

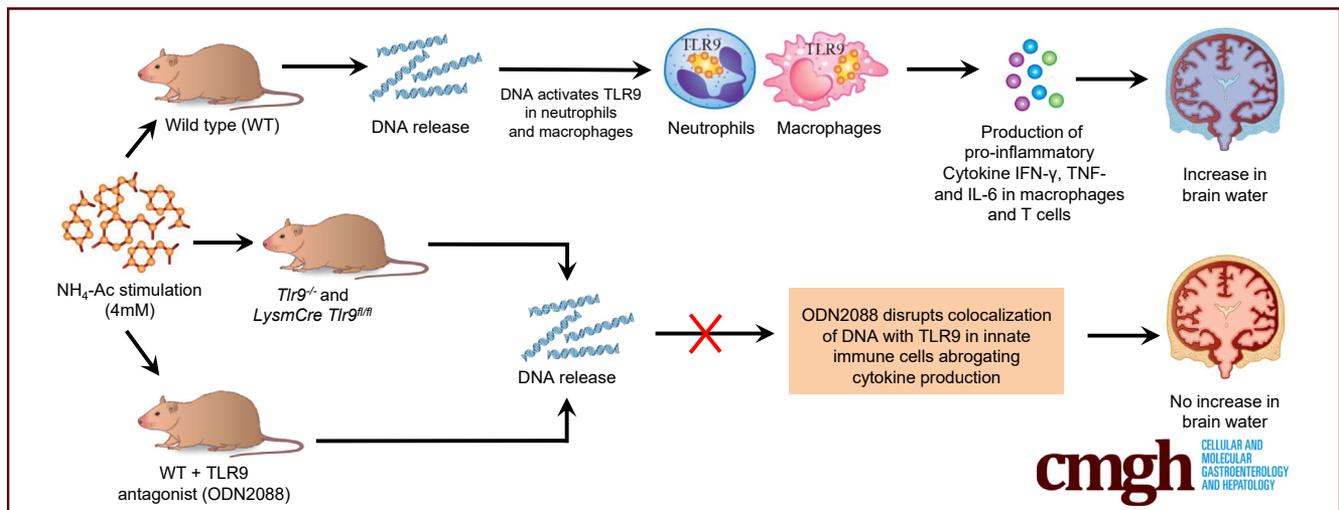
ORIGINAL RESEARCH

Ammonia-Induced Brain Edema Requires Macrophage and T Cell Expression of Toll-Like Receptor 9



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SUMMARY

Utilizing 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.

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 (NH₄-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 NH₄-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 NH₄-Ac injection, BW, macrophage and T cell cytokine production increased ($P < .0001$) in WT but not *Tlr9*^{-/-} mice ($P < .001$). ODN2088 inhibited macrophage and T cell cytokine production ($P < .05$) and prevented an increase in BW ($P < .0001$). Following NH₄-Ac injection, macrophage cytokine production and BW were ameliorated in *Lysm-Cre Tlr9*^{fl/fl} mice compared to WT mice ($P < .05$) but there was no difference compared to *Tlr9*^{-/-} mice. Following NH₄-Ac injection, plasma tDNA levels increased in WT and *Tlr9*^{-/-} mice ($P < .05$) 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. (*Cell Mol Gastroenterol Hepatol* 2019;8:609–623; <https://doi.org/10.1016/j.jcmgh.2019.08.002>)

Keywords: Ammonia; Brain edema; Immune dysfunction; Toll-like receptor 9.

See editorial on page 649.

Ammonia plays a pivotal role in the development of hepatic encephalopathy and brain edema in acute liver failure (ALF).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.⁴⁻¹⁰ Experimental models have unequivocally associated ammonia exposure with astrocyte swelling and brain edema, potentiated by proinflammatory cytokines.¹¹⁻¹⁴

Toll-like receptor 9 (TLR9) is an innate pattern recognition receptor that binds to the CpG motif of bacterial and mammalian DNA.¹⁵ TLR9 plays an important role in acetaminophen-induced liver inflammation,¹⁶ and we recently reported that ammonia along with DNA induces neutrophil TLR9 expression in patients with acetaminophen-induced ALF and advanced hepatic encephalopathy.¹⁷ Although the evidence base supporting the relationship between ammonia, inflammation and brain edema is robust in ALF, there is a paucity of data characterizing the specific pathogenic mechanisms entailed. We hypothesized 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,¹⁶ the hypothesis could only be tested in a murine model exposed to ammonium acetate (NH₄-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

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 6 hours after a single dose of NH₄-Ac (4 mM) injection (intraperitoneal). Following NH₄-Ac stimulation, there was a significant increase in the BW content in WT mice compared with control mice; which was significantly decreased in *Tlr9*^{-/-} mice compared with 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-to-body weight ratio and liver histopathology after NH₄-Ac stimulation. In WT mice, there was a significant increase in liver-to-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 and C). We hypothesized that DNA released following NH₄-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 NH₄-Ac stimulation compared with control mice, but there was no difference in *Tlr9*^{-/-} mice compared with WT mice following NH₄-Ac stimulation (Figure 1D).

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 (interferon gamma [IFN γ], tumor necrosis factor alpha [TNF α], and interleukin [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 2A-D) and CD4⁺ and CD8⁺ T cells (Figure 3A-F) isolated from the spleen in WT mice following NH₄-Ac stimulation, compared with control mice 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).

Ammonia-Induced Brain Edema and Inflammation Were Independent of Acetate or pH

Acetate has been reported to influence inflammation in acute alcoholic hepatitis¹⁸ 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 alkalinization.¹⁹ 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 (NaCH₃CO₂) (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-to-body weight ratio, or intracellular cytokine production of various immune cell subsets in WT mice compared with control mice, unlike NH₄-Ac (Figure 6A-F). These results confirm that the observed ammonia-induced cytokine production and brain edema

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Abbreviations used in this paper: ALF, acute liver failure; BW, brain water; IFN γ , interferon gamma; IL, interleukin; mAbs, monoclonal antibodies; Na-Ac, sodium acetate; NH₄-Ac, ammonium acetate; TNF α , tumor necrosis factor alpha; TLR9, Toll-like receptor 9; WT, wild-type.



Most current article

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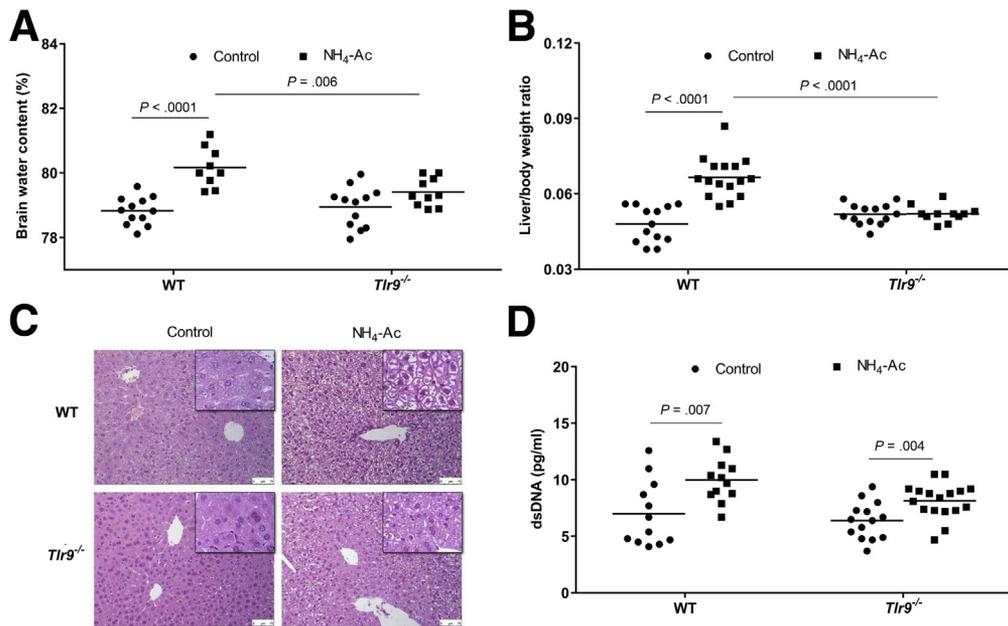


Figure 1. TLR9 stimulates ammonia-induced brain edema and liver-to-body weight ratio through DNA. (A) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the BW content in WT mice ($n = 9$) compared with control mice ($n = 12$) ($P < .0001$) (mean difference: 1.35; 95% confidence interval [CI], 0.9 to 1.8), which was ameliorated in $Tlr9^{-/-}$ mice ($n = 10$) ($P = .006$) (mean difference: -0.76 ; 95% CI, -1.3 to -0.25). (B) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the liver-to-body weight ratio in WT mice ($n = 16$) compared with control mice ($n = 13$) ($P < .0001$) (mean difference: 0.019; 95% CI, 0.013 to 0.024), which was ameliorated in $Tlr9^{-/-}$ mice ($n = 11$) ($P < .0001$) (mean difference: -0.014 ; 95% CI, -0.019 to -0.009). (C) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the hepatocyte swelling in the liver histology (hematoxylin and eosin stain, $200\times$ magnification) in WT mice compared with control mice, where the cytoplasm of cells remained intact. Following $\text{NH}_4\text{-Ac}$ stimulation, there was no difference in the hepatocyte morphology in $Tlr9^{-/-}$ mice compared with control mice but the hepatocyte swelling was reduced compared with WT mice. The images in the black box inset are the representative images at $400\times$ magnification. (D) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the total plasma DNA level in WT mice ($n = 12$) compared with control mice ($n = 12$) ($P = .007$) (mean difference: 2.97; 95% CI, 0.88 to 5) and in $Tlr9^{-/-}$ mice ($n = 17$) compared with control mice ($n = 14$) ($P = .004$) (mean difference: 1.76; 95% CI, 0.6 to 2.9).

were induced by ammonia per se, and not influenced by acetate or pH changes.

Ammonia-Induced Brain Edema and Inflammation Were Mediated by TLR9 Expressed Within Lysozyme-Expressing Cells

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

$\text{NH}_4\text{-Ac}$ induced inflammation compared with *Lysm-Cre Tlr9^{fl/fl}* mice (Figure 8A–E).

TLR9 Antagonism Abrogates Ammonia-Induced Brain Edema and Inflammation

As $Tlr9^{-/-}$ mice were protected against $\text{NH}_4\text{-Ac}$ stimulation, we tested whether an antagonist of TLR9 (ODN2088) could inhibit the ammonia-induced proinflammatory changes and brain edema observed in the WT mice. Administration of ODN2088 (50 $\mu\text{g}/\text{mouse}$) with $\text{NH}_4\text{-Ac}$ (4 mM) significantly decreased the BW content and liver-to-body weight ratio and ameliorated the hepatocyte swelling (Figure 9A–C). Administration of ODN2088 did not alter the total plasma DNA levels in WT mice following $\text{NH}_4\text{-Ac}$ stimulation but they were increased compared with control mice (Figure 9D). There was also a significant reduction in the cytokines produced by macrophages (Figures 9E–G) and T cells (Figure 10A–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

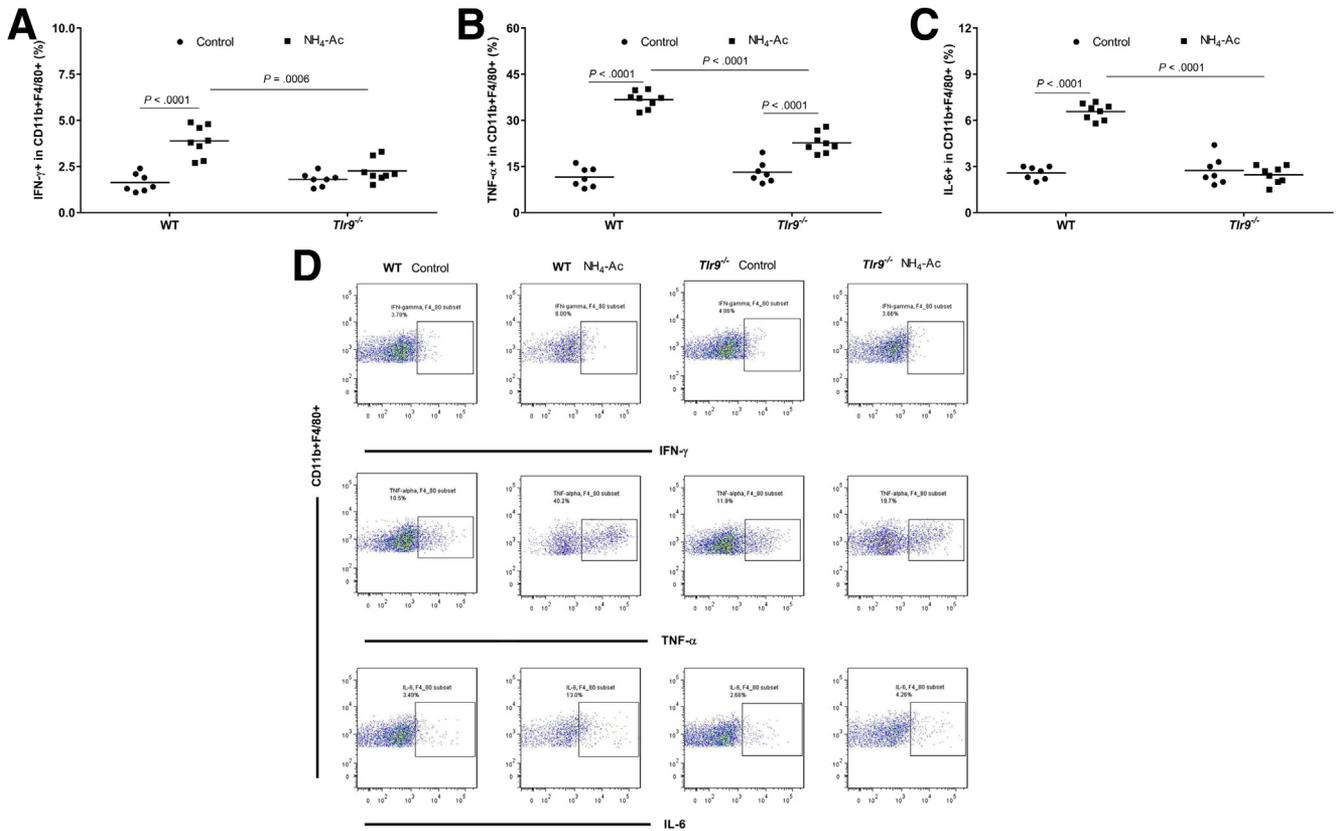


Figure 2. Ammonia-induced intracellular cytokine production by macrophages is mediated by TLR9. (A) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine IFN γ produced by splenic macrophages in WT mice (n = 8) compared with control mice (n = 7) ($P < .0001$) (mean difference: 2.3; 95% CI, 1.5 to 3), which was ameliorated in *Tlr9*^{-/-} mice (n = 8) ($P = .0006$) (mean difference: -1.6; 95% CI, -2.4 to -0.83). (B) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine TNF α produced by splenic macrophages in WT mice (n = 8) compared with control mice (n = 7) ($P < .0001$) (mean difference: 25; 95% CI, 22 to 28.5), which was ameliorated in *Tlr9*^{-/-} mice (n = 8) ($P < .0001$) (mean difference: -14; 95% CI, -17.2 to -10.8). (C) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine IL-6 produced by splenic macrophages in WT mice (n = 8) compared with control mice (n = 7) ($P < .0001$) (mean difference: 4; 95% CI, 3.5 to 4.5), which was ameliorated in *Tlr9*^{-/-} mice (n = 8) ($P < .0001$) (mean difference: -4; 95% CI, -4.7 to -3.5). (D) Representative FACS plots of intracellular cytokines IFN γ , TNF α , and IL-6 produced by splenic macrophages in WT mice and *Tlr9*^{-/-} control mice, and following NH₄-Ac stimulation.

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 γ , TNF α , 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 γ , TNF α , 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 proinflammatory 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 proinflammatory 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 α and IL-6 in response to CpG DNA and IFN γ produced by CD4⁺ T cells in response to CpG DNA and mycobacteria.^{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 nonalcoholic steatohepatitis.^{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

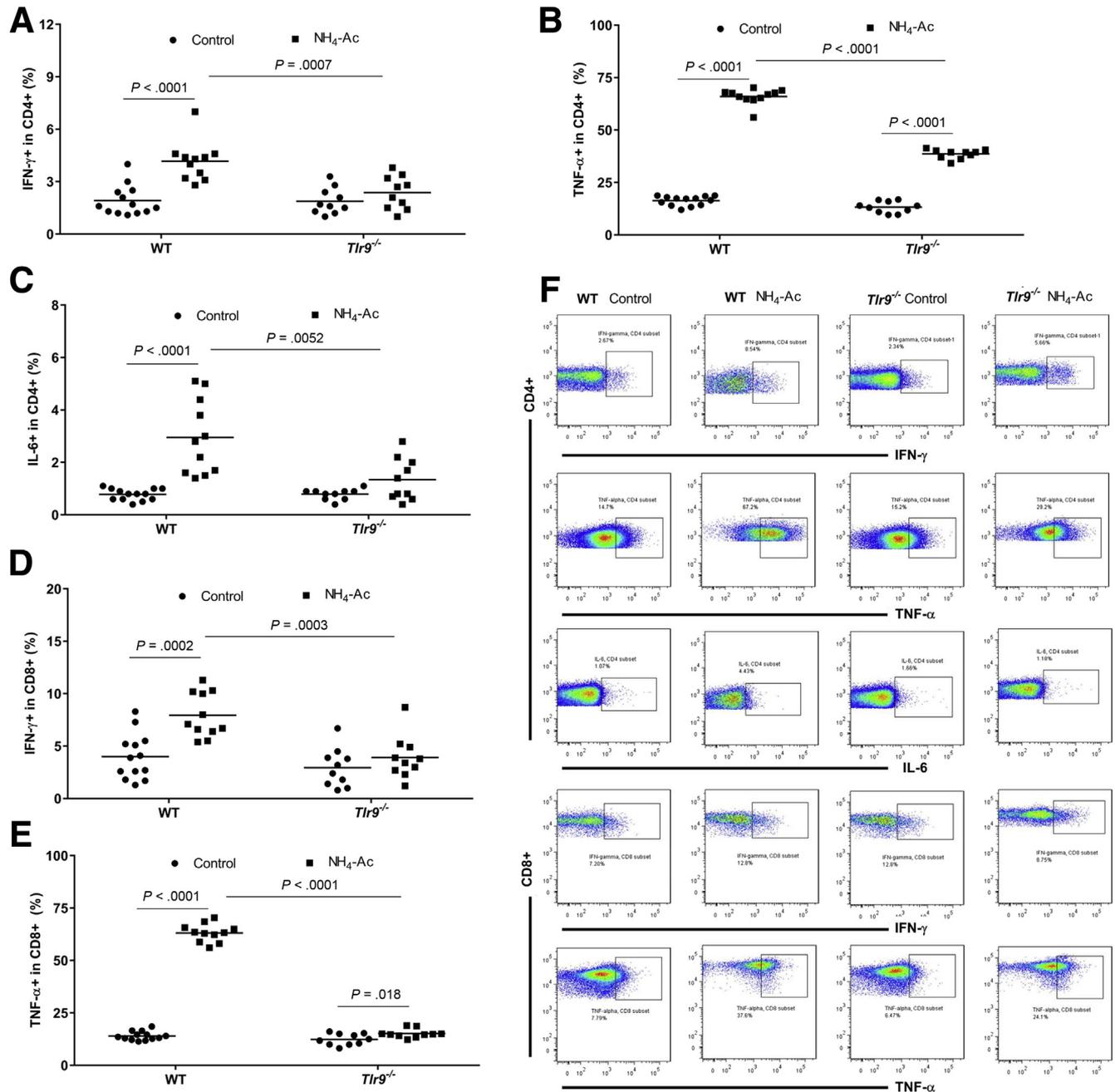


Figure 3. Ammonia-induced intracellular cytokine production by T cells is mediated by TLR9. (A) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine IFN γ produced by splenic CD4⁺ T cells in WT mice compared with control mice ($P < .0001$) (median difference: 2.7; 95% CI, 1.5 to 3), which was ameliorated in *Tlr9*^{-/-} mice compared with WT mice ($P = .0007$) (median difference: -1.9; 95% CI, -2.8 to -0.8). (B) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine TNF α produced by splenic CD4⁺ T cells in WT mice compared with control mice ($P < .0001$) (mean difference: 49.5; 95% CI, 47 to 52), which was ameliorated in *Tlr9*^{-/-} mice compared with WT mice ($P < .0001$) (mean difference: -27.5; 95% CI, -30 to -25). (C) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine IL-6 produced by splenic CD4⁺ T cells in WT mice compared with control mice ($P < .0001$) (mean difference: 2.2; 95% CI, 1.4 to 3), which was ameliorated in *Tlr9*^{-/-} mice compared with WT mice ($P = .0052$) (mean difference: -1.6; 95% CI, -2.7 to -0.5). (D) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine IFN γ produced by splenic CD8⁺ T cells in WT mice compared with control mice ($P = .0002$) (mean difference: 3.9; 95% CI, 2.1 to 5.8), which was ameliorated in *Tlr9*^{-/-} mice compared with WT mice ($P = .0003$) (median difference: -3.5; 95% CI, -6.4 to -2.1). (E) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine TNF α produced by splenic CD8⁺ T cells in WT mice compared with control mice ($P < .0001$) (mean difference: 49; 95% CI, 46 to 52), which was ameliorated in *Tlr9*^{-/-} mice compared with WT mice ($P < .0001$) (mean difference: -48; 95% CI, -51 to -45). (F) Representative FACS plots of the intracellular cytokines IFN γ , TNF α , and IL-6 produced by splenic T cells in WT mice and *Tlr9*^{-/-} control mice and following NH₄-Ac stimulation. WT control mice (n = 11) and NH₄-Ac-treated mice (n = 13); *Tlr9*^{-/-} control mice (n = 10) and NH₄-Ac-treated mice (n = 10).

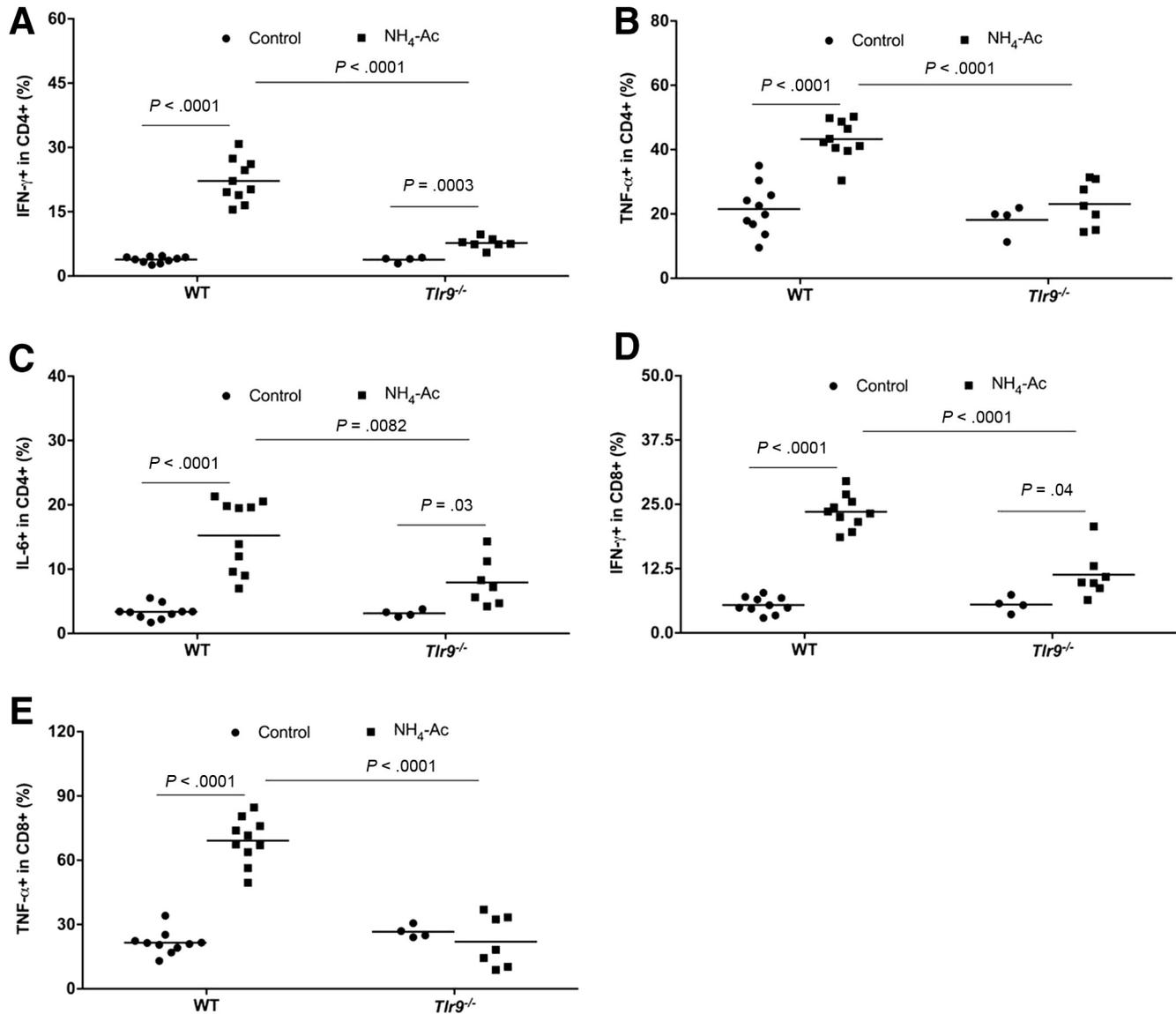


Figure 4. Ammonia-induced intracellular cytokine production by liver-infiltrated T cells. (A) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the intracellular cytokine $\text{IFN}\gamma$ produced by liver-infiltrated CD4^+ T cells in WT mice compared with control mice ($P < .0001$) (median difference: 18.3; 95% CI, 15 to 21.6), which was ameliorated in $\text{Tlr9}^{-/-}$ mice compared with WT mice ($P < .0001$) (median difference: -14.5; 95% CI, -18.6 to -10.3). (B) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the intracellular cytokine $\text{TNF}\alpha$ produced by liver-infiltrated CD4^+ T cells in WT mice compared with control mice ($P < .0001$) (mean difference: 21.4; 95% CI, 14.5 to 28.2), which was ameliorated in $\text{Tlr9}^{-/-}$ mice compared with WT mice ($P < .0001$) (mean difference: -20; 95% CI, -27 to -12.7). (C) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the intracellular cytokine IL-6 produced by liver-infiltrated CD4^+ T cells in WT mice compared with control mice ($P < .0001$) (mean difference: 11.9; 95% CI, 8.1 to 15.6), which was ameliorated in $\text{Tlr9}^{-/-}$ mice compared with WT mice ($P = .0082$) (mean difference: -7.3; 95% CI, -12.4 to -2.2). (D) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the intracellular cytokine $\text{IFN}\gamma$ produced by liver-infiltrated CD8^+ T cells in WT mice compared with control mice ($P < .0001$) (mean difference: 18.3; 95% CI, 15.8 to 20.9), which was ameliorated in $\text{Tlr9}^{-/-}$ mice compared with WT mice ($P < .0001$) (median difference: -12.4; 95% CI, -16.7 to -8.2). (E) Following $\text{NH}_4\text{-Ac}$ stimulation, there was a significant increase in the intracellular cytokine $\text{TNF}\alpha$ produced by liver-infiltrated CD8^+ T cells in WT mice compared with control mice ($P < .0001$) (mean difference: 47.3; 95% CI, 39 to 56), which was ameliorated in $\text{Tlr9}^{-/-}$ mice compared with WT mice ($P < .0001$) (mean difference: -44; 95% CI, -56 to -32). WT control mice ($n = 10$) and $\text{NH}_4\text{-Ac}$ -treated mice ($n = 10$); $\text{Tlr9}^{-/-}$ control mice ($n = 4$) and $\text{NH}_4\text{-Ac}$ -treated mice ($n = 7$).

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.^{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,¹⁷ suggesting that TLR9 mediates the ammonia-induced brain edema in a DNA-driven manner. In this study, it is not possible to identify

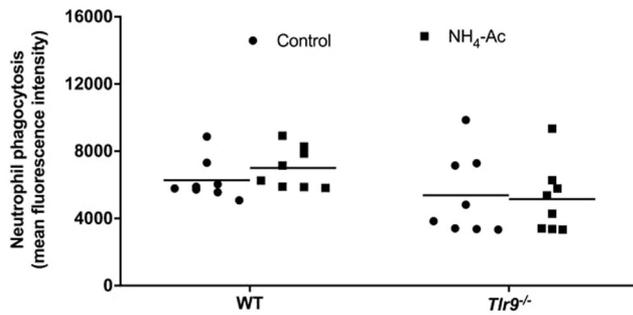


Figure 5. Neutrophil phagocytosis measured by dextran fluorescein isothiocyanate (FITC) using flow cytometry in WT and Tlr9^{-/-} mice after NH₄-Ac stimulation. Following NH₄-Ac stimulation, there was no difference in the mean fluorescence intensity of dextran FITC in neutrophils isolated from whole blood in WT mice or Tlr9^{-/-} mice compared with control mice.

the source of the DNA, and we can only speculate that nonimmune 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.²⁹ recently demonstrated that neutrophils are the predominant innate immune cells that sense DNA through the TLR9/nuclear factor-kappa B 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.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.,¹⁶ who in an acetaminophen-induced hepatotoxicity model, established that inhibition of TLR9 using ODN2088 and IRS954, a TLR7/9 antagonist, downregulated proinflammatory 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 rat³⁰ and pig,³¹ and the rat with thioacetamide-induced toxic liver injury.³⁰ While these models develop cerebral edema and exhibit intracranial hypertension, the impact of hepatic devascularization 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.¹⁶ Therefore, we could not utilize 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 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.^{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 intracranial hypertension.

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. Four strains of mice on C57BL/6 genetic background were used for the experiments. C57BL/6 (WT) were originally obtained from the Jackson Laboratory (Bar Harbor, ME). The original Tlr9^{-/-} and Tlr9^{fl/fl} breeders were kindly provided by Professor Shizuo Akira (Japan)¹⁵ and Professor Mark Shlomchik (University of Pittsburgh),²⁰ respectively. We bred *Lysm-Cre* mice with Tlr9^{fl/fl} mice to generate *Lysm-Cre Tlr9^{fl/fl}* mice with TLR9 specific deletion in lysozyme expressing cells.²⁰ All the mice used in the study were bred and kept at the Yale animal facility in specific pathogen-free 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, NH₄-Ac (NH₄CH₃CO₂) (4 mmol/kg of bodyweight) was injected intraperitoneally in WT mice, Tlr9^{-/-} mice, *Lysm-Cre Tlr9^{fl/fl}* mice, and Tlr9^{fl/fl} mice that were sacrificed 6 hours after the injection. The concentration and duration of NH₄-Ac used for this experiment was optimized in a pilot experiment. Na-Ac (NaCH₃CO₂) (J.T. Baker) (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 homogenized using rough sides of 2 grinding slides and red blood cells were lysed by hypotonic solution and quickly restored in isotonic phosphate-buffered saline. The single suspension of splenocytes (10⁶) was stained with monoclonal antibodies (mAbs) conjugated with different fluorochromes before flow cytometry analysis. Liver tissue was homogenized using a plunge through a wire mesh and

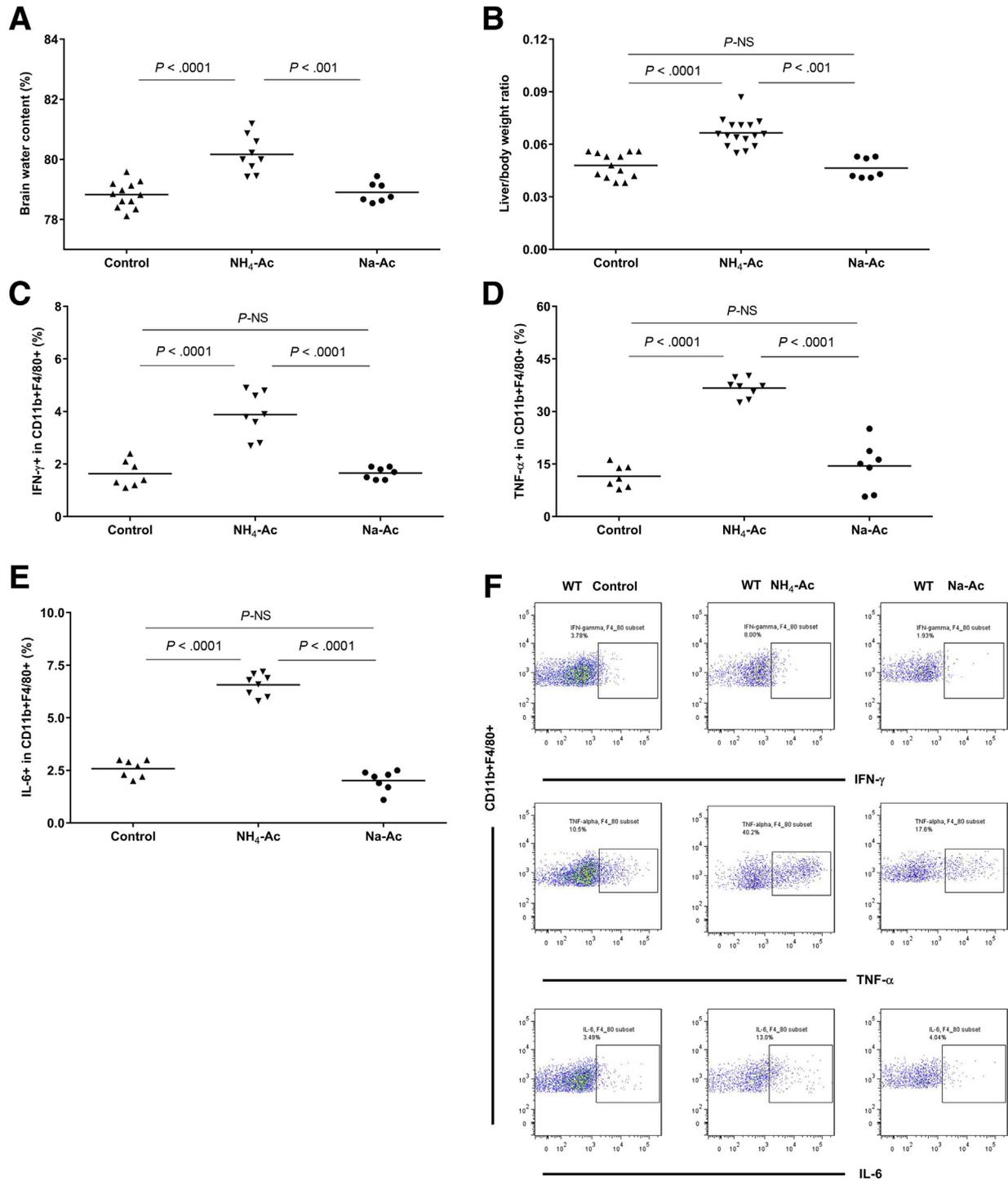
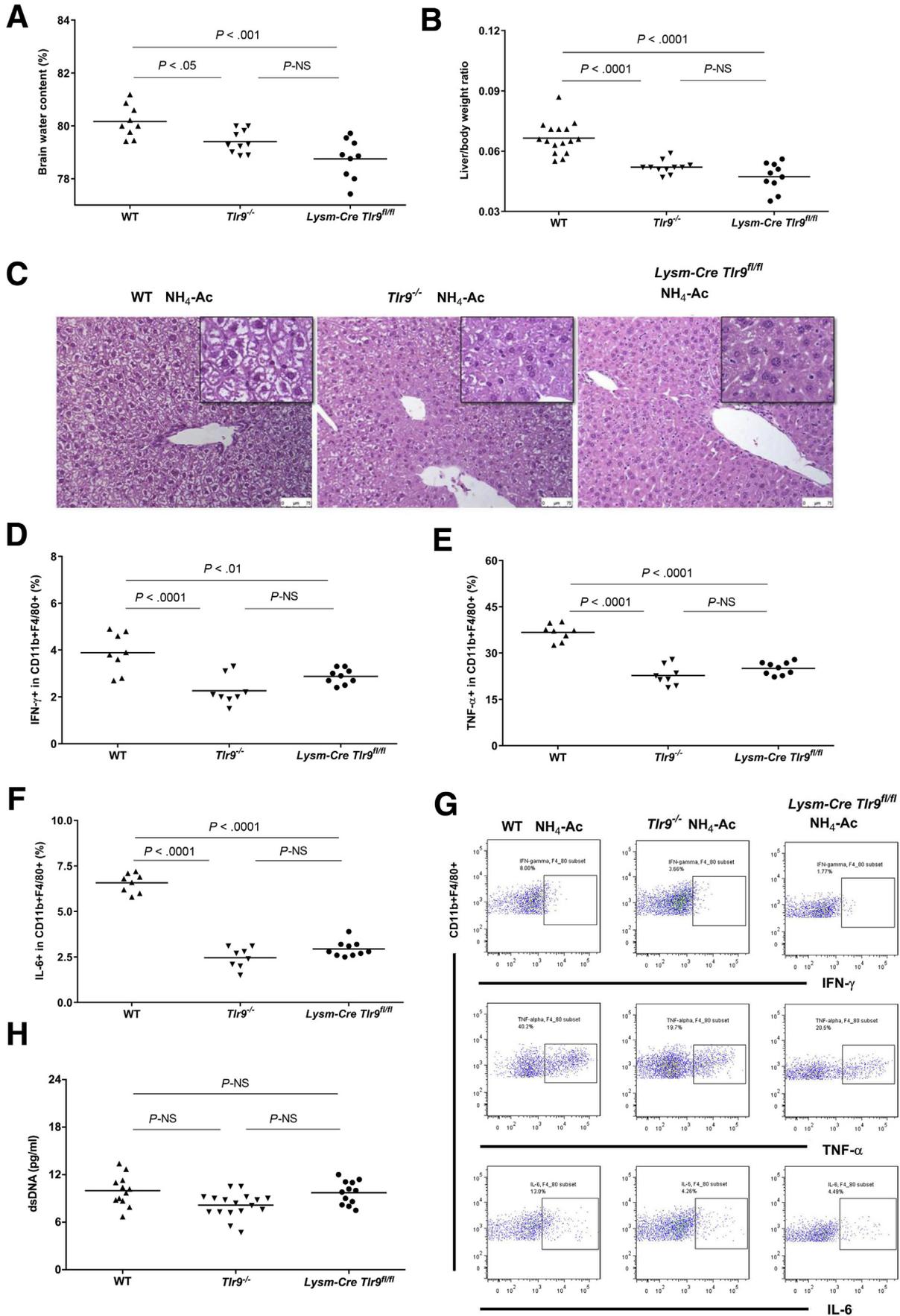


Figure 6. Unaltered brain edema and intracellular cytokine production by macrophages after Na-Ac stimulation. (A) Following Na-Ac stimulation, BW content remained unaltered in WT mice ($n = 7$) compared with control mice ($n = 12$), but was significantly reduced compared with the NH₄-Ac-stimulated WT mice ($n = 9$) ($P < .001$) (mean difference: -1.1 ; 95% CI, -1.7 to -0.47). (B) Following Na-Ac stimulation, liver-to-body weight ratio remained unaltered in WT mice ($n = 7$) compared with control mice ($n = 13$), but was significantly reduced compared with the NH₄-Ac-stimulated WT mice ($n = 16$) ($P < .001$) (mean difference: -0.02 ; 95% CI, -0.028 to -0.012). Following Na-Ac stimulation, the intracellular cytokine (C) IFN γ ($P < .0001$) (mean difference: -2.2 ; 95% CI, -3 to -1.4), (D) TNF α ($P < .0001$) (mean difference: -22.3 ; 95% CI, -28.3 to -16.3), and (E) IL-6 ($P < .0001$) (mean difference: -4.5 ; 95% CI, -5.2 to -3.9) produced by splenic macrophages were significantly reduced in WT mice ($n = 7$) compared with the NH₄-Ac-stimulated WT mice ($n = 8$) but remained unaltered compared with control mice ($n = 7$). (F) Representative FACS plots of the intracellular cytokines IFN γ , TNF α , and IL-6 produced by splenic macrophages in WT control mice, NH₄-Ac-stimulated WT mice and Na-Ac-stimulated WT mice.



digested using collagenase-I and DNase-I (Sigma-Aldrich, St Louis, MO). Liver-infiltrated immune cells were isolated from the homogenized tissue using the density-gradient Polymorphprep solution (Axis Shield, Oslo, Norway), stained with mAbs and analyzed 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 formula³²:

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

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. Then, 6- μ M tissue sections were stained with hematoxylin and eosin 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, UK) as detailed previously.²⁰

TLR9 Antagonist Injection

To determine whether an inhibitor of TLR9 offers protection against NH₄-Ac stimulation, the TLR9 antagonist (ODN2088) (InvivoGen, San Diego, CA) (50 μ g/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 on a recently established mouse model of acetaminophen hepatotoxicity.¹⁶

Stimulation of Intracellular Cytokine Production

To determine the intracellular cytokine production of mononuclear cells from spleen and liver, up to 5.0×10^6 cells per mL were stimulated with phorbol 12-myristate 13-acetate (50 ng) and ionomycin (InvivoGen, San Diego, CA) (500 pg) in complete media with Golgi plug (BD Biosciences, San Jose, CA) (1 μ L) 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 analyzed using flow cytometry.

Cell Staining and Flow Cytometry

Up to 1 million (1×10^6) cells were resuspended in 100 μ L of staining buffer and stained with different fluorochrome conjugated mAbs in a tube followed by incubation at room temperature in darkness for 30 minutes and the stained cells were washed with phosphate-buffered saline. For intracellular cytokine staining, 100 μ L of cytofix/cytoperm solution (BD Biosciences, San Jose, CA) was added to the cell pellet after staining with surface markers and kept at room temperature for 20 minutes. The cells were washed with 1 mL of permeabilization wash buffer (BD Biosciences) followed by resuspension in 300 μ L of phosphate-buffered saline and acquired in a LSRII flow cytometry (BD Biosciences) using BD FACS DIVA software V6.0 (BD Biosciences).

Neutrophil Phagocytosis

The phagocytic ability of neutrophils was determined by incubating the whole blood with Dextran FITC (1 mg/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

Figure 7. (See previous page). 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) ($P < .05$) (mean difference: -0.76; 95% CI, -1.5 to -0.06) and *Lysm-Cre Tlr9*^{fl/fl} mice (n = 9) ($P < .001$) (mean difference: -1.4; 95% CI, -2 to -0.7) compared with WT mice (n = 9). (B) Following NH₄-Ac stimulation, liver-to-body weight ratio was significantly ameliorated in *Tlr9*^{-/-} mice (n = 11) ($P < .0001$) (mean difference: -0.015; 95% CI, -0.021 to -0.008) and *Lysm-Cre Tlr9*^{fl/fl} mice (n = 10) ($P < .0001$) (mean difference: -0.019; 95% CI, -0.026 to -0.013) compared with WT mice (n = 16). (C) Following NH₄-Ac stimulation, there was a significant increase in the hepatocyte swelling in the liver histology (hematoxylin and eosin stain, 200 \times magnification) in WT mice compared with *Tlr9*^{-/-} mice and *Lysm-Cre Tlr9*^{fl/fl} mice, where the cytoplasm of cells remained intact. The images in the black box inset are the representative images at 400 \times magnification. (D) Following NH₄-Ac stimulation, the intracellular cytokine IFN γ produced by splenic macrophages was significantly ameliorated in *Tlr9*^{-/-} mice (n = 8) ($P < .0001$) (mean difference: -1.6; 95% CI, -2.4 to -0.84) and *Lysm-Cre Tlr9*^{fl/fl} mice (n = 9) ($P < .01$) (mean difference: -1; 95% CI, -1.75 to -0.25) compared with WT mice (n = 8). (E) Following NH₄-Ac stimulation, the intracellular cytokine TNF α produced by splenic macrophages was significantly ameliorated in *Tlr9*^{-/-} mice (n = 8) ($P < .0001$) (mean difference: -14; 95% CI, -17.4 to -10.6) and *Lysm-Cre Tlr9*^{fl/fl} mice (n = 9) ($P < .0001$) (mean difference: -11.7; 95% CI, -15 to -8.4) compared with WT mice (n = 8). (F) Following NH₄-Ac stimulation, the intracellular cytokine IL-6 produced by splenic macrophages was significantly ameliorated in *Tlr9*^{-/-} mice (n = 8) ($P < .0001$) (mean difference: -4.1; 95% CI, -4.7 to -3.4) and *Lysm-Cre Tlr9*^{fl/fl} mice (n = 9) ($P < .0001$) (mean difference: -3.6; 95% CI, -4.2 to -3) compared with WT mice (n = 8). (G) Representative FACS plots of the intracellular cytokines IFN γ , TNF α , and IL-6 produced by splenic macrophages in WT mice, *Tlr9*^{-/-} mice and *Lysm-Cre Tlr9*^{fl/fl} mice following NH₄-Ac stimulation. (H) There was no difference in the total plasma DNA level between WT mice (n = 12), *Tlr9*^{-/-} mice (n = 17) and *Lysm-Cre Tlr9*^{fl/fl} mice (n = 12).

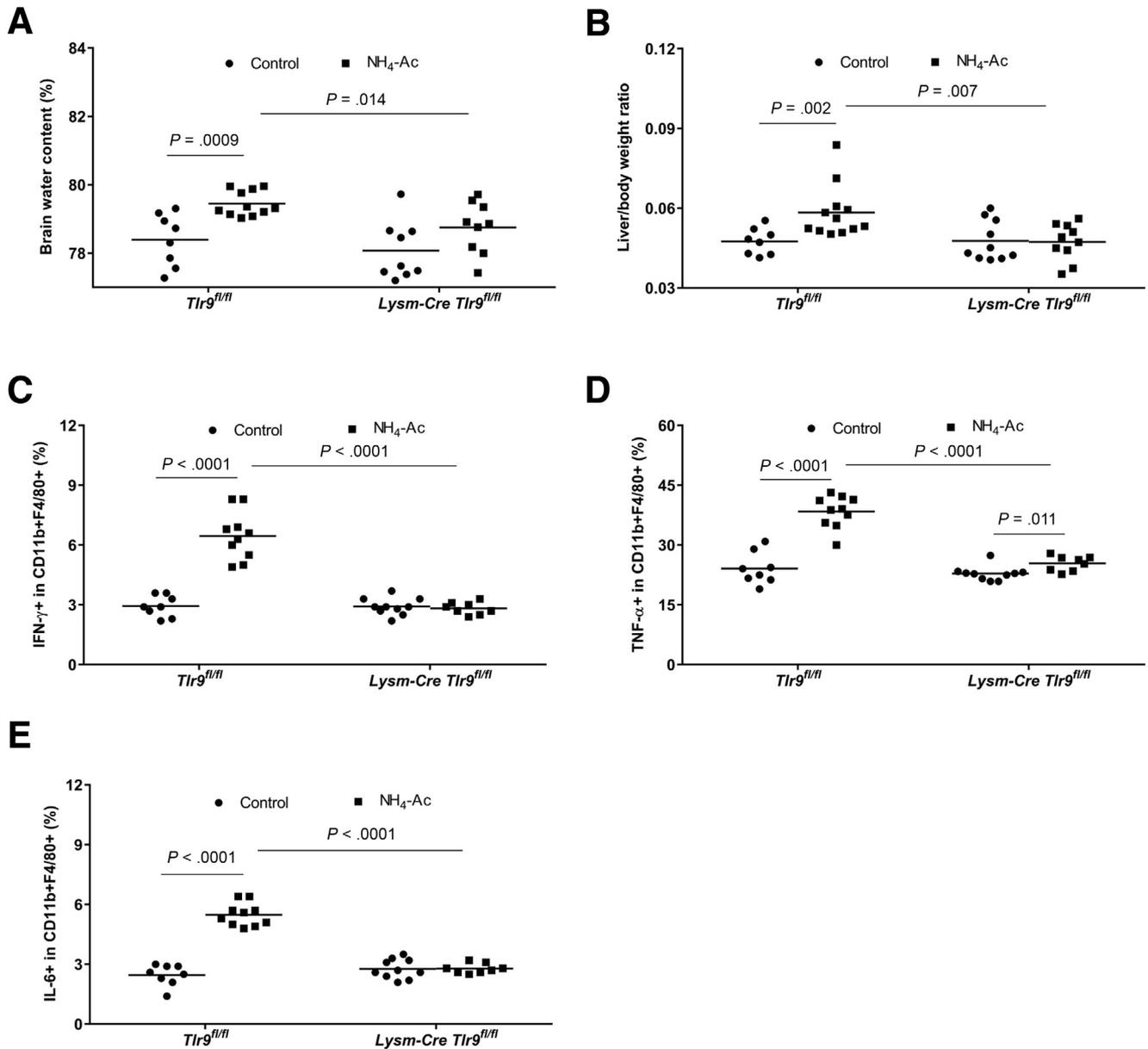
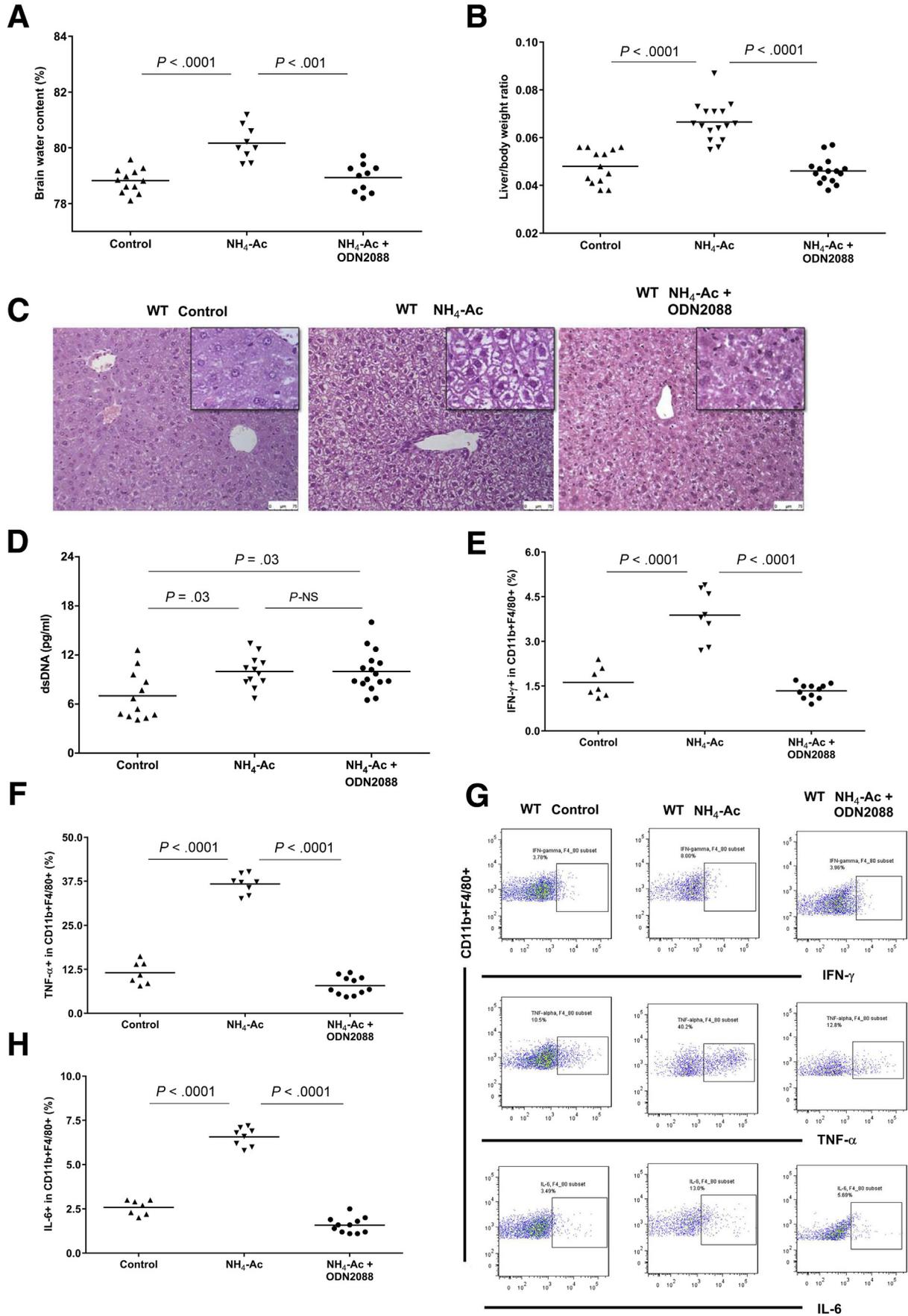


Figure 8. Ammonia-induced brain edema and cytokine production in *Lysm-Cre Tlr9^{fl/fl}* mice compared with *Tlr9^{fl/fl}* mice. (A) Following NH₄-Ac stimulation, there was a significant increase in the BW content in *Tlr9^{fl/fl}* mice (n = 11) compared with control mice (n = 8) ($P = .0009$) (mean difference: 1.05; 95% CI, 0.5 to 1.6), which was ameliorated in *Lysm-Cre Tlr9^{fl/fl}* mice (n = 9) ($P = .014$) (mean difference: -0.7; 95% CI, -1.2 to -0.16). (B) Following NH₄-Ac stimulation, there was a significant increase in the liver-to-body weight ratio in *Tlr9^{fl/fl}* mice (n = 12) compared with control mice (n = 8) ($P = .002$) (mean difference: 0.007; 95% CI, 0.003 to 0.015), which was ameliorated in *Lysm-Cre Tlr9^{fl/fl}* mice (n = 10) ($P = .007$) (mean difference: -0.008; 95% CI, -0.017 to -0.002). (C) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine IFN γ produced by splenic macrophages in *Tlr9^{fl/fl}* mice (n = 10) compared with control mice (n = 8) ($P < .0001$) (mean difference: 3.5; 95% CI, 2.5 to 4.5), which was ameliorated in *Lysm-Cre Tlr9^{fl/fl}* mice (n = 8) ($P < .0001$) (mean difference: -3.6; 95% CI, -4.6 to -2.7). (D) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine TNF α produced by splenic macrophages in *Tlr9^{fl/fl}* mice (n = 10) compared with control mice (n = 8) ($P < .0001$) (mean difference: 14.3; 95% CI, 10.2 to 18.3), which was ameliorated in *Lysm-Cre Tlr9^{fl/fl}* mice (n = 8) ($P < .0001$) (mean difference: -13; 95% CI, -16.3 to -9.7). (E) Following NH₄-Ac stimulation, there was a significant increase in the intracellular cytokine IL-6 produced by splenic macrophages in *Tlr9^{fl/fl}* mice (n = 10) compared with control mice (n = 8) ($P < .0001$) (mean difference: 3; 95% CI, 2.5 to 3.5), which was ameliorated in *Lysm-Cre Tlr9^{fl/fl}* mice (n = 8) ($P < .0001$) (mean difference: -2.7; 95% CI, -3.2 to -2.2).



CD3 (17A2); CD4 (GK1.5) and CD8 (53-6.7) markers were used to characterize the different subsets of T cells and macrophages were identified using F4/80 (BM8) and CD11b (M1/70) markers. Intracellular cytokine production (IL-6 [MP5-20F3], IFN γ [XMG1.2], and TNF α [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 (San Diego, CA).

Statistics

For comparisons between 2 groups, Student's *t* test (parametric data) or Mann-Whitney *U* test (nonparametric data) were used; for comparisons among 3 or more groups, 1-way analysis of variance with Tukey's multiple comparison tests (parametric data) or Kruskal Wallis with Dunn's multiple comparison tests (nonparametric 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. Hypothesis testing was 2 tailed at an alpha level of .05. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA); *P* < .05 was considered as statistically significant.

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Figure 9. (See previous page). ODN2088 inhibits the ammonia-induced brain edema and cytokine production by macrophages in WT mice. (A) Administration of the TLR9 antagonist, ODN2088, along with NH₄-Ac prevented the rise in BW content (*n* = 10) (*P* < .001) (mean difference: -1.07; 95% CI, -1.7 to -0.48) compared with the NH₄-Ac alone-stimulated mice (*n* = 9) and there was no difference compared with control mice (*n* = 12). (B) Administration of ODN2088, along with NH₄-Ac prevented the rise in the liver-to-body weight ratio in WT mice (*n* = 15) (*P* < .0001) (mean difference: -0.02; 95% CI, -0.026 to -0.014) compared with the NH₄-Ac alone-stimulated mice (*n* = 16) and there was no difference compared with control mice (*n* = 13). (C) Following NH₄-Ac stimulation, there was a significant increase in the hepatocyte swelling in the liver histology (hematoxylin and eosin stain, 200 \times magnifications) in WT mice compared with control mice, where the cytoplasm of cells remained intact. Administration of ODN2088, ameliorated the hepatocyte swelling in WT mice. The images in the black box inset are the representative images at 400 \times magnification. (D) Following NH₄-Ac stimulation, there was a significant increase in the total plasma DNA level in WT mice (*n* = 12) (*P* = .02) (mean difference: 12.58; 95% CI, 0.5 to 5.4) and in WT mice administered with ODN2088 (*n* = 16) (mean difference: 11.4; 95% CI, 0.6 to 5.2) compared with control mice (*n* = 12). There was no difference in the total plasma DNA level in NH₄-Ac alone WT mice stimulated compared with ODN2088 treated group. (E) Administration of ODN2088, along with NH₄-Ac prevented the rise in the intracellular cytokine IFN γ produced by splenic macrophages in WT mice (*n* = 11) (*P* < .0001) (mean difference: -2.5; 95% CI, -3.1 to -1.9) compared with the NH₄-Ac alone-stimulated mice (*n* = 8) and there was no difference compared with control mice (*n* = 7). (F) Administration of ODN2088, along with NH₄-Ac prevented the rise in the intracellular cytokine TNF α produced by splenic macrophages in WT mice (*n* = 11) (*P* < .0001) (mean difference: -28.9; 95% CI, -32.1 to -25.6) compared with the NH₄-Ac alone-stimulated mice (*n* = 8) and there was no difference compared with control mice (*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* < .0001) (mean difference: -5; 95% CI, -5.5 to -4.5) compared with the NH₄-Ac alone-stimulated mice (*n* = 8) and there was no difference compared with control mice (*n* = 7). (H) Representative FACS plots of the intracellular cytokines IFN γ , TNF α , and IL-6 produced by splenic macrophages in WT control mice and NH₄-Ac-stimulated WT mice with and without ODN2088.

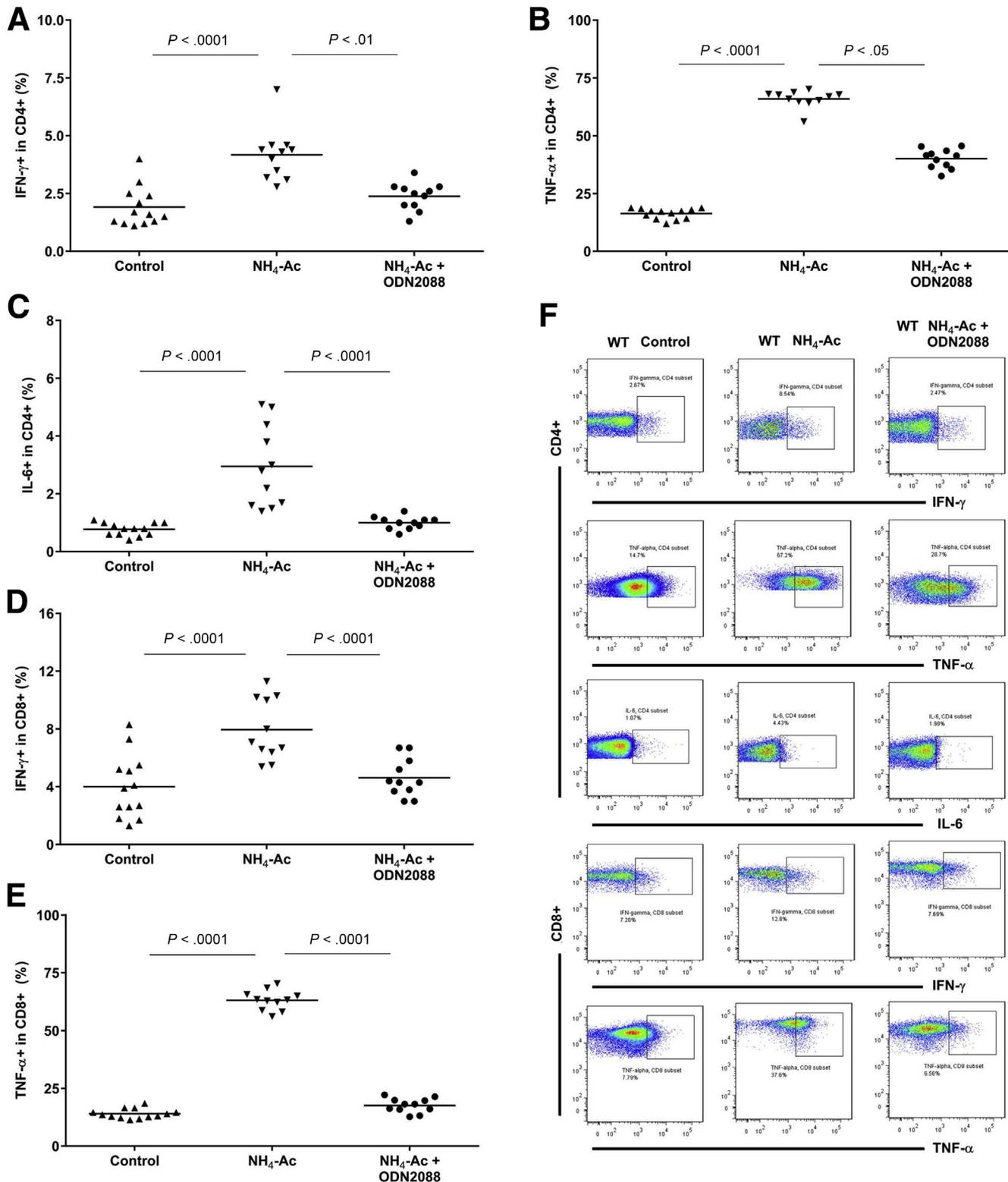


Figure 10. ODN2088 inhibits the ammonia-induced cytokine production by T cells in WT mice. Administration of the TLR9 antagonist, ODN2088, along with NH₄-Ac significantly inhibited (A) the intracellular cytokine IFN γ ($P < .01$) (median difference: -1.8 ; 95% CI, -2.7 to -0.9), (B) the intracellular cytokine TNF α ($P < .05$) (median difference: -26 ; 95% CI, -29.5 to -22), and (C) the intracellular cytokine IL-6 ($P < .001$) (mean difference: -2 ; 95% CI, -2.8 to -1.1) produced by splenic CD4⁺ T cells in WT mice ($n = 11$) compared with the NH₄-Ac alone-stimulated WT mice ($n = 11$). ODN2088 along with NH₄-Ac also significantly inhibited (D) the intracellular cytokine IFN γ ($P < .0001$) (mean difference: -3.3 ; 95% CI, -5.4 to -1.3) and (E) the intracellular cytokine TNF α ($P < .0001$) (mean difference: -45.5 ; 95% CI, -49 to -42) produced by splenic CD8⁺ T cells in WT mice ($n = 11$) compared with the NH₄-Ac alone-stimulated WT mice ($n = 11$). (F) Representative FACS plots of the intracellular cytokines IFN γ , TNF α , and IL-6 produced by splenic T cells in WT control mice and NH₄-Ac-stimulated WT mice with and without ODN2088.

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Conflicts of interest

The authors disclose no conflicts.

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