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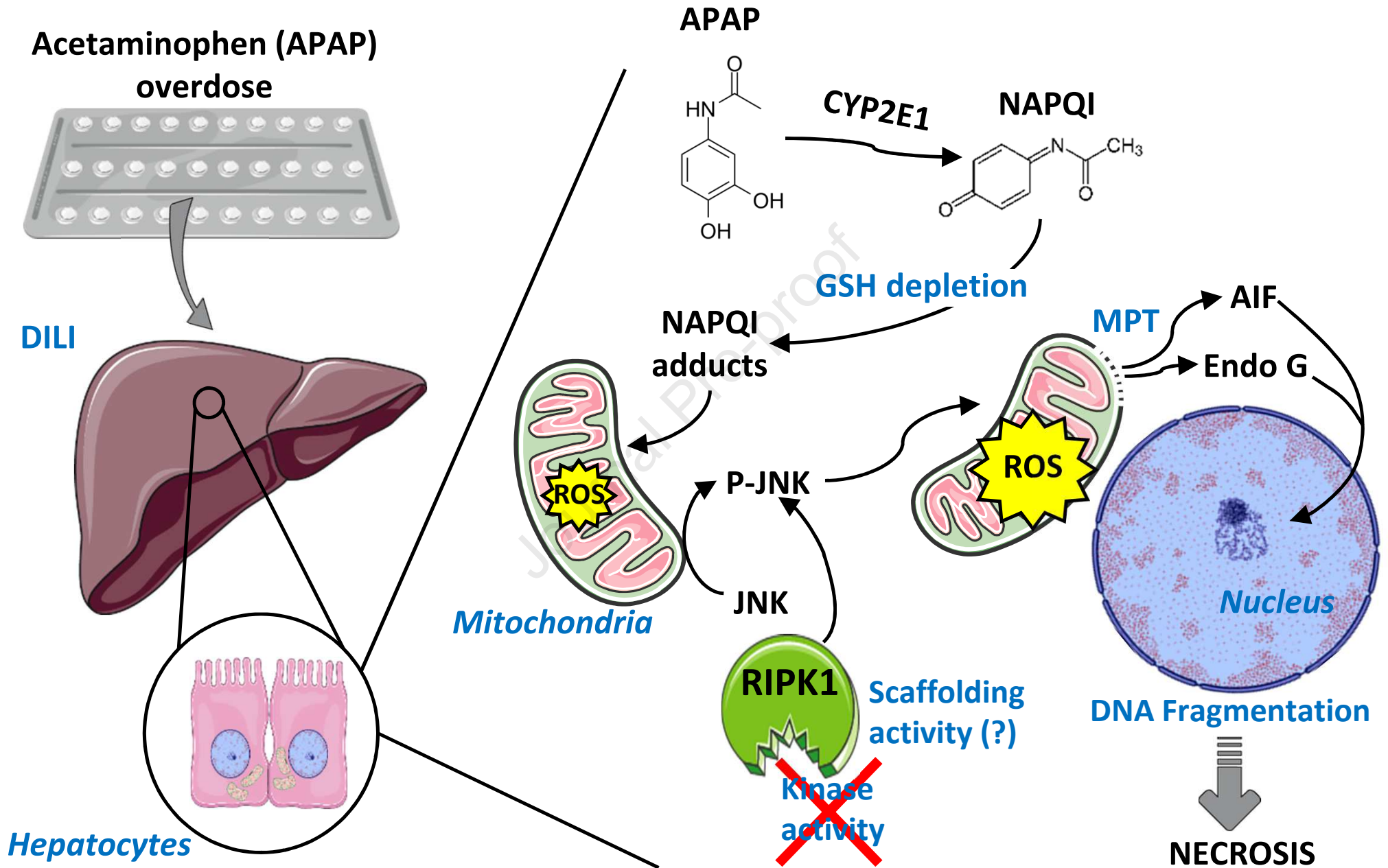
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Questioning the RIPK1 kinase activity involvement in acetaminophen-induced hepatotoxicity in mouse.

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To the Editor:

In a recent previous issue, Yi and its collaborators tackled a research subject related to a worldwide public health problem, the hepatotoxicity of a widely used antipyretic and analgesic drug which is listed under the category of over-the-counter medicines: acetaminophen (paracetamol or N-acetyl-p-aminophenol; APAP) [1]. Based on complementary genetic and pharmaceutical experimental approaches, authors concluded that inactivation of the kinase activity of the receptor interacting protein kinase 1 (RIPK1) would be protective against liver injury induced by APAP in mouse. However, by performing similar experiments, we did not find any therapeutic interest in targeting the kinase activity of RIPK1, questioning the real implication of RIPK1 kinase activity in APAP hepatotoxicity in mouse.

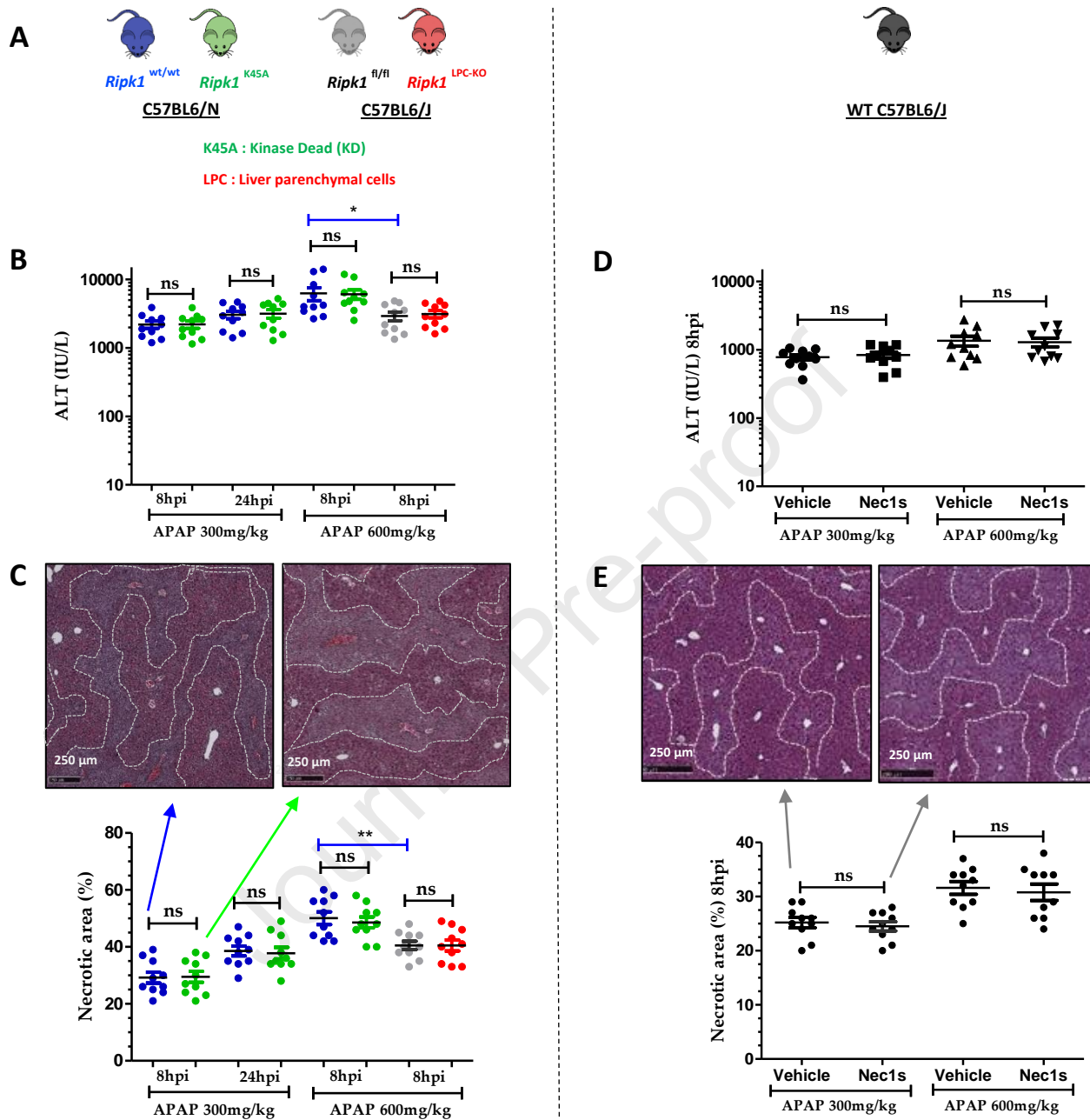
Thus, two different mouse lines, either deficient for the kinase activity of RIPK1 (*Ripk1*^{K45A}) or for *Ripk1* specifically in liver parenchymal cells (*Ripk1*^{LPC-KO}) plus their respective WT littermates (*Ripk1*^{wt/wt} and *Ripk1*^{fl/fl}) were challenged with toxic doses of APAP (Fig. 1A). To have appropriate littermates, the *Ripk1*^{K45A} mice were obtained from heterozygous breeding. The *Ripk1*^{LPC-KO} colony has a *Ripk1*^{fl/fl} background with half of the population carrying the *Cre* recombinase transgene under the control of the mouse albumin regulatory elements and α -fetoprotein enhancers (*Alfp-Cre* transgene). In accordance with other published data [2,3] genetic inactivation of its kinase activity (*Ripk1*^{K45A}) or *Ripk1* depletion specifically in liver parenchymal cells (*Ripk1*^{LPC-KO}) never affected APAP-induced hepatotoxicity. These experiments were performed as studied in the literature at sub-lethal and lethal doses of APAP (300 and 600 mg/kg, respectively) [2,4]. Liver damage remained similar between genetically modified mice and their respective littermate controls as shown by the measurement of serum transaminases (Fig. 1B) and of the necrotic liver tissue (Fig. 1C). Unlike the previous study in which *Ripk1*^{K45A} mice had a C57BL/6J genetic background, those used in our study were C57BL/6N mice. Data from the literature

clearly underlined the influence of the genetic background on the susceptibility of mouse strains to APAP-induced hepatitis. As previously described [5], symptoms were exacerbated in *Ripk1*^{wt/wt} (N background) compared to their congeners *Ripk1*^{fl/fl} mice (J background) (Fig. 1B and C). Besides the genetic background, and most importantly, other genetic or epigenetic variations existing between different colonies of mice can influence a phenotype [6,7]. Appropriate littermate controls, also born in the same colony under similar breeding conditions, should be used whenever possible to minimize the risk of compromising experimental results. Otherwise, in addition to the kinase activity of RIPK1, its scaffolding properties did not appear to contribute to APAP-induced liver injury in our investigations. However, previous works reported that knockdown of *Ripk1* through antisense oligonucleotide treatment and adult hepatocyte-specific *Ripk1* conditional knockout (*Ripk1*^{HepCKO}) partly reduced liver damage, conversely arguing for a potential harmful role of RIPK1 scaffolding properties in APAP-induced liver damage [2,4]. Dara's team attempted to propose a hypothesis to explain these conflicting results. Knowing the importance of *Ripk1* during mouse embryogenesis [8], interfering compensatory phenomena would be less likely to occur in knockout models induced later in development (*Ripk1*^{HepCKO} versus *Ripk1*^{LPC-KO}) [2]. Note, nonetheless, that this *Ripk1*^{LPC-KO} mice exhibit an aggravated acute hepatitis phenotype when TNF or FasL are involved [9-12].

In addition to the genetic approach, we also tested the impact of chemical inhibition of the RIPK1 kinase activity using a generation of more specific inhibitor with better *in-vivo* pharmacokinetic properties, Nec-1s. As mentioned by Yi and colleagues [1], DMSO is known to interfere with the metabolism of APAP on its own partly improving liver injury. Therefore, our investigations with Nec-1s included the essential control of the vehicle alone, which only included 6% of DMSO. The inhibitory activity of the batch of Nec-1s used in our investigations was also verified in parallel in an *in vitro* assay (data not shown). Thus, WT C57Bl6/J mice received vehicle alone or with Nec-1s prior to APAP injection. The severity of the hepatitis induced in the two groups of mice was similar, as evidenced by the evaluation of serum transaminase levels and of percentages of necrotic liver tissue (Fig. 1D and E). The inhibitory effect observed in Yi's works most probably resulted from the known off-target effect of Nec-1 which inhibits the indoleamine-2,3-dioxygenase (IDO) that restricts inflammation, a central process in APAP-induced hepatitis [13].

Therefore, the mitochondrial permeability transition (MPT)-driven necrosis, the cell death pathway that has been established to occur in APAP-overdose [14], did not appear to be influenced by the kinase activity of RIPK1.

Figure 1



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Legend

Figure 1: RIPK1 scaffolding and kinase properties without influence on APAP-induced hepatotoxicity in mouse. Injected APAP doses were either 300 or 600 mg/ kg. When used, Nec-1s or vehicle (PBS-DMSO 6%) was intravenously injected at 6.25 mg/kg 1 hour before APAP treatment. Analysis were conducted 8 or 24 hours post APAP intraperitoneal injection (hpi) as indicated on graphs. Each animal groups consisted of 10 individuals with an equal number of males and females, all aged 8-10 weeks. (A) *Ripk1*^{K45A} (green) and *Ripk1*^{LPC-KO} (red) mice with their respective littermate controls (*Ripk1*^{wt/wt}, blue and *Ripk1*^{fl/fl}, grey), as well as WT C57Bl6/J mice (black), were included in experiments of APAP-induced hepatotoxicity. Genetic backgrounds of each colony are indicated. (B and D) Levels of serum alanine aminotransferase (ALT). (C and E) Quantification of necrotic area with 2 representative pictures of liver tissue sections stained by H&E. For all graphs, each dot, whose colour is defined according to its genotype (A), represents an individual. Errors bars are expressed as means \pm SEM. Mean differences between experimental groups were assessed using the non-parametric Mann–Whitney U-test. Significance is shown as follows: *P<0.05, **P<0.01; ns: non-significant.

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