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Comment on "A Role for Immature Myeloid Cells in Immune Senescence"

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Letters to the Editor

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Comment on "A Role for Immature Myeloid Cells in Immune Senescence"

We recently read an article published in *The Journal of Immunology* by Enioutina et al. (1) and found an experimental procedure we believe should be discussed. In this article, as well as in many others by the same authors—also in *The Journal of Immunology* and other renowned journals (2, 3)—serum-specific Abs are quantified by ELISA. According to our knowledge, this could be a misconception, given that we believe ELISA cannot be used for that purpose.

Polyclonal antisera raised against the same Ag could differ in both the affinity and the concentration of the specific Abs. The main premise of quantification is that sample and reference standards must share all biological and physicochemical properties because the analyte concentration is the only unknown factor. The authors have not declared what they have used as the "reference standard," not in the article that gives rise to this letter, nor in the others published before. Considering that the samples are polyclonal antisera, we believe there is no way to construct such a standard curve that allows expressing the results as a concentration of specific Igs. Therefore, we think that the only reliable information that can be obtained from this sort of assay is a "relative potency" value, commonly expressed as titer of specific Abs.

We certainly think that this issue does not affect the quality of the work by Enioutina et al., but we do think that the quantitative aspects deserve further discussion.

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Response to Comment on "A Role for Immature Myeloid Cells in Immune Senescence"

Numerous investigators have employed an ELISA to evaluate differences in the levels of Ag-binding Abs present in the serum of immunized humans or animals (1–3). However, we agree with the concern raised by Drs. Ferrari, Cela, and Maglio that this type of assay might underestimate the actual quantity of specific Abs present in serum samples, through a possible loss of low affinity Abs during the exhaustive washing procedures needed to eliminate nonspecific background. For our evaluation of Ag-specific IgG Abs in serum, a reference curve was created with defined concentrations of purified murine IgG plated onto wells coated with affinity-purified goat anti-mouse IgG (H+L chain–specific) Abs. This was followed by treatment with secondary HRP-labeled goat anti-mouse IgG (γ-chain–specific) Abs. Serial dilutions of test serum samples collected from young and old OVA-immunized animals were then plated onto OVA-coated wells followed by treatment with HRP-labeled goat anti-mouse IgG (γ-chain–specific) Abs. The reference curve was then used to report Ab quantity. This protocol is always validated for specificity using serum samples collected from naive/unimmunized mice (negative control) and with hyperimmune serum collected from mice repeatedly immunized with OVA (positive control). Use of this ELISA protocol to quantify Ag-specific Abs offers the additional advantage, with minor modification, of being able to detect differences in relative potency of various Ab classes and subclasses present in serum or mucosal secretions of immunized animals, thereby providing a means to evaluate influences of distinct adjuvants or therapeutic treatments, or immune modulators on the nature of elicited immune responses. We thank Drs. Ferrari, Cela, and Maglio for their concern; however, reporting "quantity" or "relative potency" does not alter the differences being observed in specific Ab levels between experimental groups.

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Comment on "Cyclophilin A Is a Damage-Associated Molecular Pattern Molecule That Mediates Acetaminophen-Induced Liver Injury"

After reading the article by Dear et al. (1) in the September 15 issue of *The Journal of Immunology*, we have several serious concerns. First, there was extreme variation of injury in both wild-type and cyclophilin A-deficient mice with >50% of animals having no injury. We assume that this is caused by the use of both male and female mice, which substantially differ in their susceptibility to acetaminophen. As the composition of the individual groups is not disclosed, it is difficult to interpret these data. The authors also conclude that there was no difference in metabolic activation based on similar glutathione levels at 6 h. However, this time point is too late to justify this conclusion (2). Furthermore, acetaminophen caused dramatically less injury in IgG-treated mice compared to wild-type animals. As a pretreatment regimen with immunological agents was used, this may have caused impaired metabolic activation or enhanced resistance to cell injury (3).

The second concern is the role of damage-associated molecular patterns (DAMPs) in the pathophysiology. DAMPs act on immune cells to generate cytokines, which activate neutrophils during the first 24 h after acetaminophen overdose (3). Thus, if cyclophilin A mediates acetaminophen-induced injury, this mediation would depend on a neutrophil-induced injury. However, neutrophils are not activated after acetaminophen overdose (4) and a large number of diverse intervention strategies against neutrophils did not result in any effect on liver injury (4–7). The data of the few studies that claim involvement of neutrophils or inflammatory mediators such as IL-1β can be explained by off-target effects of the immunological agents (8) or are simply not reproducible (9, 10). Acetaminophen-induced liver injury is determined by intracellular signaling events in hepatocytes (11); any involvement of DAMPs released by necrotic cells has to modulate these pathways in hepatocytes, not neutrophils, to have an impact on the overall injury (3).

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Response to Comment on "Cyclophilin A Is a Damage-Associated Molecular Pattern Molecule That Mediates Acetaminophen-Induced Liver Injury"

Dr. Jaeschke and Dr. Williams are experts in the field of acetaminophen hepatotoxicity and we are grateful for the opportunity to respond to their comments regarding our recent article (1). However, we feel they have no grounds for serious concern.

We agree that there was variability in the serum alanine aminotransferase activity after acetaminophen injection and we presented the data for individual mice to allow the reader to appreciate this variability. This highlights the importance of performing a prestudy power calculation based on relevant pilot studies, and our power calculations are included in the article's *Materials and Methods* section. We included males and females in our experiments, and while individual mice of both sexes developed liver injury, we agree that this may have contributed to the variability in alanine aminotransferase activity. However, all our study groups are age- and sex-matched, and, therefore we do not believe treatment effects can be explained by sex differences. We measured liver glutathione concentration at 6 h after acetaminophen injection as in previous published studies (2), so our data do not allow us to comment on whether the wild-type and knockout mice differed at earlier time points. Drs. Jaeschke and Williams are correct in stating that our control Ab may have reduced liver injury, although this was not formally tested by direct comparison with vehicle pretreatment. This possible reduction

in injury was the reason that an appropriate control Ab was essential when we studied the effect of the anti-CD147 Ab. Our data demonstrate a difference between anti-CD147 and control, supporting the role of this receptor in acetaminophen hepatotoxicity.

The role of the immune system in acetaminophen hepatotoxicity is unclear and controversial. This is mentioned in the *Discussion* section of our article (1): "However, the exact role of inflammation is still an area of controversy [e.g., there is conflicting evidence on the importance of neutrophil infiltration into the liver in acetaminophen poisoning (3, 4)]." We hope that continued study will clarify the situation in mice and, more importantly, produce new therapeutic targets in humans.

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