Ammonia-induced brain edema requires macrophage and T cell expression of Toll-like receptor 9

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**NH₄-Ac stimulation (4mM)**

DNA release

DNA activates TLR9 in neutrophils and macrophages

Neutrophils

Macrophages

Production of pro-inflammatory Cytokine IFN-γ, TNF-α and IL-6 in macrophages and T cells

Increase in brain water

Wild type (WT)

WT + TLR9 antagonist (ODN2088)

*Tlr9⁻/⁻* and *LysmCre Tlr9⁻/⁻*

DNA release

ODN2088 disrupts colocalization of DNA with TLR9 in innate immune cells abrogating cytokine production

No increase in brain water

WT + TLR9 antagonist (ODN2088)
Ammonia-induced brain edema requires macrophage and T cell expression of Toll-like receptor 9

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Key Words: Ammonia; Brain edema; Immune dysfunction; Toll-Like Receptor 9.

Synopsis: Utilising a TLR9-deficient mouse model and the TLR9 antagonist ODN2088, we have shown that ammonia-induced brain edema requires macrophage and T cell expression of TLR9 supporting exploration of TLR9 antagonism in acute liver failure.
Abstract

**Background & Aim:** Ammonia is central in the pathogenesis of brain edema in acute liver failure (ALF) with infection and systemic inflammation expediting development of intracranial hypertension (ICH). Patients with acetaminophen-induced ALF have increased neutrophil TLR9 expression which can be induced by ammonia. We determined whether ammonia-induced brain edema and immune dysfunction are mediated by TLR9 and if this could be prevented in a TLR9-deficient mouse model.

**Methods:** Ammonium acetate (NH$_4$-Ac; 4mmol/kg) was injected intraperitoneally in wild type (WT), Tlr9$^{-/-}$ and Lysm-Cre Tlr9$^{fl/fl}$ mice (TLR9 absent in neutrophils and macrophages including Kupffer cells) and compared to controls. Six hours after NH$_4$-Ac injection, intracellular cytokine production was determined in splenic macrophages, CD4+ and CD8+ T cells. Brain water (BW) and total plasma DNA (tDNA) were also measured. The impact of the TLR9 antagonist ODN2088 (50µg/mouse) was evaluated.

**Results:** Following NH$_4$-Ac injection, BW, macrophage and T cell cytokine production increased (p<0.0001) in WT but not Tlr9$^{-/-}$ mice (p<0.001). ODN2088 inhibited macrophage and T cell cytokine production (p<0.05) and prevented an increase in BW (p<0.0001). Following NH$_4$-Ac injection, macrophage cytokine production and BW were ameliorated in Lysm-Cre Tlr9$^{fl/fl}$ mice compared to WT mice (p<0.05) but there was no difference compared to Tlr9$^{-/-}$ mice. Following NH$_4$-Ac injection, plasma tDNA levels increased in WT and Tlr9$^{-/-}$ mice (p<0.05) suggesting that TLR9 may be activated by DNA released from ammonia-stimulated cells.

**Conclusion:** Ammonia-induced brain edema requires macrophage and T cell expression of TLR9. Amelioration of brain edema and lymphocyte cytokine production by ODN2088 supports exploration of TLR9 antagonism in early ALF to prevent progression to ICH.
Introduction

Ammonia plays a pivotal role in the development of hepatic encephalopathy (HE) and brain edema in acute liver failure (ALF).\textsuperscript{1-3} A robust systemic inflammatory response and susceptibility to developing infection are common in ALF, exacerbate the development of ammonia-induced brain edema and are major prognosticators.\textsuperscript{4-10} Experimental models have unequivocally associated ammonia exposure with astrocyte swelling and brain edema, potentiated by proinflammatory cytokines.\textsuperscript{11-14}

Toll-like receptor 9 (TLR9) is an innate pattern recognition receptor that binds to the CpG motif of bacterial and mammalian DNA.\textsuperscript{15} TLR9 plays an important role in acetaminophen-induced liver inflammation\textsuperscript{16} and we recently reported that ammonia along with DNA induces neutrophil TLR9 expression in patients with acetaminophen-induced ALF and advanced HE.\textsuperscript{17} Although the evidence base supporting the relationship between ammonia, inflammation and brain edema is robust in ALF, there is a paucity of data characterising the specific pathogenic mechanisms entailed. We hypothesised that ammonia-induced brain edema and immune dysfunction are mediated by TLR9. As TLR9 is necessary for the development of acetaminophen-induced acute liver injury in murine models,\textsuperscript{16} the hypothesis could only be tested in a murine model exposed to ammonium acetate ($\text{NH}_4$-Ac) without liver injury.

Using an acute hyperammonemic mouse model, we demonstrated that ammonia-induced brain edema and immune dysfunction, as measured by increased brain water (BW) content and intracellular cytokine production of macrophages and T cells, are mediated through TLR9. In mice, hyperammonemia resulted in DNA release and activation of TLR9 inducing downstream inflammatory cytokine production. TLR9 in lysozyme expressing cells was
critical for the development of brain edema and immune dysfunction. Administration of a TLR9 antagonist abrogated inflammation and prevented brain edema.

Results

(i) Ammonia-induced brain edema and changes in the liver were dependent on TLR9

To determine whether TLR9 plays a role in ammonia-induced brain edema, we evaluated the BW content of wild type (WT) and \( Tlr9^{-/-} \) mice six hours after a single dose of ammonium acetate (NH\(_4\)-Ac, 4 mM) injection [I.P]. Following NH\(_4\)-Ac stimulation, there was a significant increase in the BW content in WT mice compared to controls; which was significantly decreased in \( Tlr9^{-/-} \) mice compared to WT mice [Figure 1a] indicating that TLR9 plays an important role in the development of brain edema. To determine whether the increased BW content was associated with any changes in the liver, we assessed the liver/body weight ratio and liver histopathology after NH\(_4\)-Ac stimulation. In WT mice, there was a significant increase in liver/body weight ratio with evidence of hepatocyte swelling but not necrosis (there was no rise in serum aspartate and alanine transaminases), a finding which was abrogated in \( Tlr9^{-/-} \) mice [Figure 1b-c]. We hypothesized that DNA released following NH\(_4\)-Ac stimulation can bind to TLR9 resulting in activation of the innate immune system. To test our hypothesis, we measured plasma DNA. Total plasma dsDNA levels were significantly increased in WT mice and \( Tlr9^{-/-} \) mice following NH\(_4\)-Ac stimulation compared to controls, but there was no difference in \( Tlr9^{-/-} \) mice compared to WT mice following NH\(_4\)-Ac stimulation [Figure 1d].

(ii) Ammonia altered the function of macrophages and T cells in a TLR9-dependent manner
To determine whether systemic inflammation contributed to the ammonia-induced increase in BW content through a TLR9-mediated pathway, we measured intracellular cytokine (IFN-gamma, TNF-alpha and IL-6) production in T cells and macrophages isolated from spleen and liver in WT mice and *Tlr9*−/− mice following NH₄-Ac stimulation. There was a significant increase in the intracellular cytokines produced by macrophages [Figure 2 a-d] and CD4+ and CD8+ T cells [Figure 3 a-f] isolated from the spleen in WT mice following NH₄-Ac stimulation, compared to controls which was abrogated in *Tlr9*−/− mice. A similar trend was observed in the immune T cells isolated from the liver [Figure 4]. Neutrophil phagocytic activity was unaltered following NH₄-Ac stimulation [Figure 5].

(iii) **Ammonia-induced brain edema and inflammation were independent of acetate or pH**

Acetate has been reported to influence inflammation in acute alcoholic hepatitis and the increased toxicity of ammonium salts promotes ammonium gas transfer across the blood brain barrier due to the rise in blood pH and the direct effect of alkalinisation. Therefore, to confirm whether the TLR9-mediated brain edema and inflammation were solely induced by ammonia and not by the acetate or changes in pH, an alternate salt of acetate, sodium acetate (NaCH₃C0₂) (Na-Ac) was injected in WT mice after adjusting for pH (same as NH₄-Ac). Na-Ac (4 mM) did not alter the BW content, liver bodyweight ratio or intracellular cytokine production of various immune cell subsets in WT mice compared to controls unlike NH₄-Ac [Figure 6 a-f]. These results confirm that the observed ammonia-induced cytokine production and brain edema were induced by ammonia *per se*, and not influenced by acetate and/or pH changes.

(iv) **Ammonia-induced brain edema and inflammation were mediated by TLR9 expressed within lysozyme-expressing cells**
Our results showed that deletion of TLR9 tempered the pro-inflammatory state and abrogated the development of brain edema following ammonia stimulation. To examine the role of TLR9 in macrophages, we used Lysm-Cre Tlr9\(^{0/0}\) mice that have TLR9 specifically deleted in lysozyme-expressing cells, namely macrophages and neutrophils\(^{20}\). NH\(_4\)-Ac (4 mM) was therefore injected into Lysm-Cre Tlr9\(^{0/0}\) mice. Interestingly, we found that BW content, liver/body weight ratio, hepatocyte swelling and cytokines produced by macrophages were significantly ameliorated in Lysm-Cre Tlr9\(^{0/0}\) mice compared to WT mice following NH\(_4\)-Ac stimulation but with no difference compared to Tlr9\(^{-/-}\) mice [Figure 7 a-g]. There was no difference in the total plasma dsDNA levels in Tlr9\(^{-/-}\) mouse and Lysm-Cre Tlr9\(^{0/0}\) mice compared to WT mice following NH\(_4\)-Ac stimulation [Figure 7h]. We also tested another control mouse strain, Tlr9\(^{0/0}\) mice (TLR9 is sufficient in all the cell types). As expected, Tlr9\(^{0/0}\) mice were not protected from NH\(_4\)-Ac induced inflammation compared to Lysm-Cre Tlr9\(^{0/0}\) mice [Figure 8 a-e].

(v) TLR9 antagonism abrogates ammonia-induced brain edema and inflammation

Since Tlr9\(^{-/-}\) mice were protected against NH\(_4\)-Ac stimulation, we tested whether an antagonist of TLR9 [ODN2088] could inhibit the ammonia-induced pro-inflammatory changes and brain edema observed in the WT mice. Administration of ODN2088 (50 \(\mu\)g/mouse) with NH\(_4\)-Ac (4 mM) significantly decreased the BW content and liver/body weight ratio and ameliorated the hepatocyte swelling [Figure 9 a-c]. Administration of ODN2088 did not alter the total plasma DNA levels in WT mice following NH\(_4\)-Ac stimulation but they were increased compared to controls [Figure 9d]. There was also a significant reduction in the cytokines produced by macrophages [Figures 9 e-g] and T cells [Figure 10 a-f].
Discussion

In this study, we have demonstrated a novel link between ammonia-induced inflammation and the subsequent development of brain edema mediated by TLR9 using different mouse models. In addition, we showed that TLR9 expressed by lysozyme-producing cells in mice was critical for ammonia-induced brain edema and inflammation to develop. Importantly, we also showed that administration of a TLR9 antagonist abrogated the inflammation and prevented brain edema supporting therapeutic exploration of TLR9 antagonism in early ALF.

Ammonia stimulation upregulated the production of the cytokines IFN-gamma, TNF-alpha and IL-6 by macrophages and T cells. The observation of increased cytokine production from the hepatic infiltrated T cells indicates that the immune response originates from the liver. The cytokines IFN-gamma, TNF-alpha and IL-6 produced by T cells are predominantly responsible for mediating inflammation within the liver.²¹⁻²³ In this study ammonia activated cytokine production in macrophages indicated that those cells, along with CD4⁺ and CD8⁺ T cells, are responsible for promoting the proinflammatory environment. It is possible that ammonia and systemic inflammation induce brain edema by inducing astrocyte swelling. This is supported by the previously published observation that astrocyte swelling can be induced when astrocytes are exposed to pro-inflammatory cytokines after being exposed to ammonia.¹¹ Though deficiency of TLR9 exacerbates cognitive impairment and severity of seizures in the brain,²⁴ it has been demonstrated that activation of TLR9 in microglia and astrocytes induces the production of various pro-inflammatory cytokines in response to CpG DNA²⁵ and activation of TLR9 in astrocytes leads to synaptic protein loss in the brain in chronic hyperglycemia.²⁶ Activation of Tlr9 results in the upregulation of cytokine production and increase in the BW content, and the critical role of TLR9 in this mechanism has been demonstrated by using genetically modified mice. TLR9 is essential for macrophage
production of TNF-alpha and IL-6 in response to CpG DNA and IFN-gamma produced by CD4+ T cells in response to CpG DNA and mycobacteria.\textsuperscript{15, 27} The observation that ammonia-induced TLR9 activation induces systemic inflammation and brain edema is well supported by recently published studies that show TLR9 induces inflammation in acetaminophen-induced ALF and non-alcoholic steatohepatitis.\textsuperscript{16, 20}

Ammonia stimulation increased the total plasma DNA levels in all the different mouse models, thereby suggesting that ammonia activates TLR9 through DNA release. In acetaminophen-induced ALF, DNA fragments released by apoptotic hepatocytes have been shown to be responsible for the activation of TLR9 and induction of systemic inflammation.\textsuperscript{16, 28} These findings support the results of our recent human study, which showed profound TLR9 activation in neutrophils in the presence of plasma DNA in patients with acetaminophen-induced ALF, systemic inflammation and brain edema\textsuperscript{17} suggesting that TLR9 mediates the ammonia-induced brain edema in a DNA-driven manner. In this study, it is not possible to identify the source of the DNA and we can only speculate that non-immune cells are the source of DNA.

TLR9 expressed in the lysozyme-producing cells played an important role in the ammonia-induced cytokine production of macrophages and brain edema. Marques et al\textsuperscript{29} recently demonstrated that neutrophils are the predominant innate immune cells that sense DNA through the TLR9/NF-κB pathway and induce inflammation in the context of acetaminophen toxicity. These data are also well supported by the strong correlation demonstrated between neutrophil TLR9 expression, ammonia and IL-8 in acetaminophen-induced ALF and the abrogation of neutrophil TLR9 upregulation and cytokine production in acetaminophen-induced ALF plasma by DNAse-I.\textsuperscript{17}
Administration of the TLR9 antagonist, ODN2088 abrogated the cytokine production and prevented the increase in BW content induced by stimulation of ammonia. These data are well supported by the findings of Imaeda et al., who in an acetaminophen-induced hepatotoxicity model, established that inhibition of TLR9 using ODN2088 and IRS954, a TLR7/9 antagonist, downregulated pro-inflammatory cytokine release and reduced mortality. The amelioration of brain edema and cytokine production by ODN2088 supports exploration of TLR9 antagonism as a therapeutic modality in early ALF to prevent the development of brain edema and intracranial hypertension.

There are pros and cons of using an ammonia-induced murine model of cerebral edema without acute liver injury. Some of the best-characterized animal models of cerebral edema in ALF include the hepatic devascularized rat and pig, and the rat with thioacetamide-induced toxic liver injury. Whilst these models develop cerebral edema and exhibit intracranial hypertension, the impact of hepatic devascularisation and necrosis bring other sequelae including systemic inflammation, bacterial and fungal sepsis and coagulopathy. This makes it hard to tease out the specific mechanisms that underpin the development of ammonia-induced brain edema per se and how this relates to innate immune dysfunction. Furthermore, we know that murine acetaminophen-induced ALF is mediated by TLR9 and can be abrogated by a TLR9 antagonist. Therefore, we could not utilise an acetaminophen-induced model of ALF to assess TLR9-mediated cerebral edema in this study and thus chose to examine this in a model of ammonia-induced cerebral edema.

In summary, this study demonstrates that the development of ammonia-induced brain edema requires macrophage and T cell expression of TLR9 which may be stimulated by DNA release. The importance of TLR9 expressing neutrophils and/or Kupffer cells in the induction of brain edema and inflammation was confirmed by the deletion of the TLR9 gene only in
lysozyme expressing cells. We have therefore demonstrated that TLR9 is necessary for the development of ammonia-induced brain edema.\textsuperscript{16,29} The observation that systemic inflammation and brain edema can be prevented following administration of a TLR9 antagonist supports the exploration of TLR9 antagonism as a therapeutic modality in early ALF to prevent the progression to ICH.
Materials and methods

Animals used for this study

All the procedures and protocols used in the studies with animals were approved by the Institutional Animal Care and Use Committee at Yale University, USA. Four strains of mice on C57BL/6 genetic background were used for the experiments. C57BL/6 (wild type, WT) were originally obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The original Tlr9−/− and Tlr9fl/fl breeders were kindly provided by Prof Shizuo Akira, Japan 15 and Prof Mark Shlomchik, University of Pittsburgh, USA 20, respectively. We bred Lysm-Cre mice with Tlr9fl/fl mice to generate Lysm-Cre Tlr9fl/fl mice with TLR9 specific deletion in lysozyme expressing cells. 32 All the mice used in the study were bred and kept at the Yale animal facility in specific pathogen free (SPF) conditions with autoclaved food, bedding and filtered cage. The mice were fed on a regular chow diet and on a 12 hour light/dark cycle. Male mice (7-9 weeks old) were used in the study.

Stimulation with ammonium acetate

To determine whether TLR9 plays a role in brain edema in the mice, ammonium acetate (NH₄CH₃CO₂) (NH₄-Ac) (4 mmol/kg of bodyweight) was injected intraperitoneally in WT mice, Tlr9−/− mice, Lysm-Cre Tlr9fl/fl mice and Tlr9fl/fl mice that were sacrificed 6 hours after the injection. The concentration and duration of NH₄-Ac used for this experiment was optimised in a pilot experiment. Sodium acetate (NaCH₃CO₂) (Na-Ac) [J.T. Baker, USA] (4 mmol/kg of bodyweight) was injected intraperitoneally in WT mice to demonstrate that any effect was due to ammonia and not the acetate moiety.

Blood collection and tissue harvesting

Six hours after the NH₄-Ac stimulation, blood was collected from the mice and liver, spleen and brain were harvested. Plasma was collected and stored in -80°C. Spleen was
homogenised using rough sides of two grinding slides and red blood cells were lysed by hypotonic solution and quickly restored in isotonic PBS. The single suspension of splenocytes (10⁶) was stained with monoclonal antibodies (mAbs) conjugated with different fluorochromes prior to flow cytometry analysis. Liver tissue was homogenised using a plunge through a wire mesh and digested using Collagenase-I and DNAse-I [Sigma Aldrich, USA]. Liver infiltrated immune cells were isolated from the homogenised tissue using the density-gradient Polymorphprep™ solution [Axis Shield, Norway], stained with mAbs and analysed using flow cytometry. The entire brain was weighed immediately after sacrifice using an electronic balance to determine the wet weight. The brain was then dried in an oven at 100°C for 24 hours to obtain the dry weight. The BW content was then calculated according to the formula⁴:

\[
\text{BW content (\%)} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100
\]

**Preservation of tissues for histopathological examination**

For histopathological examination, tissues were harvested and stored in 10% formalin at room temperature. Specimens were then embedded in paraffin. Six µM tissue sections were then stained with hematoxylin and eosin (H&E) and examined under a light microscope.

**Total DNA estimation**

Total DNA was measured in the plasma samples stored at -80°C using the Quant-iT PicoGreen® dsDNA quantitation kit (Life Technologies, USA) as detailed previously.²⁰

**TLR9 antagonist injection**

To determine whether an inhibitor of TLR9 offers protection against NH₄Ac stimulation, the TLR9 antagonist (ODN2088) [Invivogen, USA] (50 µg/mouse) was injected intraperitoneally
in WT mice immediately following NH₄-Ac injection. Six hours later, blood was collected and organs were harvested as mentioned above. The time and concentration of ODN2088 were chosen based upon a recently established mouse model of acetaminophen hepatotoxicity.

**Stimulation of intracellular cytokine (ICC) production**

To determine the intracellular cytokine production of mononuclear cells from spleen and liver, up to 5.0 x 10⁶ cells per mL were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng) and ionomycin [Invivogen, USA] (500 pg) in complete media with Golgi plug [BD, USA] (1 µL) and incubated at 37°C for 5 hours in the presence of 5% CO₂. At the end of 5 hours, stimulated cells were washed, stained with fluorochrome conjugated mAbs and analysed using flow cytometry.

**Cell staining and flow cytometry**

Up to 1 million (1 x 10⁶) cells were re-suspended in 100 µL of staining buffer and stained with different fluorochrome conjugated mAbs in a tube followed by incubation at room temperature (RT) in darkness for 30 minutes and the stained cells were washed with PBS. For ICC staining, 100 µL of cytofix/cytoperm solution [BD, USA] was added to the cell pellet after staining with surface markers and kept at RT for 20 minutes. The cells were washed with 1 mL of permeabilization wash buffer [BD, USA] followed by re-suspension in 300 µL of PBS and acquired in a LSRII flow cytometry [BD, San Jose, California, USA] using BD FACS DIVA software V6.0 [BD, San Jose, California, USA].

**Neutrophil phagocytosis**

The phagocytic ability of neutrophils was determined by incubating the whole blood with Dextran FITC (1mg/mL) at 37°C for 20 minutes in a water bath and measuring the mean
fluorescence intensity of the neutrophils in a flow cytometer. Neutrophils were identified using Lys6-G (1A8) and CD11b (M1/70) antibodies.

**Identification and characterization of various immune cell subsets**

The different subsets of lymphocytes were identified using their specific markers. T cells were identified using CD3 (17A2); CD4 (GK1.5) and CD8 (53-6.7) markers were used to characterise the different subsets of T cells and macrophages were identified using F4/80 (BM8) and CD11b (M1/70) markers. ICC production [IL-6 (MP5-20F3), IFN-gamma (XMG1.2) and TNF-alpha (MP6-XT22)] was determined in the CD4⁺ and CD8⁺ T cell subsets and F4/80⁺ and CD11b⁺ macrophages. Flow cytometry antibodies were purchased from Biolegend, USA.

**Statistics**

For comparisons between two groups, Student’s t-test (parametric data) or Mann-Whitney U test (non-parametric data) were used; for comparisons between three or more groups, One-way ANOVA with Tukey’s multiple comparison tests (parametric data) or Kruskal Wallis with Dunn’s multiple comparison tests (non-parametric data) were used based on the normal distribution of the data. All the results are presented as mean or median differences with 95% confidence intervals (C.I.). Hypothesis testing was two-tailed at an alpha level 0.05. All statistical analyses were performed using GraphPad Prism 7.0; p<0.05 was considered as statistically significant.


**Figure 1: TLR9 stimulates ammonia-induced brain edema and liver bodyweight ratio through DNA.**

(a) Following NH$_4$-Ac stimulation, there was a significant increase in the BW content in WT mice (n=9) compared to controls (n=12) (p<0.0001) [mean difference: 1.35; 95% C.I.: 0.9 to 1.8], which was ameliorated in $Tlr9^{-/-}$ mice (n=10) (p=0.006) [mean difference: -0.76; 95% C.I.: -1.3 to -0.25]. (b) Following NH$_4$-Ac stimulation, there was a significant increase in the liver bodyweight ratio in WT mice (n=16) compared to controls (n=13) (p<0.0001) [mean difference: 0.019; 95% C.I.: 0.013 to 0.024], which was ameliorated in $Tlr9^{-/-}$ mice (n=11) (p<0.0001) [mean difference: -0.014; 95% C.I.: -0.019 to -0.009]. (c) Following NH$_4$-Ac stimulation, there was a significant increase in the hepatocyte swelling in the liver histology (H&E stained – 200x magnifications) in WT mice compared to controls, where the cytoplasm of cells remained intact. Following NH$_4$-Ac stimulation, there was no difference in the hepatocyte morphology in $Tlr9^{-/-}$ mice compared to controls but the hepatocyte swelling was reduced compared to WT mice. The images in the black box inset are the representative images at 400x magnification. (d) Following NH$_4$-Ac stimulation, there was a significant increase in the total plasma DNA level in WT mice (n=12) compared to controls (n=12) (p=0.007) [mean difference: 2.97; 95% C.I.: 0.88 to 5] and in $Tlr9^{-/-}$ mice (n=17) compared to controls (n=14) (p=0.004) [mean difference: 1.76; 95% C.I.: 0.6 to 2.9].

**Figure 2: Ammonia-induced intracellular cytokine production by macrophages is mediated by TLR9.**

(a) Following NH$_4$-Ac stimulation, there was a significant increase in the intracellular cytokine IFN-gamma produced by splenic macrophages in WT mice (n=8) compared to
controls (n=7) (p<0.0001) [mean difference: 2.3; 95% C.I.: 1.5 to 3], which was ameliorated in $Tlr9^{-/-}$ mice (n=8) (p=0.0006) [mean difference: -1.6; 95% C.I.: -2.4 to -0.83]. (b) Following NH$_4$-Ac stimulation, there was a significant increase in the intracellular cytokine TNF-alpha produced by splenic macrophages in WT mice (n=8) compared to controls (n=7) (p<0.0001) [mean difference: 25; 95% C.I.: 22 to 28.5], which was ameliorated in $Tlr9^{-/-}$ mice (n=8) (p<0.0001) [mean difference: -14; 95% C.I.: -17.2 to -10.8]. (c) Following NH$_4$-Ac stimulation, there was a significant increase in the intracellular cytokine IL-6 produced by splenic macrophages in WT mice (n=8) compared to controls (n=7) (p<0.0001) [mean difference: 4; 95% C.I.: 3.5 to 4.5], which was ameliorated in $Tlr9^{-/-}$ mice (n=8) (p<0.0001) [mean difference: -4; 95% C.I.: -4.7 to -3.5]. (d) Representative FACS plots of intracellular cytokines IFN-gamma, TNF-alpha and IL-6 produced by splenic macrophages in WT mice and $Tlr9^{-/-}$ mice controls, and following NH$_4$-Ac stimulation.

**Figure 3: Ammonia-induced intracellular cytokine production by T cells is mediated by TLR9.**

(a) Following NH$_4$-Ac stimulation, there was a significant increase in the intracellular cytokine IFN-gamma produced by splenic CD4$^+$ T cells in WT mice compared to controls (p<0.0001) [median difference: 2.7; 95% C.I.: 1.5 to 3], which was ameliorated in $Tlr9^{-/-}$ mice compared to WT mice (p=0.0007) [median difference: -1.9; 95% C.I.: -2.8 to -0.8]. (b) Following NH$_4$-Ac stimulation, there was a significant increase in the intracellular cytokine TNF-alpha produced by splenic CD4$^+$ T cells in WT mice compared to controls (p<0.0001) [mean difference: 49.5; 95% C.I.: 47 to 52], which was ameliorated in $Tlr9^{-/-}$ mice compared to WT mice (p<0.0001) [mean difference: -27.5; 95% C.I.: -30 to -25]. (c) Following NH$_4$-Ac stimulation, there was a significant increase in the intracellular cytokine IL-6 produced by splenic CD4$^+$ T cells in WT mice compared to controls (p<0.0001) [mean difference: 2.2;
95% C.I.: 1.4 to 3], which was ameliorated in \textit{Tlr9}^{/-} mice compared to WT mice (p=0.0052) [mean difference: -1.6; 95% C.I.: -2.7 to -0.5]. (d) Following NH\textsubscript{4}-Ac stimulation, there was a significant increase in the intracellular cytokine IFN-gamma produced by splenic CD8\textsuperscript{+} T cells in WT mice compared to controls (p=0.0002) [mean difference: 3.9; 95% C.I.: 2.1 to 5.8], which was ameliorated in \textit{Tlr9}^{/-} mice compared to WT mice (p=0.0003) [median difference: -3.5; 95% C.I.: -6.4 to -2.1]. (e) Following NH\textsubscript{4}-Ac stimulation, there was a significant increase in the intracellular cytokine TNF-alpha produced by splenic CD8\textsuperscript{+} T cells in WT mice compared to controls (p<0.0001) [mean difference: 49; 95% C.I.: 46 to 52], which was ameliorated in \textit{Tlr9}^{/-} mice compared to WT mice (p<0.0001) [mean difference: -48; 95% C.I.: -51 to -45]. (f) Representative FACS plots of the intracellular cytokines IFN-gamma, TNF-alpha and IL-6 produced by splenic T cells in WT mice and \textit{Tlr9}^{/-} mice controls and following NH\textsubscript{4}-Ac stimulation. [WT mice controls (n=11) \& NH\textsubscript{4}-Ac treated (n=13); \textit{Tlr9}^{/-} mice controls (n=10) \& NH\textsubscript{4}-Ac treated (n=10)].

\textbf{Figure 4: Ammonia-induced intracellular cytokine production by liver infiltrated T cells.}

(a) Following NH\textsubscript{4}-Ac stimulation, there was a significant increase in the intracellular cytokine IFN-gamma produced by liver infiltrated CD4\textsuperscript{+} T cells in WT mice compared to controls (p<0.0001) [median difference: 18.3; 95% C.I.: 15 to 21.6], which was ameliorated in \textit{Tlr9}^{/-} mice compared to WT mice (p<0.0001) [median difference: -14.5; 95% C.I.: -18.6 to -10.3]. (b) Following NH\textsubscript{4}-Ac stimulation, there was a significant increase in the intracellular cytokine TNF-alpha produced by liver infiltrated CD4\textsuperscript{+} T cells in WT mice compared to controls (p<0.0001) [mean difference: 21.4; 95% C.I.: 14.5 to 28.2], which was ameliorated in \textit{Tlr9}^{/-} mice compared to WT mice (p<0.0001) [mean difference: -20; 95% C.I.: -27 to -12.7]. (c) Following NH\textsubscript{4}-Ac stimulation, there was a significant increase in the intracellular cytokine IL-6 produced by liver infiltrated CD4\textsuperscript{+} T cells in WT mice compared
to controls (p<0.0001) [mean difference: 11.9; 95% C.I.: 8.1 to 15.6], which was ameliorated in Tlr9⁻/⁻ mice compared to WT mice (p=0.0082) [mean difference: -7.3; 95% C.I.: -12.4 to -2.2]. (d) Following NH₄Ac stimulation, there was a significant increase in the intracellular cytokine IFN-gamma produced by liver infiltrated CD8⁺ T cells in WT mice compared to controls (p<0.0001) [mean difference: 18.3; 95% C.I.: 15.8 to 20.9], which was ameliorated in Tlr9⁻/⁻ mice compared to WT mice (p<0.0001) [median difference: -12.4; 95% C.I.: -16.7 to -8.2]. (e) Following NH₄Ac stimulation, there was a significant increase in the intracellular cytokine TNF-alpha produced by liver infiltrated CD8⁺ T cells in WT mice compared to controls (p<0.0001) [mean difference: 47.3; 95% C.I.: 39 to 56], which was ameliorated in Tlr9⁻/⁻ mice compared to WT mice (p<0.0001) [mean difference: -44; 95% C.I.: -56 to -32]. [WT mice controls (n=10) & NH₄Ac treated (n=10); Tlr9⁻/⁻ mice controls (n=4) & NH₄Ac treated (n=7)].

Figure 5: Neutrophil phagocytosis measured by dextran FITC using flow cytometry in WT and Tlr9⁻/⁻ mice after NH₄Ac stimulation.

Following NH₄Ac stimulation, there was no difference in the mean fluorescence intensity of dextran FITC in neutrophils isolated from whole blood in WT mice or Tlr9⁻/⁻ mice compared to controls.

Figure 6: Unaltered brain edema and intracellular cytokine production by macrophages after Na-Ac stimulation.

(a) Following Na-Ac stimulation, BW content remained unaltered in WT mice (n=7) compared to controls (n=12), but was significantly reduced compared to the NH₄Ac stimulated WT mice (n=9) (p<0.001) [mean difference: -1.1; 95% C.I.: -1.7 to -0.47]. (b) Following Na-Ac stimulation, liver bodyweight ratio remained unaltered in WT mice (n=7)
compared to controls (n=13), but was significantly reduced compared to the NH₄-Ac stimulated WT mice (n=16) (p<0.001) [mean difference: -0.02; 95% C.I.: -0.028 to -0.012]. Following Na-Ac stimulation, the intracellular cytokine (c) IFN-gamma (p<0.0001) [mean difference: -2.2; 95% C.I.: -3 to -1.4], (d) TNF-alpha (p<0.0001) [mean difference: -22.3; 95% C.I.: -28.3 to -16.3], and (e) IL-6 (p<0.0001) [mean difference: -4.5; 95% C.I.: -5.2 to -3.9] produced by splenic macrophages were significantly reduced in WT mice (n=7) compared to the NH₄-Ac stimulated WT mice (n=8) but remained unaltered compared to controls (n=7). (f) Representative FACS plots of the intracellular cytokines IFN-gamma, TNF-alpha and IL-6 produced by splenic macrophages in WT mice controls, NH₄-Ac stimulated WT mice and Na-Ac stimulated WT mice.

**Figure 7: TLR9 in lysosomal expressed cells stimulates ammonia-induced brain edema and cytokine production by macrophages.**

(a) Following NH₄-Ac stimulation, BW content was significantly ameliorated in *Tlr9*⁻/⁻ mice (n=10) (p<0.05) [mean difference: -0.76; 95% C.I.: -1.5 to -0.06] and *Lysm-Cre Tlr9*⁻/⁻ mice (n=9) (p<0.001) [mean difference: -1.4; 95% C.I.: -2 to -0.7] compared to WT mice (n=9). (b) Following NH₄-Ac stimulation, liver bodyweight ratio was significantly ameliorated in *Tlr9*⁻/⁻ mice (n=11) (p<0.0001) [mean difference: -0.015; 95% C.I.: -0.021 to -0.008] and *Lysm-Cre Tlr9*⁻/⁻ mice (n=10) (p<0.0001) [mean difference: -0.019; 95% C.I.: -0.026 to -0.013] compared to WT mice (n=16). (c) Following NH₄-Ac stimulation, there was a significant increase in the hepatocyte swelling in the liver histology (H&E stained – 200x magnifications) in WT mice compared to *Tlr9*⁻/⁻ mice and *Lysm-Cre Tlr9*⁻/⁻ mice, where the cytoplasm of cells remained intact. The images in the black box inset are the representative images at 400x magnification. (d) Following NH₄-Ac stimulation, the intracellular cytokine IFN-gamma produced by splenic macrophages was significantly ameliorated in *Tlr9*⁻/⁻ mice.
(n=8) (p<0.0001) [mean difference: -1.6; 95% C.I.: -2.4 to -0.84] and Lysm-Cre Tlr9^fl/fl^ mice (n=9) (p<0.01) [mean difference: -1; 95% C.I.: -1.75 to -0.25] compared to WT mice (n=8).

(e) Following NH\(_4\)-Ac stimulation, the intracellular cytokine TNF-alpha produced by splenic macrophages was significantly ameliorated in Tlr9^-/-^ mice (n=8) (p<0.0001) [mean difference: -14; 95% C.I.: -17.4 to -10.6] and Lysm-Cre Tlr9^fl/fl^ mice (n=9) (p<0.0001) [mean difference: -11.7; 95% C.I.: -15 to -8.4] compared to WT mice (n=8). (f) Following NH\(_4\)-Ac stimulation, the intracellular cytokine IL-6 produced by splenic macrophages was significantly ameliorated in Tlr9^-/-^ mice (n=8) (p<0.0001) [mean difference: -4.1; 95% C.I.: -4.7 to -3.4] and Lysm-Cre Tlr9^fl/fl^ mice (n=9) (p<0.0001) [mean difference: -3.6; 95% C.I.: -4.2 to -3] compared to WT mice (n=8). (g) Representative FACS plots of the intracellular cytokines IFN-gamma, TNF-alpha and IL-6 produced by splenic macrophages in WT mice, Tlr9^-/-^ mice and Lysm-Cre Tlr9^fl/fl^ mice following NH\(_4\)-Ac stimulation.

Figure 8: Ammonia-induced brain edema and cytokine production in Lysm-Cre Tlr9^fl/fl^ mice compared to Tlr9^fl/fl^ mice.

(a) Following NH\(_4\)-Ac stimulation, there was a significant increase in the BW content in Tlr9^fl/fl^ mice (n=11) compared to controls (n=8) (p=0.0009) [mean difference: 1.05; 95% C.I.: 0.5 to 1.6], which was ameliorated in Lysm-Cre Tlr9^fl/fl^ mice (n=9) (p=0.014) [mean difference: -0.7; 95% C.I.: -1.2 to -0.16]. (b) Following NH\(_4\)-Ac stimulation, there was a significant increase in the liver bodyweight ratio in Tlr9^fl/fl^ mice (n=12) compared to controls (n=8) (p=0.002) [mean difference: 0.007; 95% C.I.: 0.003 to 0.015], which was ameliorated in Lysm-Cre Tlr9^fl/fl^ mice (n=10) (p=0.007) [mean difference: -0.008; 95% C.I.: -0.017 to -0.002]. (c) Following NH\(_4\)-Ac stimulation, there was a significant increase in the intracellular
cytokine IFN-gamma produced by splenic macrophages in Tlr9<sup>fl/fl</sup> mice (n=10) compared to controls (n=8) (p<0.0001) [mean difference: 3.5; 95% C.I.: 2.5 to 4.5], which was ameliorated in Lysm-Cre Tlr9<sup>fl/fl</sup> mice (n=8) (p<0.0001) [mean difference: -3.6; 95% C.I.: -4.6 to -2.7]. (d) Following NH<sub>4</sub>-Ac stimulation, there was a significant increase in the intracellular cytokine TNF-alpha produced by splenic macrophages in Tlr9<sup>fl/fl</sup> mice (n=10) compared to controls (n=8) (p<0.0001) [mean difference: 14.3; 95% C.I.: 10.2 to 18.3], which was ameliorated in Lysm-Cre Tlr9<sup>fl/fl</sup> mice (n=8) (p<0.0001) [mean difference: -13; 95% C.I.: -16.3 to -9.7]. (e) Following NH<sub>4</sub>-Ac stimulation, there was a significant increase in the intracellular cytokine IL-6 produced by splenic macrophages in Tlr9<sup>fl/fl</sup> mice (n=10) compared to controls (n=8) (p<0.0001) [mean difference: 3; 95% C.I.: 2.5 to 3.5], which was ameliorated in Lysm-Cre Tlr9<sup>fl/fl</sup> mice (n=8) (p<0.0001) [mean difference: -2.7; 95% C.I.: -3.2 to -2.2].

**Figure 9: ODN2088 inhibits the ammonia-induced brain edema and cytokine production by macrophages in WT mice.**

(a) Administration of the TLR9 antagonist, ODN2088, along with NH<sub>4</sub>-Ac prevented the rise in BW content (n=10) (p<0.001) [mean difference: -1.07; 95% C.I.: -1.7 to -0.48] compared to the NH<sub>4</sub>-Ac alone stimulated mice (n=9) and there was no difference compared to controls (n=12). (b) Administration of ODN2088, along with NH<sub>4</sub>-Ac prevented the rise in the liver bodyweight ratio in WT mice (n=15) (p<0.0001) [mean difference: -0.02; 95% C.I.: -0.026 to -0.014] compared to the NH<sub>4</sub>-Ac alone stimulated mice (n=16) and there was no difference compared to controls (n=13). (c) Following NH<sub>4</sub>-Ac stimulation, there was a significant increase in the hepatocyte swelling in the liver histology (H&E stained – 200x magnifications) in WT mice compared to controls, where the cytoplasm of cells remained intact. Administration of ODN2088, ameliorated the hepatocyte swelling in WT mice. The
Following NH₄-Ac stimulation, there was a significant increase in the total plasma DNA level in WT mice (n=12) (p=0.02) [mean difference: 12.58; 95% C.I.: 0.5 to 5.4] and in WT mice administered with ODN2088 (n=16) [mean difference: 11.4; 95% C.I.: 0.6 to 5.2] compared to controls (n=12). There was no difference in the total plasma DNA level in NH₄-Ac alone WT mice stimulated compared to ODN2088 treated group. (e) Administration of ODN2088, along with NH₄-Ac prevented the rise in the intracellular cytokine IFN-gamma produced by splenic macrophages in WT mice (n=11) (p<0.0001) [mean difference: -2.5; 95% C.I.: -3.1 to -1.9] compared to the NH₄-Ac alone stimulated mice (n=8) and there was no difference compared to controls (n=7). (f) Administration of ODN2088, along with NH₄-Ac prevented the rise in the intracellular cytokine TNF-alpha produced by splenic macrophages in WT mice (n=11) (p<0.0001) [mean difference: -28.9; 95% C.I.: -32.1 to -25.6] compared to the NH₄-Ac alone stimulated mice (n=8) and there was no difference compared to controls (n=7). (g) Administration of ODN2088, along with NH₄-Ac prevented the rise in the intracellular cytokine IL-6 produced by splenic macrophages in WT mice (n=11) (p<0.0001) [mean difference: -5; 95% C.I.: -5.5 to -4.5] compared to the NH₄-Ac alone stimulated mice (n=8) and there was no difference compared to controls (n=7). (h) Representative FACS plots of the intracellular cytokines IFN-gamma, TNF-alpha and IL-6 produced by splenic macrophages in WT mice controls and NH₄-Ac stimulated WT mice with and without ODN2088.

Figure 10: ODN2088 inhibits the ammonia-induced cytokine production by T cells in WT mice.

Administration of the TLR9 antagonist, ODN2088, along with NH₄-Ac significantly inhibited (a) the intracellular cytokine IFN-gamma (p<0.01) [median difference: -1.8; 95% C.I.: -2.7 to -0.9], (b) the intracellular cytokine TNF-alpha (p<0.05) [median difference: -26; 95% C.I.: -
29.5 to -22] and (c) the intracellular cytokine IL-6 (p<0.001) [mean difference: -2; 95% C.I.: -2.8 to -1.1] produced by splenic CD4$^+$ T cells in WT mice (n=11) compared to the NH$_4$-Ac alone stimulated WT mice (n=11). ODN2088 along with NH$_4$-Ac also significantly inhibited (d) the intracellular cytokine IFN-gamma (p<0.0001) [mean difference: -3.3; 95% C.I.: -5.4 to -1.3], (e) the intracellular cytokine TNF-alpha (p<0.0001) [mean difference: -45.5; 95% C.I.: -49 to -42] produced by splenic CD8$^+$ T cells in WT mice (n=11) compared to the NH$_4$-Ac alone stimulated WT mice (n=11). (f) Representative FACS plots of the intracellular cytokines IFN-gamma, TNF-alpha and IL-6 produced by splenic T cells in WT mice controls and NH$_4$-Ac-stimulated WT mice with and without ODN2088.
Figure 1

(a) Brain water content (%)
- Control
- $\text{NH}_4\text{-Ac}$

(b) Liver/body weight ratio
- Control
- $\text{NH}_4\text{-Ac}$

(c) Histological images of liver:
- WT
- $Tlr9^{-/-}$

(d) dsDNA (pg/ml)
- Control
- $\text{NH}_4\text{-Ac}$
Figure 2

(a) IFN-γ^+ in CD11b+F4/80^+ (%)
   - Control
   - NH₂-Ac
   - WT
   - Tlr9^−/−
   - p<0.0001
   - p=0.0006

(b) TNF-α^+ in CD11b+F4/80^+ (%)
   - Control
   - NH₂-Ac
   - WT
   - Tlr9^−/−
   - p<0.0001
   - p<0.0001

(c) IL-6 in CD11b+F4/80^+ (%)
   - Control
   - NH₂-Ac
   - WT
   - Tlr9^−/−
   - p<0.0001
   - p<0.0001

(d) Flow cytometry plots:
   - WT Control
   - WT NH₂-Ac
   - Tlr9^−/− Control
   - Tlr9^−/− NH₂-Ac

- IFN-γ
- CD11b+F4/80^+
- TNF-α
- IL-6
Figure 5

Neutrophil phagocytosis (mean fluorescence intensity)

- Control
- NH₄-Ac

WT

Tlr9⁻/⁻
Figure 7

(a) Brain water content percentage

(b) Liver/body weight ratio

(c) Histological images of WT, NH₄Ac, Tlr9⁻/⁻, and Lysm-Cre Tlr9⁻/⁻ under NH₄Ac treatment

(d) IFN-γ+ in CD11b+ F4/80+ (%)

(e) TNF-α+ in CD11b+ F4/80+ (%)

(f) IL-6+ in CD11b+ F4/80+ (%)

(g) Flow cytometry analysis of CD11b-F4/80+ and cytokines (IFN-γ, TNF-α, IL-6) in different groups

(h) dsDNA levels (μg/mL)

Legend:

- WT
- Tlr9⁻/⁻
- Lysm-Cre Tlr9⁻/⁻