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.

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

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, depite the use of the doses inducing similar levels of liver injury (Seki 2017). In this study we further compared these models.

Methods

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 rabbitanti-thrombocyte serum (CLA31440, Cedarlane) or the control serum 24 h before TLRL/GalN. We confirmed that these

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 (Figure 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 (Figure 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 (Figure 2B). Consistent with this, IL-6 levels were higher in the Gr-1$^+$ cell-depleted mice compared to those in the isotype-matched control (Figure 2E). Following the IL-6 rise, IL-10 increased in Gr-1$^+$-depleted R848/GalN mice (7 h of Figure 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 (Figure 2B) and a marked decrease in IL-6 level (Figure 2E), lowering the IL-6/IL-10 ratio (to <1.0 at 7 h), suggestive of the tolerance (anti-inflammatory) state at 7 h (Figure 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) (Figure 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 mice (Klintman *et al*. 2004). However, the blocking with anti-P-selectin Ab tended to aggravate liver injury relative to the isotype-matched control for both models (Figure 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 (Figure 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 (Figure 2G,H).

When the effect of platelet depletion was examined, its effect on liver injury was not consistent between the models (Figure 2D). However, for both models, the tolerance-inducing effect of both the anti-platelet and control serum was observed (Figure 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 (Figure 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.

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

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 (Figure 1) and proneness to tolerance induction relative to the R848/GalN model (Figure 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.

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Legends for Figures

Figure 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 m
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Figure 2 Blocking/depletion analyses. The serum levels of ALT (A–D), IL-6 (E,G,I),
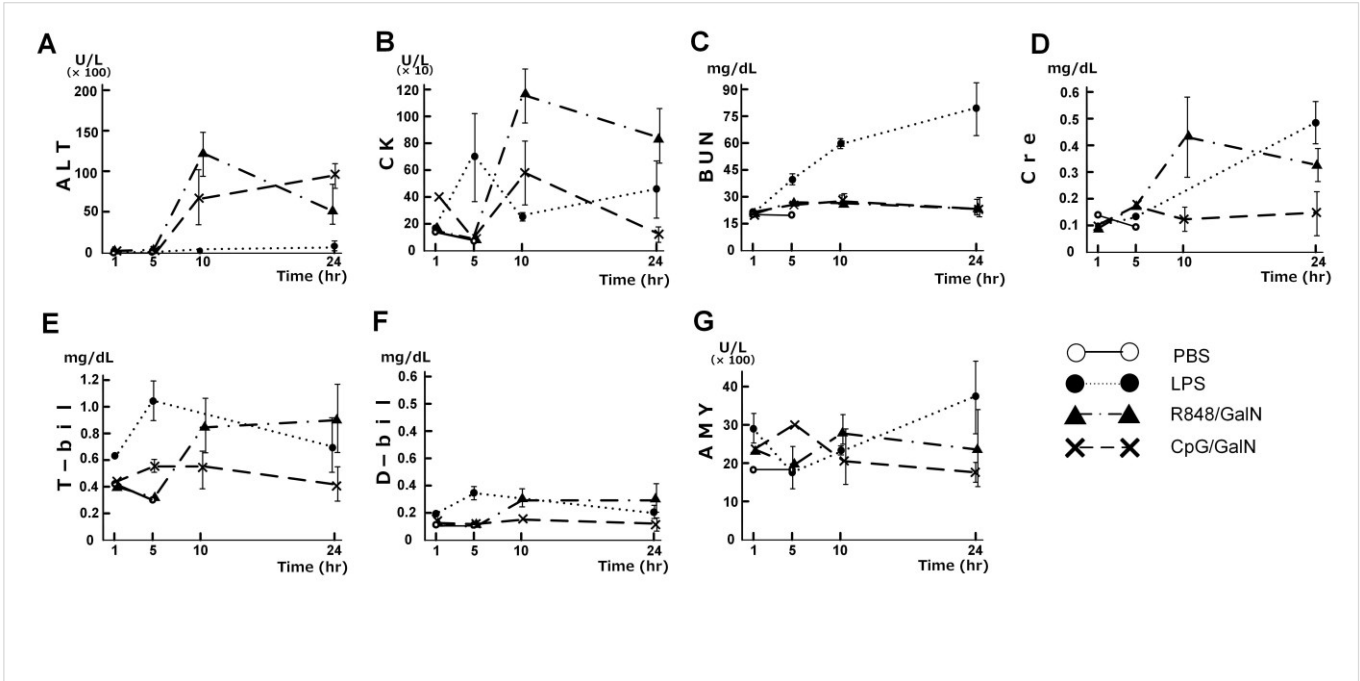a
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Figure 1

Figure 2