Published in final edited form as:

Gut. 2017 April ; 66(4): 565–566. doi:10.1136/gutjnl-2016-312966.

A Small RNA in Neutrophils Protects Against Acute-on-chronic Alcoholic Liver Injury

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Keywords

miRNA; neutrophil; alcohol; liver injury; inflammation

Alcoholic liver disease (ALD) is a major cause of chronic liver injury worldwide.^{1 2} Clinically, jaundice and liver failure are hallmark presentations of patients with severe alcoholic hepatitis (AH) who typically have been drinking up to the time of admission or have stopped drinking within 4-6 weeks of their presentation of symptoms. The liver histology of AH has features of steatohepatitis, hepatic neutrophil infiltration, cholestasis and florid inflammation³. Among ALD patients, liver-related mortality is much higher in patients with AH than those with only simple steatosis.³ Current recommended treatments for AH include corticosteroids or pentoxifylline and nutritional supplements, but they only have moderate therapeutic effects.³

A major barrier preventing drug development against AH is our lack of understanding of the regulatory pathways involved in the pathogenesis of AH despite decades of research on ALD. One reason for this knowledge gap is a lack of proper animal models that reflect the exact pathogenesis of human ALD. The commonly used rodent models, including mice or rats administered with acute oral gavage, ad libitum oral alcohol in drinking water or chronic Lieber-DeCarli diet ethanol feeding, only show mild elevation of serum alanine aminotransferase (ALT) and increased hepatic levels of triglyceride (TG). These models only mimic early pathogenic changes in human ALD, and neutrophil infiltration in the liver is not evident. Dr. Bin Gao's group recently developed a mouse model in which mice were fed with the Lieber-DeCarli ethanol diet for 10 days followed by an acute gavage of alcohol (hereafter referred to as Gao-binge model).⁴ Mice administered with Gao-binge have elevated serum ALT levels, increased hepatic TG, and more importantly, increased circulating and hepatic infiltrated neutrophils, making it possible to determine the mechanisms and role of neutrophils in AH. Indeed, using this Gao-binge model, Dr. Gao's group demonstrated that infiltrated neutrophils promote progression of ALD by inducing

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Competing interests None declared.

hepatocyte death in mice.⁵ However, the mechanisms by which alcohol increases neutrophil infiltration and induces liver injury and their relevance to human AH are largely unknown.

One of the major hurdles in the study of early stage of ALD is lack of liver biopsy samples due to ethical reasons. In this issue of *Gut*, Li *et al.*⁶ opens a new direction to study the early stage of ALD by analyzing circulating neutrophils in a large human alcoholic cohort. Because binge drinking has been increasingly suggested/proven to play a critical role in promoting AH in humans and enhancing neutrophil infiltration and liver injury in mice administered Gao-binge alcohol, authors divided the alcoholics into two groups: alcoholics without recent drinking (no drinking within 10 days before enrollment) and alcoholics with recent drinking (excessive drinking within 10 days before enrollment). Importantly, the authors found a positive correlation of the level of circulating neutrophils with serum ALT and AST only in the alcoholics with recent drinking but not in alcoholics without recent drinking or healthy controls, underscoring a critical role of binge drinking in potentiating alcohol-induced neutrophil infiltration and an important role of neutrophils in liver injury. Moreover, this study may lead to a breakthrough on the prediction of the susceptibility of AH, which is known extremely variable among alcoholics, by studying blood neutrophils. Increasing evidence implicates miRNAs in the pathogenesis of ALD.^{7,8} Using an elegantly designed serum microRNA (miRNA) array profiling study from a large human alcoholic cohort, Li *et al.*⁶ also provide a new insight into the regulatory pathways of neutrophil infiltration in ALD. They provide evidence of experimental translation and therapeutic manipulation of miR-223 identified from the human studies using the Gao-binge mouse model. Authors found that the levels of serum miR-223 significantly increased in alcoholics compared to healthy controls. The expression of miR-223 also increased in peripheral blood and hepatic neutrophils in Gao-binge alcohol-treated mice. MiR-223 is the most abundant miRNA in neutrophils, and it negatively regulates Mef2c, a transcription factor promoting myeloid progenitor proliferation.⁹ Subsequently, the authors found that miR-223^{-/-} mice had increased levels of circulating neutrophils and exacerbated liver injury compared to their matched wild type (WT) control mice after Gao-binge alcohol. It remains to be determined whether Mef2c would contribute to the elevated levels of circulating neutrophils in mice and humans after alcohol consumption. Nevertheless, findings from this study indicate that increased miR-223 in neutrophils may serve as an adaptive protective mechanism against alcohol-induced liver injury. Indeed, authors found that Gao-binge alcohol-induced liver injury was ameliorated by overexpressing miR-223 using a lentivirus vector. Neutrophils have abundant nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which produces reactive oxygen species (ROS) to help kill bacteria as an innate immune response. However, excessive levels of ROS produced from hepatic infiltrated neutrophils can also induce death of hepatocytes. Authors found that miR-223^{-/-} mice had increased expression of p47^{phox} in neutrophils a key component of NADPH oxidase, resulting in increased ROS production and hepatocyte injury. Surprisingly, the authors did not find a direct binding of miR-223 with the p47^{phox} gene. Instead, they found that miR-223 directly bound with the 3' UTR region of the IL-6 gene to repress IL-6 expression. Direct incubation of isolated circulating neutrophils with IL-6 markedly increased expression of the p47^{phox} gene, suggesting that miR-223 inhibits expression of the p47^{phox} gene by indirectly downregulating IL-6. Taken together, this study uncovers a novel miR-223-IL-6-p47^{phox}

NADPH-ROS signaling pathway in neutrophils that contributes to the pathogenesis of AH (Figure 1).

In addition to the key findings on neutrophil miR-223 in ALD, several other findings in this study also add important perspectives to our understanding of the pathogenesis of ALD. For instance, it has been generally thought that females are more susceptible to ALD than males,¹⁰ although there are no comprehensive studies directly comparing female and male alcoholics in the early stages of ALD. Authors found that there was no difference in the number of neutrophil counts, but the serum levels of ALT and AST were higher in male alcoholics than female alcoholics. Interestingly, female alcoholics had a higher number of drinks than males, although it did not reach statistical difference. While these results should be interpreted with caution due to a smaller sample size of female alcoholics, it argues against the notion that females are more susceptible than males to ALD. In addition to neutrophils, macrophages and the liver resident Kupffer cells are also important players of innate immunity for defense against infection and tissue injury. Both detrimental and beneficial roles of macrophages have been reported in ALD, which seem to depend on the stage of ALD.¹¹ In the early phase of ALD, macrophages may contribute to alcohol-induced liver injury since removal of macrophages prevents experimental ALD.¹² In the late stage of ALD and in particular decompensated AH, hepatic macrophages may have impaired phagocytic or pinocytic activity. In addition, hepatic macrophages may also promote liver repair in AH by stimulating liver regeneration.¹³ Therefore, increased hepatic macrophages in AH or late stage ALD may be beneficial to control inflammation and stimulate liver regeneration in these patients.¹³ In the study by Li *et al.*, the authors found that the number of hepatic F4/80 positive macrophages markedly decreased in both WT and miR-223^{-/-} mice after Gao-binge compared with pair-fed mice. These results further support that neutrophils, but not macrophages, may play a more important role in the pathogenesis of ALD in this chronic-plus-binge ethanol feeding model. Another additional notable interesting finding of this study was that miR-223^{-/-} mice had lower hepatic steatosis despite their increased liver injury compared to WT mice. These results suggest that steatosis may not necessarily always be associated with liver injury during ALD.

Perhaps one intriguing paradoxical finding in this study was that the serum miR-223 levels increased, whereas miR-223 levels in neutrophils decreased in human alcoholics. These findings were also in great contrast with the findings from Gao-binge-treated mice, in which the levels of miR-223 increased in both serum and neutrophils. The exact mechanisms behind these differences remain unknown but it could be due to different exposure time to alcohol. Increased neutrophil miR-223 in mice after Gao-binge may reflect an adaptive response, whereas human alcoholics might have impaired adaptive response due to prolonged exposure to alcohol. While Gao-binge alcohol increased liver injury in miR-223^{-/-} mice, either chronic ethanol feeding or acute ethanol gavage alone failed to exacerbate liver injury in miR-223^{-/-} mice. One of the fundamental differences among these experimental conditions is that increased neutrophil infiltration was observed only in Gao-binge model but not in either chronic ethanol feeding or acute ethanol gavage alone models. These findings suggest that the levels of hepatic neutrophils may be a critical factor for determining the outcome of potential therapeutic implications of miR-223 for treating ALD.

Therefore, activation of miR-223 may only be beneficial to patients with increased hepatic neutrophils, such as AH patients.

In conclusion, the current studies highlight the important role of miR-223-mediated NADPH oxidase activation and ROS production in neutrophils, which may promote the progress of early ALD to AH. These studies expand our knowledge to further understand the role of neutrophils in the pathogenesis of ALD in addition to the liver parenchymal cells. Studying the changes of blood neutrophils such as miR-223 may be a better marker to predict the susceptibility of AH for the early stage of ALD patients, of which liver biopsy is not feasible.

Acknowledgments

Grant support: R01 AA020518, R01 DK102142 and 8 P20 GM103549-07.

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Abbreviations

AH	alcoholic hepatitis
ALD	Alcoholic Liver Disease

ALT	alanine aminotransferase
AST	aspartate aminotransferase
G-CSF	granulocyte-stimulating factor
4HNE	4-hydroxynonenal
MDA	malondialdehyde
miRNAs	microRNAs
NADPH	nicotinamide adenine dinucleotide phosphate
ROS	reactive oxygen species
TG	triglyceride

Figure 1. Signaling events mediated by neutrophil miR-223 in protecting against ALD

Chronic alcohol + acute binge drinking (Gao-binge) increases circulating and hepatic infiltrated neutrophils likely via increased release of neutrophils from bone marrow. Gao-binge increases expression of miR-223 in neutrophils, which represses IL-6 expression and acts as an adaptive protective mechanism in response to Gao-binge in mice. In the absence or reduction of miR-223 such as in miR-223 knockout mice or human alcoholics with recent binge drinking, IL-6 expression is markedly upregulated in neutrophils. IL-6 further increases expression of p47^{phox}, a key component of neutrophil NADPH oxidase, which leads to elevated ROS production in neutrophils. ROS induce hepatic lipid peroxidation (MDA and 4HNE) resulting in hepatocyte death and subsequent liver inflammation, which are key features of AH.