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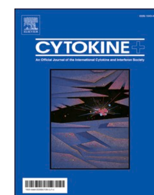

Short communication

# Myeloid cell tet methylcytosine dioxygenase 2 does not affect the host response during gram-negative bacterial pneumonia and sepsis

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ABSTRACT

Tet methylcytosine dioxygenase 2 (Tet2) is an important enzyme in the demethylation of DNA. Recent evidence has indicated a role for Tet2 in the regulation of macrophage activation by lipopolysaccharide (LPS) and mice with a myeloid cell Tet2 deficiency showed enhanced lung inflammation upon local LPS administration. However, mice with a global Tet2 deficiency showed reduced systemic inflammation during abdominal sepsis. Here, we sought to determine the role of myeloid cell Tet2 in the host response during gram-negative bacterial pneumonia. To this end we infected myeloid cell specific Tet2 deficient and control mice with two common gram-negative respiratory pathogens via the airways: *Pseudomonas aeruginosa* (PAK, causing acute infection that remains confined in the lungs) or *Klebsiella pneumoniae* (causing a gradually evolving pneumonia with subsequent dissemination and sepsis) and compared bacterial loads and host response parameters between mouse strains. Bone marrow derived macrophages from myeloid Tet2 deficient mice released more interleukin-6 than control macrophages upon stimulation with PAK or *K. pneumoniae*. However, bacterial loads did not differ between mouse strains upon infection with viable PAK or *K. pneumoniae*, and neither did cytokine levels or neutrophil recruitment. In addition, in the *K. pneumoniae* pneumosepsis model myeloid Tet2 deficiency did not affect systemic inflammation or organ injury. Together these data strongly argue against a role for myeloid cell Tet2 in the host response during gram-negative bacterial pneumonia and pneumosepsis.

## 1. Introduction

Tet methylcytosine dioxygenase 2 (Tet2) is a key enzyme in the demethylation of DNA [1]. Ample evidence indicates that Tet2 is involved in regulating immune cell development and activation [2]. Previous studies have shown that Tet2 represses inflammatory responses of macrophages and contributes to controlling inflammation in chronic inflammatory diseases such as atherosclerosis [3].

Sepsis is defined as organ dysfunction caused by a dysregulated host response to an infection. Sepsis represents a major health burden worldwide, responsible for an estimated 11 million deaths annually [4]. The two most common sources of sepsis are pneumonia and peritonitis [5]. Recent studies have implicated Tet2 in the pathogenesis of sepsis. Global *Tet2* knockout mice demonstrated a reduced mortality during abdominal sepsis, which was associated with an attenuated systemic cytokine storm [6]. We recently showed that mice with Tet2 deficiency restricted to myeloid cells demonstrate increased proinflammatory cytokine release in the bronchoalveolar space upon administration of lipopolysaccharide (LPS) via the airways when compared with littermate control mice [7]. Similarly, myeloid cell Tet2 deficient mice displayed enhanced proinflammatory cytokine release into the peritoneal cavity during LPS-induced peritonitis [7]. Furthermore, in a model of abdominal sepsis caused by intraperitoneal infection with *Escherichia coli* bacterial clearance was impaired in myeloid cell Tet2 deficient mice, which was accompanied by a reduced capacity of Tet2 deficient macrophages to limit the growth of this bacterium [7]. We recently started to

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investigate the role of Tet2 in bacterial pneumonia, reporting that mice with Tet2 deficiency restricted to bronchial epithelial cells had an impaired lung barrier function during acute *Pseudomonas aeruginosa* respiratory tract infection without alterations in the inflammatory or antibacterial response [8]. At present it is not known whether myeloid cell Tet2 contributes to the host response during bacterial pneumonia and pneumonia derived sepsis. To study this we infected myeloid cell specific Tet2 deficient mice and control littermates with two common gram-negative respiratory pathogens via the airways: *P. aeruginosa* (causing acute infection that remains confined in the lungs) or *Klebsiella pneumoniae* (causing a gradually evolving pneumonia with subsequent dissemination and sepsis).

## 2. Materials and methods

### 2.1. Animals

Homozygous *Tet2*$^{fl/fl}$ mice [9] were crossed with *LysM*$^{Cre}$ mice [10] (Jackson Laboratory, Bar Harbor, ME) to generate myeloid cell specific Tet2 deficient (*Tet2*$^{fl/fl}$*LysM*$^{Cre}$) mice. *Tet2*$^{fl/fl}$ Cre-negative littermates (*Tet2*$^{fl/fl}$ mice) were used as controls in all experiments. The mice were housed in specific pathogen-free condition. All genetically modified mice were backcrossed at least eight times to a C57BL/6 background and age and sex matched when used in experiments. Mice were used at 8–12 weeks of age. All animal studies were conducted according to the guidelines of the Declaration of Helsinki, and the animal care and use protocol adhered to the Dutch Experiments on Animals Act and European Directive of 22 September 2010 (Directive 2010/63/EU). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam (approval codes DIX17-4125–1-02 and DIX17-4125–1-26; approved in 2018).

### 2.2. Macrophage preparation and stimulation.

To obtain bone marrow derived macrophages (BMDMs) bone marrow was isolated and cultured with 15% of L929-conditioned medium for 7 days for differentiation [7]. BMDMs were then plated in 24-well plates (Greiner Bio-One, Alphen aan den Rijn, Netherlands) overnight at the density of one million cells per milliliter L929 free medium before stimulation with heat-killed *P. aeruginosa* PAK or *K. pneumoniae* serotype 2 (American Type Culture Collection no. 43816) (both at MOI = 10) for defined time periods. Cell supernatants were collected and stored at − 20 °C until further analysis. Alveolar macrophages (AMs) and peritoneal macrophages (PMs) were collected from *Tet2*$^{fl/fl}$*LysM*$^{Cre}$ and *Tet2*$^{fl/fl}$ mice are previously described [7].

### 2.3. 2.3. Quantitative reverse transcription PCR (qRT-PCR)

*Tet2* expression in BMDMs, AMs and PMs was measured by qRT-PCR as previous described [8]. *Hprt* expression was used as an endogenous control for normalization. The following specific primer pairs were used. *Tet2* forward sequence: AGCTGATGGAAAATGCAAGC; *Tet2* reverse sequence: AAGGTGCCTCTGGAGTGTTG; *Hprt* forward sequence: AGTCAAGGGCATATCCAACA; *Hprt* reverse sequence: CAAACTTTGCTT TCCGGGT.

### 2.4. Assays

Interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF) and myeloperoxidase (MPO) were measured by enzyme-linked immunosorbent assays (ELISA; RnD Systems, Minneapolis, MN) according to manufacturer's description. Plasma levels of IL-6 and TNF were measured by cytometric bead array (CBA; BD Biosciences, San Jose, CA) following manufacturer's introduction. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were measured using a c702 Roche Diagnostics analyzer (Roche Diagnostics, Almere, the Netherlands).

### 2.5. Induction of pneumonia and sampling of organs

Pneumonia was induced by intranasal inoculation with $5 \times 10^6$ CFU PAK or $10^4$ CFU *K. pneumoniae* serotype 2 (American Type Culture Collection no. 43816) as described [11]. Briefly, bacteria were collected at exponential phase and washed with cold phosphate-buffered saline. Bacteria number were calculated basing on OD620nm using a spectrophotometer. Mice were anesthetized via inhalation of 2–3% isoflurane and then were infected with bacteria intranasally. At defined time periods after inoculation, mice are anesthetized by intraperitoneal injection with 200 μL of ketamine/dexmedetomidine solution. Bronchoalveolar lavage fluid (BALF) and lung homogenates were obtained as described [11].

### 2.6. Flow cytometry

Flow cytometry was done on FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed using FlowJo software (Becton Dickinson) as described [7].

### 2.7. Statistical analysis

Non-parametric variables were analyzed using the Mann-Whitney *U* test. Analysis was done using GraphPad Prism version 8 (Graphpad Software, San Diego, CA). Statistical significance is shown as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

## 3. Results and discussion

Tet2 has been widely studied as a regulator of DNA methylation [1]. More recent studies implicated Tet2 in the regulation of inflammatory responses of macrophages and the pathogenesis of sepsis [6,7,12]. Knowledge of the potential role of Tet2 in sepsis is derived from investigations using mouse models of peritonitis [6,7]. Here we sought to determine the role Tet2 in the host response to pneumonia, the most common cause of sepsis. For this we generated mice with myeloid cell specific Tet2 deficiency and utilized established mouse models of respiratory tract infection by two common gram-negative pathogens.

*P. aeruginosa* is one of the most common causative pathogens in hospital-acquired pneumonia [13]. To obtain a first insight into the possible role of myeloid Tet2 in the host response to this bacterium, we first measured *Tet2* expression in BMDMs stimulated with heat-killed *P. aeruginosa* PAK (Fig. 1**A**). *Tet2* was induced by PAK, peaking at 6 h, thereafter decreasing to baseline levels at 24 h. To examine the functional role of Tet2 in macrophages activated by PAK, we prepared BMDMs from myeloid cell specific *Tet2* knockout (*Tet2*$^{fl/fl}$ *LysM*$^{Cre}$) mice and littermate control (*Tet2*$^{fl/fl}$) mice and stimulated these with heat-killed PAK. We first established that BMDMs, as well as resident macrophages from the alveolar space and peritoneal cavity, from *Tet2*$^{fl/fl}$ *LysM*$^{Cre}$ mice had strongly reduced Tet2 mRNA expression as compared to *Tet2*$^{fl/fl}$ control mice (Supplementary Fig. 1). In agreement with previous results showing that Tet2 represses IL-6 production by macrophages exposed to LPS [7,12], *Tet2*$^{fl/fl}$ *LysM*$^{Cre}$ BMDMs incubated with PAK produced more IL-6 than control BMDMs, whilst TNF levels were similar between genotypes (Fig. 1**B**). To evaluate the function of myeloid Tet2 during *Pseudomonas* pneumonia *in vivo*, we infected *Tet2*$^{fl/fl}$ *LysM*$^{Cre}$ and *Tet2*$^{fl/fl}$ mice with viable PAK via the airways. Bacterial loads in BALF were similar in both mouse strains at 6 and 24 h after infection (Fig. 1**C**), indicating that myeloid cell Tet2 did not directly affect antibacterial defense and allowing comparison of inflammatory responses without the potential bias of differential stimulation of the immune system due to differences in bacterial numbers. Unlike in LPS induced lung inflammation [7], myeloid Tet2 deficiency did not affect IL-1β, IL6 or TNF release into the bronchoalveolar space during PAK

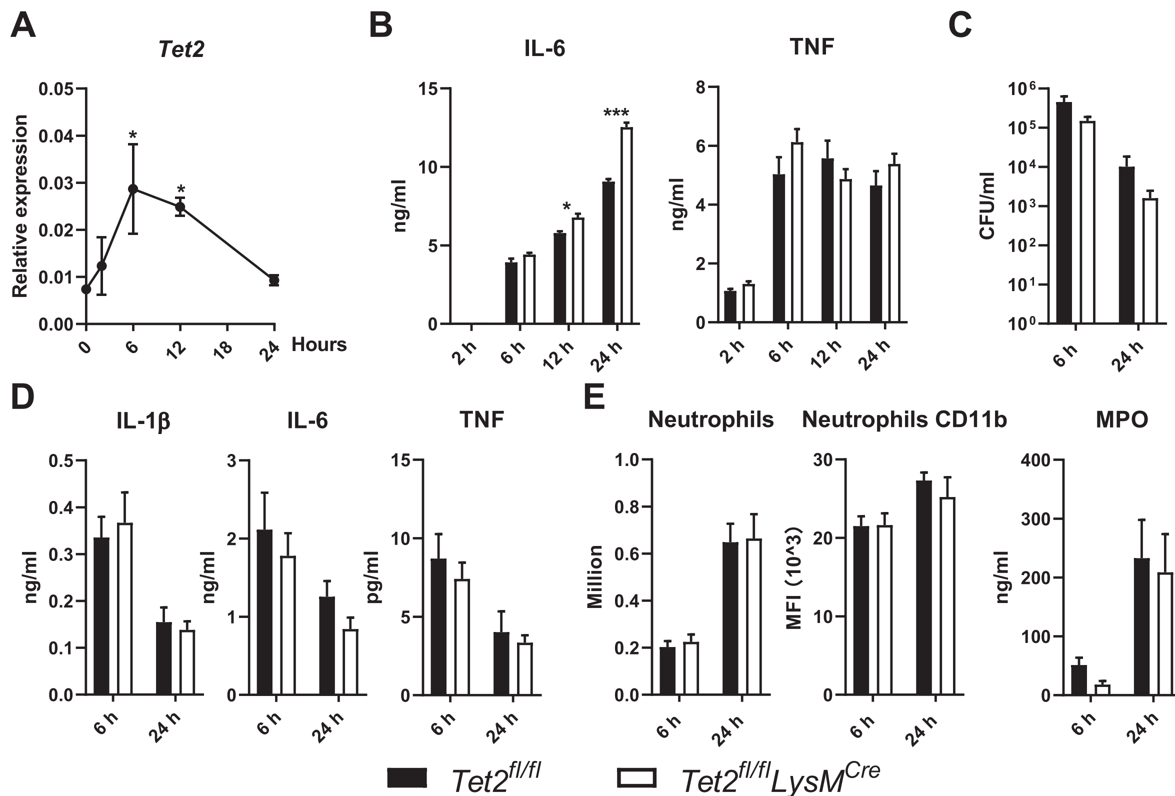


**Fig. 1. Myeloid cell Tet2 deficiency does not affect the host response during acute *Pseudomonas aeruginosa* pneumonia.** (A) *Tet2* expression in bone marrow derived macrophages (BMDMs) after stimulation with heat-killed *Pseudomonas aeruginosa* PAK (MOI = 10), mRNA expression was normalized to Hprt mRNA; (B) IL-6 and TNF production by BMDMs after stimulation with heat-killed PAK (MOI = 10) for 2, 6, 12 or 24 h; (C-G) bacterial counts (colony forming units, CFU), IL-β, IL-6, TNF levels, neutrophil numbers, neutrophil CD11b expression, and myeloperoxidase (MPO) levels in bronchoalveolar lavage fluid (BALF) of mice 6 or 24 h after infection with viable PAK ($5 \times 10^6$ CFU) via the airways. N = 6 for A and B, n = 8 for C-G. Data is shown as bar graphs with mean + SEM. *$p < 0.05$, ***$p < 0.001$.

infection *in vivo* (Fig. 1**D**). Likewise, neither the recruitment of neutrophils into the bronchoalveolar space nor their activation state (measured as neutrophil CD11b expression and MPO levels) differed between $Tet2^{fl/fl}$ $LysM^{Cre}$ and $Tet2^{fl/fl}$ mice (Fig. 1**E**). Together these results argue against a role for myeloid cell Tet2 in the host response during acute *P. aeruginosa* pneumonia *in vivo*.

In the model of *Pseudomonas* pneumonia bacteria remain confined to the lungs; the infection does not disseminate to distant organs and does not result in sepsis [8]. A previous study documented a role for Tet2 in a model of sepsis originating from the abdomen induced by cecal ligation and puncture (CLP) [6,14]. Specifically, mice with a global Tet2 deficiency demonstrated a reduced mortality during CLP induced sepsis when compared control mice, which was associated with a diminished influx of neutrophils to the primary site of infection and reduced systemic cytokine release [6,7,14]. To investigate the role of Tet2 in sepsis originating from the lungs, we made use of a well-described model of *K. pneumoniae* pneumosepsis [15]. This model is characterized by infection with a relatively low dose of a virulent *K. pneumoniae* strain, which gradually grows in the lungs with subsequent bacterial spreading and sepsis [15]. First, we studied the responsiveness of BMDMs to heat-killed *K. pneumoniae in vitro*. Similar to PAK, *K. pneumoniae* induced *Tet2* expression in BMDMs, peaking after 6 h (Fig. 2**A**) and BMDMs from $Tet2^{fl/fl}$ $LysM^{Cre}$ mice (relative to BMDMs from control mice) produced more IL-6 but equal amounts of TNF upon stimulation with *K. pneumoniae* (Fig. 2**B**). Next, we infected $Tet2^{fl/fl}$ $LysM^{Cre}$ and $Tet2^{fl/fl}$ mice with viable *K. pneumoniae* via the airways and compared bacterial burdens and inflammatory responses at an early time point after infection (12 h), when the infection is still largely confined to the lungs, and at a late time point (42 h), during fulminant sepsis. Myeloid Tet2 deficiency did not affect antibacterial defense, as reflected by comparable bacterial burdens in lungs (12 and 42 h) and distant body sites (42 h) in both mouse strains (Fig. 2**C**). Similarly, myeloid Tet2 deficiency did not modify lung cytokine (IL-1β, IL-6, TNF) (Fig. 2**D**) or MPO levels (Fig. 2**E**) during *K. pneumoniae* infection. To investigate the role of myeloid Tet2 in the systemic response to sepsis we measured IL-6 and TNF in plasma (Fig. 2**F**) and markers of organ damage, i.e. AST, ALT (reflecting hepatocellular injury) and LDH (indicating cellular injury in general) (Fig. 2**G**). None of these parameters were different between $Tet2^{fl/fl}$ $LysM^{Cre}$ and $Tet2^{fl/fl}$ mice. Together these data suggest that myeloid Tet2 does not play a significant role in the host response during pneumonia derived sepsis caused by *K. pneumoniae* infection.

Earlier investigations implicated Tet2 as a mediator in the host response to abdominal sepsis [6,7]. To the best of our knowledge this is the first study that examined the role of Tet2 in pneumonia. To this end we used two distinct mouse models of lower respiratory tract infection, one caused by *P. aeruginosa* (resulting in an acute pneumonia in which the infection remains confined to the lung) and one caused by *K. pneumoniae* (resulting in a gradually evolving pneumonia with subsequent bacterial dissemination to distant body sites and sepsis). In both models none of the host response parameters differed between $LysM^{Cre}$ and $Tet2^{fl/fl}$ control mice, either locally in the lungs or (in the *Klebsiella* model) systemically, strongly arguing against a role for myeloid Tet2 in gram-negative bacterial pneumonia. The models used are relatively acute; thus, it remains to be established whether myeloid Tet2 impacts the host response during more protracted infections of the airways. Of note, BMDMs from $LysM^{Cre}$ mice did release more IL-6 than control BMDMs when stimulated with PAK or *K. pneumoniae,* which is in agreement with previous studies that reported increased IL-6 production by Tet2 deficient BMDMs upon stimulation with LPS. Tet2 has been shown to affect IL-6 production through a mechanism that involves histone deacetylases [7,12]; Unlike IL-6, the production of other cytokines like TNF is not affected by such a mechanism. Here we did not

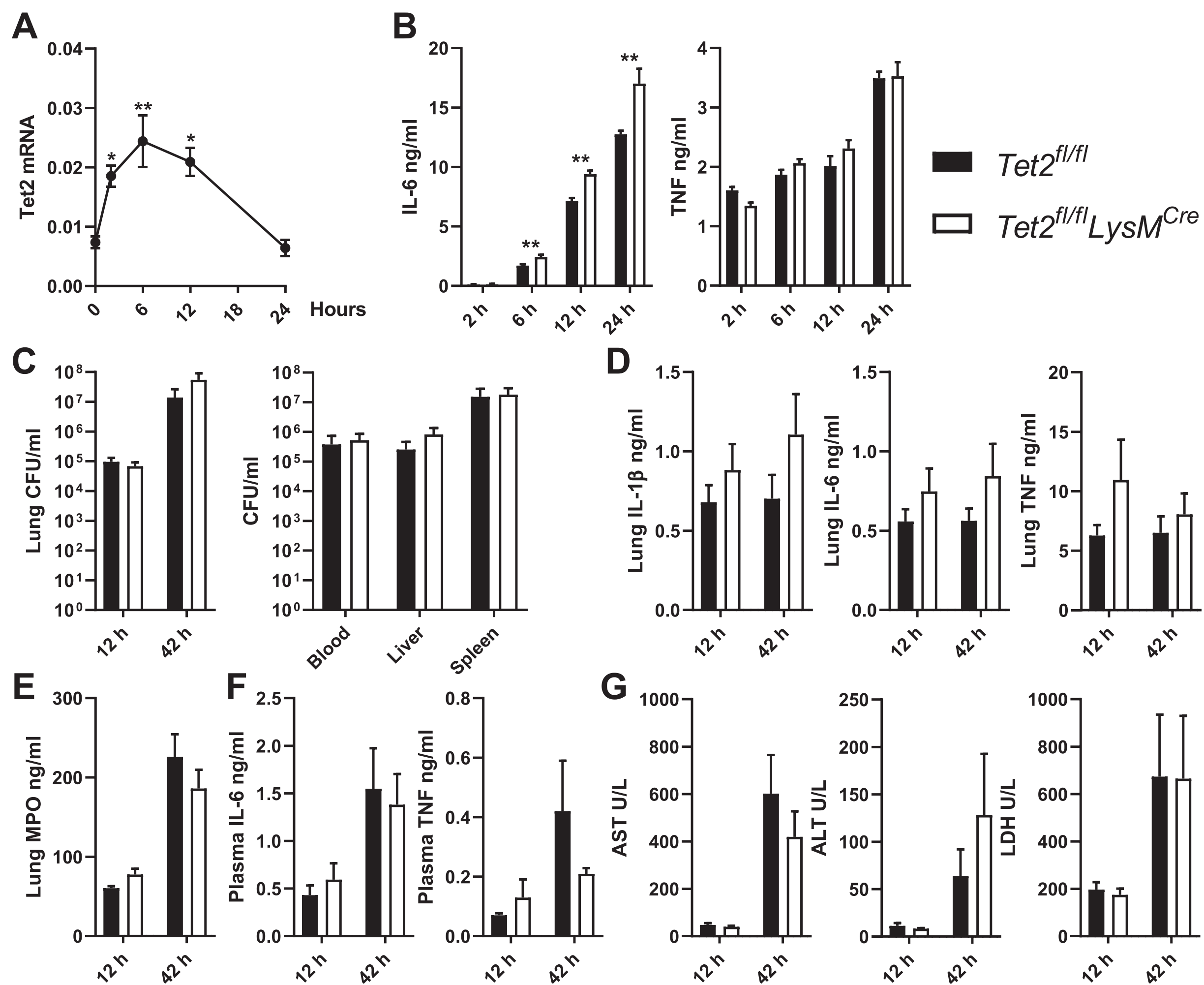


**Fig. 2. Myeloid cell Tet2 deficiency does not affect the host response during *K. pneumoniae* pneumonia derived sepsis.** (A) *Tet2* expression in bone marrow derived macrophages (BMDMs) after stimulation with heat-killed *Klebsiella (K.) pneumoniae* (MOI = 10), mRNA expression was normalized to Hprt mRNA; (B) IL-6 and TNF production by BMDMs after stimulation with heat-killed *K. pneumoniae* (MOI = 10) for 2, 6, 12 or 24 h; (C) Bacterial burdens in lung tissue (left), and in blood, liver and spleen (right) of mice 12 or 42 h after infection with *K. pneumoniae* ($10^4$ CFU) via the airways; (D-E) IL-β, IL-6, TNF protein levels and MPO protein in lung tissue of mice 12 or 42 h after infection with *K. pneumoniae* ($10^4$ CFU) via the airways; (F-G) IL-β, IL-6, TNF levels, AST, ALT and LDH levels in plasma of mice 12 or 42 h after infection with *K. pneumoniae* ($10^4$ CFU) via the airways. N = 6 for A and B, n = 8 for C-G. Data is shown as bar graphs with mean + SEM. *$p < 0.05$, **$p < 0.01$.

study the function of Tet2 in resident macrophages; yet, we previously showed that Tet2 deficient alveolar and peritoneal macrophages display a similar phenotype with regard to IL-6 release upon LPS stimulation as compared with BMDMs [7]. In addition, we recently reported increased proinflammatory cytokine release in the bronchoalveolar space upon LPS administration via the airways in $LysM^{Cre}$ mice when compared with $Tet2^{fl/fl}$ control mice [7]. Thus, these data suggest that in the context of infection of a complete organism (i.e., a mouse) with intact viable gram-negative bacteria (PAK or *Klebsiella*) the influence of myeloid Tet2 on the response to a common proinflammatory component of gram-negative microorganisms (LPS) is not detectable anymore. A likely explanation for this is the complexity of whole bacteria, which express multiple factors that can activate a variety of host cells.

In conclusion, this study shows that myeloid Tet2 deficiency does not have a significant impact on the host response during murine pneumonia caused by either *P. aeruginosa* or *K. pneumoniae*.

*CRediT authorship contribution statement*

**Wanhai Qin:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Xanthe Brands:** Conceptualization, Investigation, Writing – review & editing. **Cornelis van 't Veer:** Supervision, Writing – review & editing. **Alex F. de Vos:** Supervision, Writing – review & editing. **Brendon P. Scicluna:** Formal analysis, Supervision, Writing – review & editing. **Tom van der Poll:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2022.155876.

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