Loss of TLR4 Does Not Prevent Influenza A–induced Mortality

To the Editor:

Imai and colleagues reported that oxidized phospholipids generated during influenza A pneumonia induce Toll-like receptor 4 (TLR4)-dependent inflammation and contribute to the development of acute lung injury (1). In two subsequent papers, Vogel and colleagues showed that *Tlr4*$^{-/-}$ mice are resistant to influenza A–induced lung injury and mortality and that the administration of Eritoran, a potent TLR4 antagonist, blocked influenza A–induced lethality in wild-type mice (2, 3). In contrast, other investigators have reported that the genetic loss of TLR4 had either no effect or worsened influenza A–induced lung injury and mortality in mice (4, 5). These discrepancies may be important, as a clinical trial in which Eritoran was administered to patients with severe sepsis showed no benefit, even in a prespecified subgroup of patients in whom the lung was the primary site of infection (6).

We purchased *Tlr4*$^{-/-}$ mice from the Jackson Laboratory (Bar Harbor, ME). The TLR4-deficient strain commercially available from the Jackson Laboratory (C57BL/10ScN) is derived from a spontaneous mutation that was observed to confer hyporesponsiveness to LPS (7). To confirm LPS unresponsiveness, we injected wild-type and *Tlr4*$^{-/-}$ mice intraperitoneally with LPS (*Escherichia coli* O111:B4, 54 mg/kg; Sigma-Aldrich, St. Louis, MO) (8). We found that the *Tlr4*$^{-/-}$ mice were unresponsive to LPS whereas the wild-type mice underwent 66% mortality by Day 3 (data not shown). This shows that the strain has not regained an intact *TLR4* gene from the C57BL/6 background.

We then infected the *Tlr4*$^{-/-}$ and wild-type mice with a murine-adapted influenza A virus (A/WSN/33 [H1N1]) (WSN) (500 plaque-forming units per mouse). Weight loss and mortality were similar in *Tlr4*$^{-/-}$ mice and wild-type mice (Figures 1A and 1B). Our findings are similar to those of Abdul-Careem and colleagues, who administered another murine-adapted H1N1 influenza A virus (A/PR/8/34) (PR/8) to the same strain of mice, but contradict the results of Vogel and colleagues and call into question the conclusion that TLR4 signaling plays a role in the development of influenza A–induced lung injury (5).

We speculate that differences in murine knockout strains might explain these discordant results. Hashimoto and colleagues reported their first results with a TLR4 knockout strain created in 1999 (4, 9). The reported phenotype of these animals after influenza infection is variable, with two papers reporting that the loss of TLR4 confers resistance to influenza A infection (2, 3) and one reporting sensitization (4). In contrast, the TLR4-deficient strain commercially available from the Jackson Laboratory (C57BL/10ScN) is derived from a spontaneous mutation that was observed to confer hyporesponsiveness to LPS (7). The mutant mice lack 74,723 bp of genomic DNA encompassing the *Tlr4* gene without other identified mutations (7, 10, 11). Our results and those of Abdul-Careem and colleagues show that influenza A–induced mortality in these mice is similar to that of wild-type controls (5).

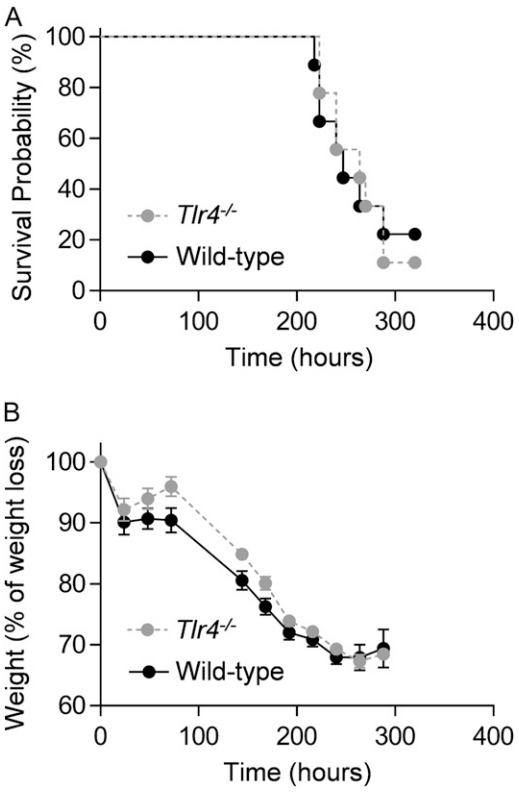

Figure 1. Influenza A infection reveals no significant difference in mortality between *Tlr4*$^{-/-}$ and wild-type mice. We treated wild-type and *Tlr4*$^{-/-}$ mice with influenza A virus (A/WSN/33 [H1N1]), using a dose predicted to kill approximately 90% of the mice (~LD_{90}) (500 plaque-forming units per mouse), and measured (*A*) mortality and (*B*) weight daily. n = 9 per group.

Furthermore, the differences between the studies are unlikely to be due to genetic variation in the influenza A virus. As circulating influenza A viruses show poor tropism for the murine lung epithelium, most laboratories use one of several murine-adapted strains. The mice generated by Hoshino and colleagues have been reported to be resistant to both the PR/8 and A/California/07/2009 strains; the C57BL/10ScN strain has been reported to show no resistance to infection with the PR/8 strain; and, in this letter, we report no protection against the WSN strain (2–5).

In summary, we independently confirmed the findings of Abdul-Careem and colleagues, using a different strain of influenza virus (5). The similarity of our findings using distinct influenza strains makes it unlikely that strain variations explain the differential sensitivity of these TLR4 knockout mice to influenza A pneumonia. Instead, we suggest that differences between TLR4 knockout strains might explain the reported resistance to influenza A pneumonia in some studies of TLR4 knockout mice. Collectively,

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our results and others suggest that more preclinical data are needed before TLR4 antagonists may be considered as therapy for patients with influenza A pneumonia, especially in light of negative clinical trials of Eritoran in patients with sepsis (6). It may be possible, however, to exploit these strain differences to identify genetic, epigenetic, or environmental factors that explain the dramatic difference in phenotype reported between these almost genetically identical mice. Such studies might identify biomarkers to identify individuals who would benefit from TLR4 antagonists. ■

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References

1. Imai Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, van Loo G, Ermolaeva M, Veldhuizen R, Leung YH, Wang H, *et al*. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* 2008;133:235–249.
2. Shirey KA, Lai W, Scott AJ, Lipsky M, Mistry P, Pletneva LM, Karp CL, McAlees J, Gioannini TL, Weiss J, *et al*. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. *Nature* 2013;497: 498–502.
3. Nhu QM, Shirey K, Teijaro JR, Farber DL, Netzel-Arnett S, Antalis TM, Fasano A, Vogel SN. Novel signaling interactions between proteinase-activated receptor 2 and Toll-like receptors *in vitro* and *in vivo*. *Mucosal Immunol* 2010;3:29–39.
4. Hashimoto Y, Moki T, Takizawa T, Shiratsuchi A, Nakanishi Y. Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice. *J Immunol* 2007;178: 2448–2457.
5. Abdul-Careem MF, Firoz Mian M, Gillgrass AE, Chenoweth MJ, Barra NG, Chan T, Al-Garawi AA, Chew MV, Yue G, van Roojen N, *et al*. FimH, a TLR4 ligand, induces innate antiviral responses in the lung leading to protection against lethal influenza infection in mice. *Antiviral Res* 2011;92:346–355.
6. Opal SM, Laterre PF, Francois B, LaRosa SP, Angus DC, Mira JP, Wittebole X, Dugernier T, Perrotin D, Tidswell M, *et al*.; ACCESS Study Group. Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial. *JAMA* 2013;309:1154–1162.
7. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, *et al*. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* 1998; 282:2085–2088.
8. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, *et al*. Non-canonical inflammasome activation targets caspase-11. *Nature* 2011;479:117–121.
9. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J Immunol* 1999;162:3749–3752.
10. Vogel SN, Hansen CT, Rosenstreich DL. Characterization of a congenitally LPS-resistant, athymic mouse strain. *J Immunol* 1979; 122:619–622.
11. Poltorak A, Smirnova I, Clisch R, Beutler B. Limits of a deletion spanning Tlr4 in C57BL/10ScCr mice. *J Endotoxin Res* 2000;6:51–56.

Eosinophilic Inflammation in Subjects with Mild-to-Moderate Asthma with and without Obesity: Disparity between Sputum and Biopsies

To the Editor:

In the September 15, 2013, issue of the *Journal*, Desai and colleagues (1) showed that, in severe asthma, sputum eosinophils do not differ between obese and nonobese patients, yet obese patients have higher sputum IL-5 levels and eosinophil numbers in the bronchial submucosa. These findings contrast with two widely held beliefs: first, that sputum cellular profiles reflect airway inflammation; and second, that the obese asthma phenotype, identified by the cluster analysis by Haldar and colleagues (2), is characterized by high symptom perception, but not eosinophilic inflammation.

The findings by Desai and colleagues (1) were obtained from two groups of subjects with asthma, one investigated with sputum and airway wall biopsies, and the other with sputum and blood differential cell counts. The investigation was restricted to severe asthma. We wondered whether these findings could be replicated in patients with mild-to-moderate asthma, in whom cell counts had been obtained from all three compartments (i.e., airway wall biopsies, sputum, and blood). To this end, we investigated a large cohort (3–5) of 147 patients with predominantly mild-to-moderate asthma, as indicated by 56% of the patients requiring treatment step 1, 10% step 2, 12% step 3, and 21% step 4, according to Global Initiative for Asthma guidelines (6). Patients in steps 2–4 were receiving a median dose of inhaled corticosteroids of 800 μg/d beclomethasone equivalent. No patient was being treated according to Global Initiative for Asthma step 5 (oral corticosteroids or omalizumab), resulting in exclusion of severe asthma. Obesity (body mass index [BMI] $>$ 30 kg/m^2) was present in 32 patients (28%). Patients had a mean prebronchodilator FEV_1 of 89% predicted (interquartile range = 79–102%), a median Asthma Control Questionnaire score of 0.57 (interquartile range = 0.29–1.3), and all had bronchial hyperresponsiveness to histamine or adenosine 5′-monophosphate.

We have previously reported differences in the relationship between clinical control and eosinophilia from biopsy compared with sputum (4). Now, we have investigated the difference between obese and nonobese patients in eosinophil counts from biopsies, sputum, and blood, and whether eosinophils in sputum, blood, and biopsies are correlated.

In our cohort, obese patients had significantly higher numbers of submucosal eosinophils and lower sputum eosinophil percentages than nonobese patients (Figures 1A and 1B; Table E1). Blood eosinophil numbers were comparable (Figure 1C). Using conventional criteria, sputum or blood eosinophilia was rarely found in the group of obese subjects, despite the higher number of submucosal eosinophils. In obese subjects with bronchial biopsy data, sputum eosinophilia (⩾3%) was present in 3 out of

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