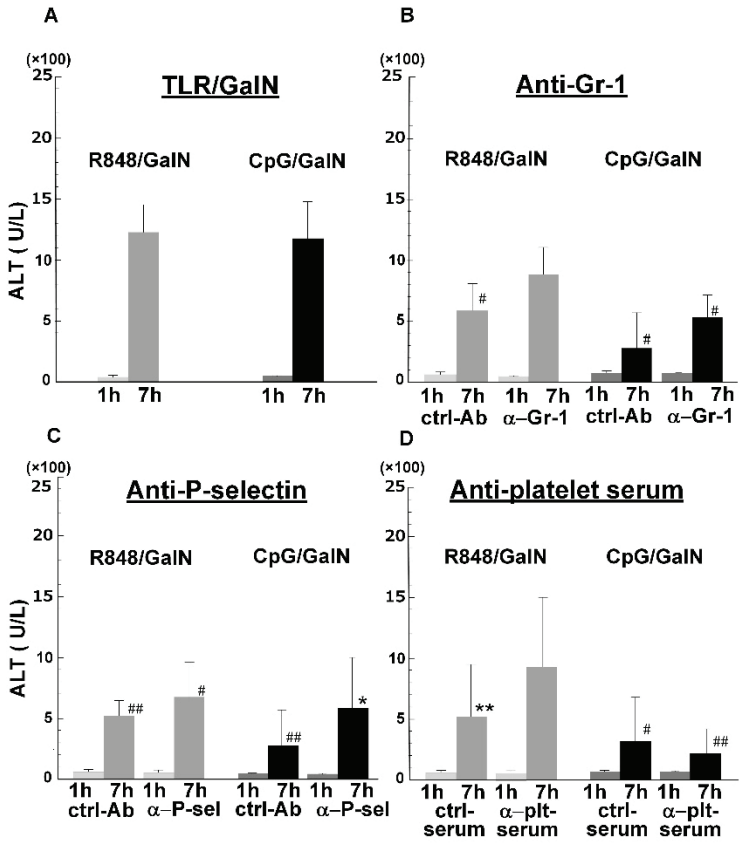
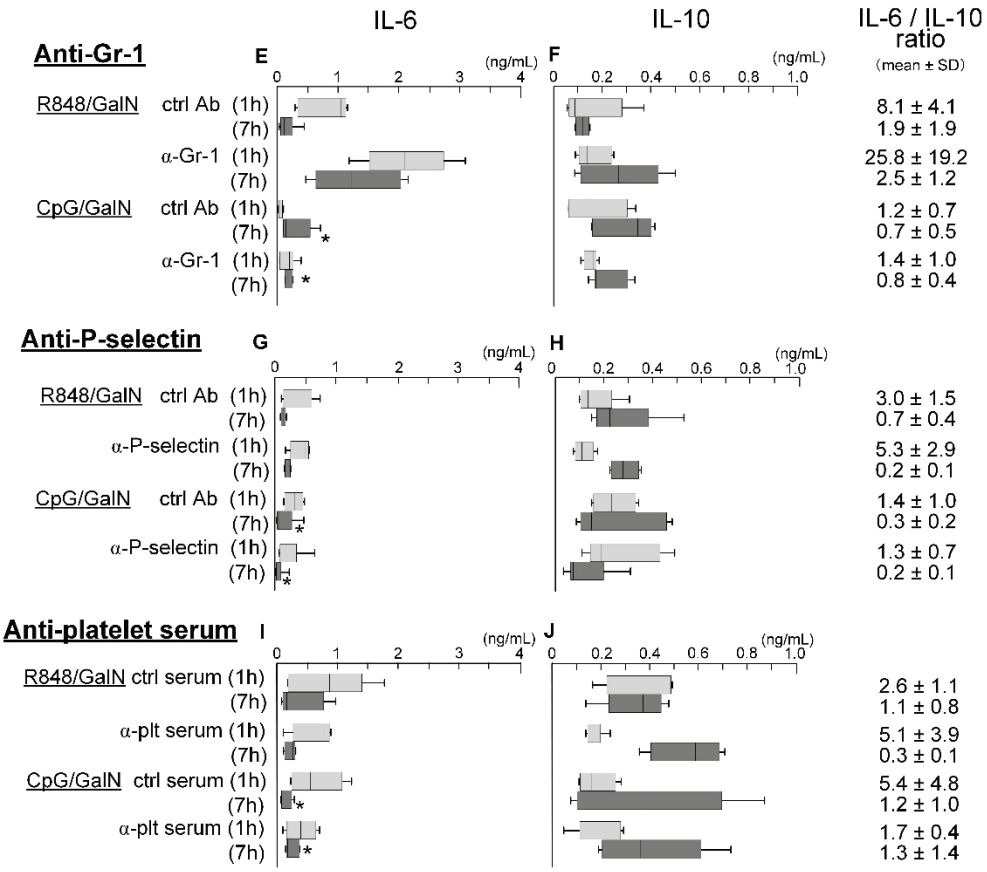


Fig. 2. Blocking/depletion analyses. The serum levels of ALT (**A–D**), IL-6 (**E, G, I**), and IL-10 (**F, H, J**) in R848/GalN and CpG/GalN mice pretreated with the indicated Abs are shown. For (A-D), the mean ± SD is shown. For (E–J), range (box) with median (line) ± SD (error bar) is shown. For all analyses, differences between control Ab (or serum) and specific Ab (or serum) were insignificant. Unpretreated (A) and Ab-pretreated groups (B-D) showed differences in ALT (*p<0.05; **p<0.01; #p<0.005, ##p<0.001). For CpG/GalN, all IL-6 data at 7 h (E, G, I) were lower than those from unpretreated mice, but none of IL-10 data showed such a difference (F, H, J). No R848/GalN analysis showed such a difference.



(Fig. 2C), suggesting a protective role of P-selectin. Cytokine analysis showed no clear effects of anti-P-selectin Ab; however, both control and anti-P-selectin Ab caused low IL-6/IL-10 ratio, suggesting tolerance-inducing effects (Fig. 2G, H). This effect was more pronounced for the CpG/GalN model than for the R848/GalN model. Interestingly, without Ab pretreatment, the IL-6/IL-10 ratio exceeded 10 (typically, IL-6 levels reached ~5 ng/ml and IL-10 ~0.3 ng/ml) at 7 h in the CpG/GalN model (Seki and unpublished data), but with the pretreatment, the ratio remained <1 (Fig. 2G, H). When the effect of platelet depletion was examined, its effect on liver injury was not consistent between the models (Fig. 2D). However, for both models, the tolerance-inducing effect of both the anti-platelet and control serum was observed (Fig. 2A, D, I, J). Once again, the tolerance-inducing effect based on the IL-6/IL-10 ratio was more pronounced in the CpG/GalN model (Fig. 2I, J). The mechanism underlying this effect of "rabbit" serum is presently unknown; however, as shown in murine model analyses of intravenous immunoglobulin therapy, if the recognition of sialic acid on Fc by SIGN-R1 plays a key role (Tjon *et al.* 2015), this may explain this cross-species phenomenon. Overall, the Ab/serum pretreatment generally induced tolerance based on the low IL-6/IL-10 ratio, and this effect was more pronounced in the CpG/GalN model. In the R848/GalN model, suppression by Ab/serum of IL-6 and IL-10 was modest, with the IL-6 levels being comparable between the mice with and without pretreatment (data not shown). However, further characterization of the tolerance induction by the control Ab/serum in the future is necessary.

Discussion

Difference in systemic toxicity between the TLR7/8 and TLR9 agonists

The R848/GalN and CpG/GalN models showed similar levels of increase in serum levels of TNF-α, an early inflammatory mediator (Seki 2017). Without Ab pretreatment, the CpG/GalN model induced higher serum IL-6 levels (~5 ng/ml at 7 h) relative to the R848/GalN model (~0.5 ng/ml) (Seki 2017). Why did the CpG/GalN model exhibit modest systemic inflammation (Fig. 1) and proneness to tolerance induction relative to the R848/GalN model (Fig. 2)? One possibility is that TLR9 signaling has a more significant impact on the liver relative to TLR7/8. Notably, TLR9 has been implicated in several liver diseases (Arrese *et al.* 2016, Imaeda *et al.* 2009, Bakker *et al.* 2015), whereas, to our knowledge, corresponding studies on TLR7/8 are limited. Another possibility is the limited systemic effect of the TLR9 agonist due to the protective functions of TLR9 in non-immune cells. TLR9 has been shown to contribute to cellular protection by reducing energy substrates and activating AMP-activated protein kinase (AMPK), subsequently inducing stress tolerance (Shintani *et al.* 2014). The TLR9/AMPK pathway can promote autophagy, which is crucial for cell protection (Ye *et al.* 2018). Possibly, efficient mechanisms responsible for tolerance toward TLR9 agonists have evolved to adjust TLR9 response to endogenous ligands, including mitochondrial DNA. Of note, pretreatment with lipoteichoic acid induced tolerance in CpG/GalN better than that in the R848/GalN model (Seki 2017).

Conclusion

This study showed that the CpG/GalN insult induced more liver-focused injury compared to R848/GalN, which caused systemic and liver injuries, and also suggested that the tolerance induction by Ab/serum pretreatment was more pronounced for the CpG/GalN insult. Further analyses of the protective effects of TLR9 on various cells and induction of tolerance toward TLR9 signaling are warranted.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviation

GalN, D-galactosamine; TLR, toll-like receptor; CpG, cytosine-guanosine dinucleotide; ODN, oligodeoxy-nucleotides; CK, creatine kinase; Ab, antibody

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