

TLR9 and IRF3 Cooperate to Induce a Systemic Inflammatory Response in Mice Injected With Liposome:DNA

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Liposome:DNA is a promising gene therapy vector. However, this vector can elicit a systemic inflammatory response syndrome (SIRS). Prior reports indicate that liposome:DNA vectors activate Toll-like receptor (TLR)9. We hypothesized that liposome:DNA vectors also activate the cytosolic DNA-sensing pathway, which signals *via* interferon (IFN) regulatory factor (IRF)3. To test this, we treated dendritic cells (DCs) with liposome:DNA *in vitro* and found that IRF3 was phosphorylated independent of TLR9. To test the contribution of this pathway *in vivo*, we injected a liposome:DNA vector into wild-type (WT), TLR9-knockout (KO), IRF3-KO, and TLR9-IRF3-double-KO (DKO) mice. WT mice exhibited a systemic inflammatory response, evidenced by elevations in serum cytokines, serum enzyme changes indicating organ damage, hypothermia, and mortality. The cytokine response was reduced in TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice and all three groups survived. We found that IFN- γ -KO mice that receive liposome:DNA had a reduced cytokine response and 100% survival. CD11c⁺ and NK1.1⁺ cells produced IFN- γ and depleting CD11c⁺ cells reduced the cytokine response in mice injected with liposome:DNA. These findings may facilitate the development of immunologically inert gene therapy vectors and may provide general insight into the mechanisms of SIRS.

Received 11 November 2009; accepted 4 January 2010; published online 9 February 2010. doi:10.1038/mt.2010.1

INTRODUCTION

Gene therapy is a promising therapeutic modality for many diseases. However, animal studies and clinical trials have revealed that gene therapy vectors activate the innate immune system, which can cause a systemic inflammatory response reminiscent of sepsis.^{1–15} This is a major barrier to the use of gene therapy in the clinic. Liposome:plasmid DNA complex is a promising nonviral gene therapy vector, which lacks viral proteins that may activate the adaptive immune system, but still elicits an innate immune response.^{1,3–5,7,11,12,14} There is a critical need to characterize the host innate immune response to viral and nonviral gene therapy vectors to facilitate the development of immunologically inert gene therapy vectors.

The innate immune system is the first line of defense against microbial invasion and is critical for immediate containment of pathogens and subsequent priming of adaptive immunity.¹⁶ An important component of innate immunity is the rapid response to conserved microbial motifs. Toll-like receptors (TLRs) are germline-encoded receptors, which recognize conserved microbial motifs and initiate a signal transduction cascade to produce an innate immune response.¹⁷ Of relevance to this study, TLR9 resides in endosomes and recognizes unmethylated CpG residues in DNA that is taken up *via* endocytosis, a common entry route for pathogens and gene therapy vectors.^{18,19} TLRs are expressed by antigen-presenting cells, such as dendritic cells (DCs) and macrophages, which play an important role in the innate immune response.¹⁷ Accumulating evidence indicates that TLRs are also expressed by a variety of other lymphocytes, including natural killer (NK) cells.^{20,21}

In addition to the TLRs, alternative receptors in the cytosol recognize nucleic acids from invading pathogens, leading to activation of interferon (IFN) regulatory factors (IRFs),^{22,23} with similar consequences to TLR activation.^{22,24} The retinoic acid-inducible gene I (*RIG-I*) receptor-like helicases recognize cytosolic RNA, leading to activation of IRF3 and IRF7 (refs. 23,25,26). Cytosolic DNA can activate IRF3 in a similar manner,^{27,28} although the mechanisms of this pathway have not been fully elucidated. DNA-dependent activator of IRF is one of the receptors involved in this pathway.^{29,30} Furthermore, recent reports indicate that RNA polymerase III can synthesize RNA from certain cytosolic DNA templates, leading to activation of IRF3 *via* RIG-I (refs. 31,32). However, prior reports indicate that additional sensors of cytosolic DNA exist, which remain to be elucidated.^{32,33}

Although the innate immune response is critical to pathogen containment, an excessive innate immune response can lead to systemic inflammatory response syndrome (SIRS).^{34,35} Clinically, SIRS is characterized by low blood pressure, elevated heart and respiratory rate, and alteration of body temperature and lymphocyte levels. In severe cases, hemodynamic changes, diffuse endothelial injury and apoptosis ensue, resulting in organ damage and mortality.

This report investigates the mechanisms of the innate immune response to a liposome:DNA vector. Prior studies indicate that TLR9 is partially responsible for the innate immune

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response to liposome:DNA.^{5,7,14,36–40} However, this response is not completely abolished in TLR9-knockout (KO) mice,³⁸ indicating that a second pathway must be involved. We hypothesized that liposome:DNA vectors also activate the cytosolic DNA-sensing pathway, which signals *via* IRF3 (refs. 27,28). We used TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice to test this hypothesis. We found that liposome:DNA induced IRF3 phosphorylation in DCs, independent of TLR9. In mice injected with the liposome:DNA vector, the TLR9 and IRF3 pathways cooperated to induce proinflammatory cytokines. Liposome:DNA also induced hypothermia, elevations in serum enzymes indicative of organ damage and mortality (signs of SIRS), which required the presence of both TLR9 and IRF3. IFN- γ was critical for this response and was produced by splenic CD11c⁺CD11b⁺ and NK1.1⁺ cells. These findings have implications for nonviral gene therapy and are relevant to the understanding of SIRS in general.

RESULTS

Liposome:DNA activated IRF3 independent of TLR9

Prior reports indicate that liposome:DNA vectors induce an innate immune response, SIRS, and mortality, and that this is partially due to CpG motifs in the plasmid DNA, which activate TLR9 (refs. 5,7,14,36–40). We hypothesized that plasmid DNA also activates the cytosolic DNA-sensing pathway, which signals *via* IRF3 (refs. 27,28).

We first investigated whether IRF3 was activated by a liposome:DNA vector *in vitro*, and whether this required TLR9. We cultured wild-type (WT), TLR9-KO, and IRF3-KO splenic CD11c⁺ DCs *in vitro* with a liposome:DNA vector (containing DOTAP:cholesterol liposomes and plasmid DNA), then performed a western blot to quantify phosphorylation of IRF3 serine-396, which is indicative of IRF3 activation.⁴¹ IRF3 serine-396 was phosphorylated in both WT and TLR9-KO DCs after 1–2 hours of liposome:DNA treatment (Figure 1a). These data

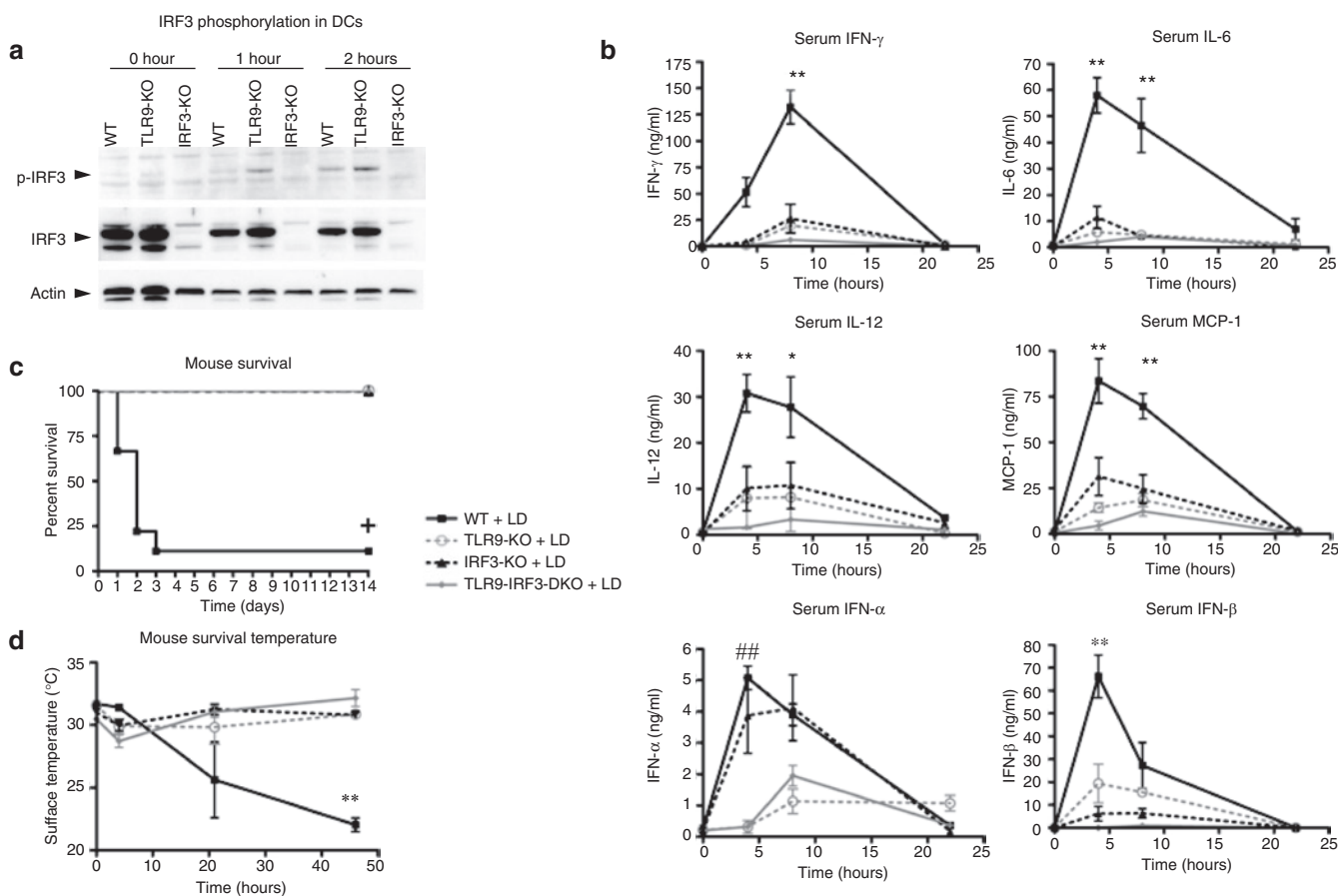


Figure 1 TLR9 and IRF3 induced serum cytokines, mortality, and hypothermia in response to liposome:DNA (LD) vector. **(a)** Western blot demonstrates that IRF3 serine-396 was phosphorylated (P-IRF3) in TLR9-KO and wild-type (WT) DCs, but not IRF3-KO DCs, following 1–2 hours of LD treatment *in vitro*. Total IRF3 and actin controls are also shown. **(b)** Serum IFN- γ , IL-6, IL-12, MCP-1, IFN- α , and IFN- β levels were reduced in TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice relative to WT mice injected with LD. Graphs show mean \pm SE. (For all graphs $P \leq 0.0061$ for 0–8 hours time points by two-way analysis of variance (ANOVA); Bonferroni post-test $*P < 0.05$ and $**P < 0.01$ for WT versus TLR9-KO, IRF3-KO and TLR9-IRF3-DKO; $##P < 0.01$ for WT versus TLR9-KO and TLR9-IRF3-DKO and IRF3-KO versus TLR9-KO and TLR9-IRF3-DKO). **(c)** There was significant mortality in WT mice following injection of LD, whereas all TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice survived. Graph shows survival fractions (Kaplan–Meier method), $^+P < 0.0001$ by Logrank test. **(d)** Surface body temperature dropped significantly in WT mice injected with LD, whereas TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice maintained normal temperature. Graph shows mean \pm SE. $P = 0.0316$ by two-way ANOVA for 0- to 46-hour time points, Bonferroni post-test $**P < 0.01$ for WT versus TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO. DC, dendritic cells; IFN, interferon; IL, interleukin; IRF, IFN regulatory factor; KO, knockout; MCP, monocyte chemoattractant protein; TLR, Toll-like receptor.

directly demonstrate that IRF3 was activated in DCs treated with liposome:DNA, independent of TLR9.

The TLR9 and IRF3 pathways contribute to the innate immune response after liposome:DNA vector administration *in vivo*

Next, we investigated the roles of TLR9 and IRF3 in the innate immune response to liposome:DNA vector *in vivo*. For this purpose, we intravenously injected WT, TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO (C57BL/6) mice with a liposome:DNA vector containing 50 µg of DNA or 5% dextrose vehicle. WT mice exhibited a rapid elevation in the serum proinflammatory cytokines IFN-γ, interleukin (IL)-6, IL-12, and the chemokine monocyte chemoattractant protein-1 as well as the type I IFN-α and IFN-β (Figure 1b). In contrast, the tumor necrosis factor-α response was weaker (Supplementary Figure S1a). The cytokine responses were dose-dependent (Supplementary Figure S1b). In comparison to WT mice, TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice injected with the vector had a significant reduction in IFN-γ, IL-6, IL-12, monocyte chemoattractant protein-1, and IFN-β (Figure 1b and Supplementary Figure S1b). There was also a trend toward a further reduction in TLR9-IRF3-DKO mice compared to TLR9-KO or IRF3-KO mice (Figure 1b and Supplementary Figure S1b). IFN-α levels were similar in the WT and IRF3-KO mice injected with the vector, whereas TLR9-KO and TLR9-IRF3-DKO mice injected with the vector showed a significant reduction compared to WT mice (Figure 1b). Proinflammatory cytokines remained at background levels throughout the experiment in mice of each genotype injected with 5% dextrose vehicle control (data not shown). These data indicate that both TLR9 and IRF3 contributed to the innate immune response to liposome:DNA.

The proinflammatory cytokine response to liposome:DNA required presence of plasmid DNA, as injection of liposomes in 5% dextrose vehicle or 5% dextrose vehicle alone, did not induce serum cytokines (Supplementary Figure S2a). Endotoxin was undetectable in our liposomes and was present at biologically insignificant levels in our plasmid DNA [<271 pg lipopolysaccharide (LPS)/50 µg DNA dose administered to a mouse]. To confirm that the endotoxin level in plasmid DNA was biologically insignificant, we intravenously injected mice with LPS mixed with liposomes: we did not observe an elevation in serum proinflammatory cytokines even at an LPS dose of 6 ng (20-fold higher than the endotoxin levels in our plasmid DNA preparations) (Supplementary Figure S2b). In fact, only a 50 µg intraperitoneal (i.p.) dose of LPS was sufficient to induce similar levels of IL-6 and IL-12 to liposome:DNA vector, and this dose of LPS failed to induce IFN-γ. Furthermore, to ensure that LPS did not synergize with plasmid DNA to induce proinflammatory cytokines, we injected mice with liposome:DNA supplemented with 6 ng LPS. These mice exhibited similar IL-6 levels to mice that received liposome:DNA only, at 4 hours (Supplementary Figure S2c). These data indicate that plasmid DNA, rather than endotoxin contamination, induced the innate immune response that we observed.

TLR9 and IRF3 induced mortality and signs of SIRS in mice injected with liposome:DNA vector

We found that 90% of the WT mice died within 3 days of liposome:DNA administration (Figure 1c); in contrast, 100% of

the TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice survived >14 days. Death was preceded by a drop in surface body temperature in WT mice, which was not observed in the TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice (Figure 1d). We did not observe mortality or hypothermia in any of the genotypes following injection of 5% dextrose vehicle control (data not shown). In summary, the hypothermia and mortality induced by liposome:DNA required both the TLR9 and IRF3 pathways.

We observed alterations in several enzymes indicative of multiple organ failure in WT mice injected with liposome:DNA vector, these changes were not observed in the 5% dextrose vehicle controls (Table 1). These included elevations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (both are released during hepatocellular injury), elevation of creatine phosphokinase (indicative of heart, skeletal muscle, or brain damage), and elevation of amylase (secreted in pancreatic, liver, or small intestinal damage). At 24 hours after administration of liposome:DNA, WT mice also had a substantially reduced percentage of CD3⁺ cells in the blood, relative to naive mice (Supplementary Figure S3). These results provide evidence that systemic administration of liposome:DNA induced organ damage and lymphopenia, which are typical signs of SIRS.

We next examined the importance of TLR9 and IRF3 in the aforementioned systemic response using AST and ALT as readouts of liver damage. There was a trend toward a reduced ALT and AST response in TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice relative to WT mice (Figure 2a,b).

The IRF3 pathway reduced transgene expression

Prior reports indicate that inhibiting the innate immune response may increase transgene expression.^{5,37,42,43} Hence, we examined the impact of the TLR9 and IRF3 pathways on transgene expression. To determine the site of transgene expression, we injected WT, TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO intravenously with liposome:DNA containing 25 µg of pGL4.13 plasmid encoding luciferase or 5% dextrose vehicle, then examined the luciferase activity at 24 hours using *in vivo* imaging. We observed transgene expression in the lungs in all genotypes after injection of

Table 1 Serum chemistry in wild-type mice at 24 hours after injection of a high dose of liposome:DNA vector or 5% dextrose vehicle

	Vehicle	Liposome:DNA
ALT (U/l)	62.5 ± 27.5	341.0 ± 192.7
AST (U/l)	262 ± 56	551 ± 189.4
CPK (U/l)	837 ± 21	2,267 ± 429
Amylase (U/l)	646 ± 11	1,797 ± 707
Total protein	5.45 ± 0.05	4.8 ± 0.17
Albumin/globulin ratio	1.55 ± 0.05	1.17 ± 0.07
Calcium (mg/dl)	10.8 ± 0.30	9 ± 0.87
Glucose (mg/dl)	441.5 ± 107.5	126 ± 10.4

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatine phosphokinase; LD, liposome:DNA; WT, wild type.

At 24 hours after injection of liposome:DNA complex WT mice had elevated ALT, AST, and CPK, reduced total protein, calcium, and glucose, and reduced albumin/globulin ratio. WT mice injected with 5% dextrose vehicle did not show similar changes, although blood glucose was elevated as a consequence of the dextrose injection.

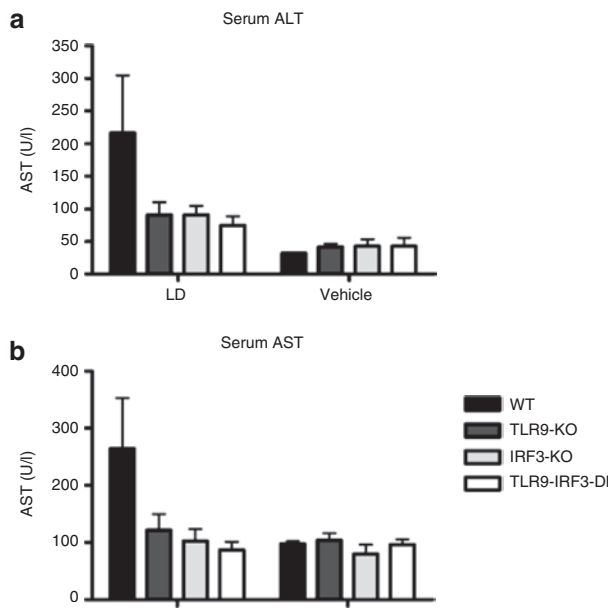


Figure 2 A trend toward reduced ALT and AST in mice lacking TLR9 or IRF3. There was a trend toward reduced serum (a) ALT and (b) AST in TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice versus WT mice 24 hours after injection of LD. AST and ALT remained at background levels in mice injected with 5% dextrose vehicle. Graphs show mean \pm SE. ALT, alanine aminotransferase; AST, aspartate aminotransferase; IRF, interferon regulatory factor; LD, liposome:DNA; TLR, Toll-like receptor; WT, wild-type.

liposome:DNA, but not after injection of 5% dextrose vehicle (**Supplementary Figure S4a**). Next, we used a biochemical assay to quantify luciferase transgene expression in the lungs 24 hours after administering liposome:DNA containing 25 or 50 μ g of pGL4.13. We observed a significantly higher level of luciferase in the lungs of IRF3-KO and TLR9-IRF3-DKO mice versus WT mice at the 25 μ g dose and there was a trend toward increased luciferase levels in TLR9-KO mice versus WT mice (**Supplementary Figure S4b**). These data indicate that the IRF3 pathway reduced transgene expression.

IFN- γ plays a key role in the response to liposome:DNA

Next, we examined which cytokines were critical to the inflammatory response and mortality induced by liposome:DNA. As both IFN- α and IFN- β (type I IFNs) exhibited a rapid rise following liposome:DNA administration, we first examined the importance of this pathway. However, liposome:DNA induced similar mortality in IFN- $\alpha\beta$ receptor (IFN- $\alpha\beta$ R)-KO and WT mice (**Figure 3a**). The IFN- $\alpha\beta$ R-KO mice had similar serum IFN- γ and IL-6 levels to WT mice, and significantly increased IL-12 levels (**Figure 3b**). We then examined the importance of IFN- γ in this model. Although this cytokine was released later than the type I IFNs, the levels of IFN- γ were the highest of all the cytokines measured (**Figure 1b**). We found that 100% of the IFN- γ -KO mice survived following liposome:DNA administration (**Figure 3a**). Furthermore, IFN- γ -KO mice had significantly reduced levels of serum IL-6, and a trend toward reduced serum IL-12 at the 6-hour time point (**Figure 3a**). The IFN- γ -KO mice maintained normal body

temperature (**Figure 3b**) and had significantly reduced serum AST levels and a trend toward reduced ALT levels, relative to WT mice (**Figure 3d**).

To verify this result, we treated WT mice with an IFN- γ -neutralizing antibody, or rat-IgG1 anti-horseradish peroxidase (rIgG1-HRP) isotype control, 18 hours before administering liposome:DNA. All (100%) the mice administered the IFN- γ neutralizing antibody survived, whereas 83% of the mice that received the isotype control died (**Figure 4a**). The mice that received the IFN- γ neutralizing antibody also had significantly reduced levels of IL-6 and IL-12 serum cytokines relative to mice administered the isotype control at the 6-hour time point (**Figure 4b**). Mice administered 5% dextrose vehicle after the IFN- γ neutralizing antibody or isotype control exhibited 100% survival and serum cytokines remained at background levels (data not shown). Together, these results demonstrate that IFN- γ played an important role in the serum cytokine response and SIRS that occurred following liposome:DNA administration.

Splenic CD11c⁺CD11b⁺ and NK1.1⁺ cells produce IFN- γ in response to liposome:DNA vector

We performed further experiments to investigate which organ and cell type produced the IFN- γ following administration of liposome:DNA vector. We injected WT mice with the liposome:DNA vector, then harvested their livers, spleens, and lungs at 3 hours. A single-cell suspension was prepared from each organ and cultured for 18 hours, and then IFN- γ was quantified in the media. The liver and spleen cells from the mice injected with liposome:DNA vector produced significantly more IFN- γ *ex vivo* than cells from naive control mice (**Figure 5a**). In contrast, lung cells from mice injected with liposome:DNA vector failed to produce more IFN- γ than lung cells from naive control mice. These data indicate that the spleen and the liver produced IFN- γ in response to liposome:DNA.

We used intracellular cytokine staining to identify the cell type that produced cytokines in the spleen. We identified an IFN- γ ⁺ population in spleen cells from WT mice that were injected with the liposome:DNA vector, which was not evident in mice injected with 5% dextrose vehicle (**Figure 5b**). This IFN- γ ⁺ population was predominantly composed of CD11c⁺CD11b⁺ and NK1.1⁺ cells, although these cell types made up only a small fraction of the total spleen cell gate (**Figure 5b**). The IFN- γ ⁺ population did not contain CD3⁺ T cells, CD19⁺B220⁺ B cells or CD1d-tetramer-binding NKT cells (data not shown).

We confirmed these results by harvesting spleen cells from WT mice injected with the liposome:DNA vector and magnetically depleting the CD11c⁺, CD11b⁺, and NK1.1⁺ subpopulations. The remaining cells were cultured for 18 hours and IFN- γ was quantified in the media. We observed that depletion of CD11c⁺ and/or CD11b⁺ cells significantly reduced IFN- γ levels (**Figure 5c**). Depletion of NK1.1⁺ cells also significantly reduced IFN- γ levels (**Figure 5c**). Cells isolated from WT mice injected with 5% dextrose vehicle control did not produce IFN- γ , either with or without magnetic depletion of the aforementioned populations (data not shown). These results demonstrate that splenic CD11c⁺CD11b⁺ and NK1.1⁺ cells produced IFN- γ following liposome:DNA administration.

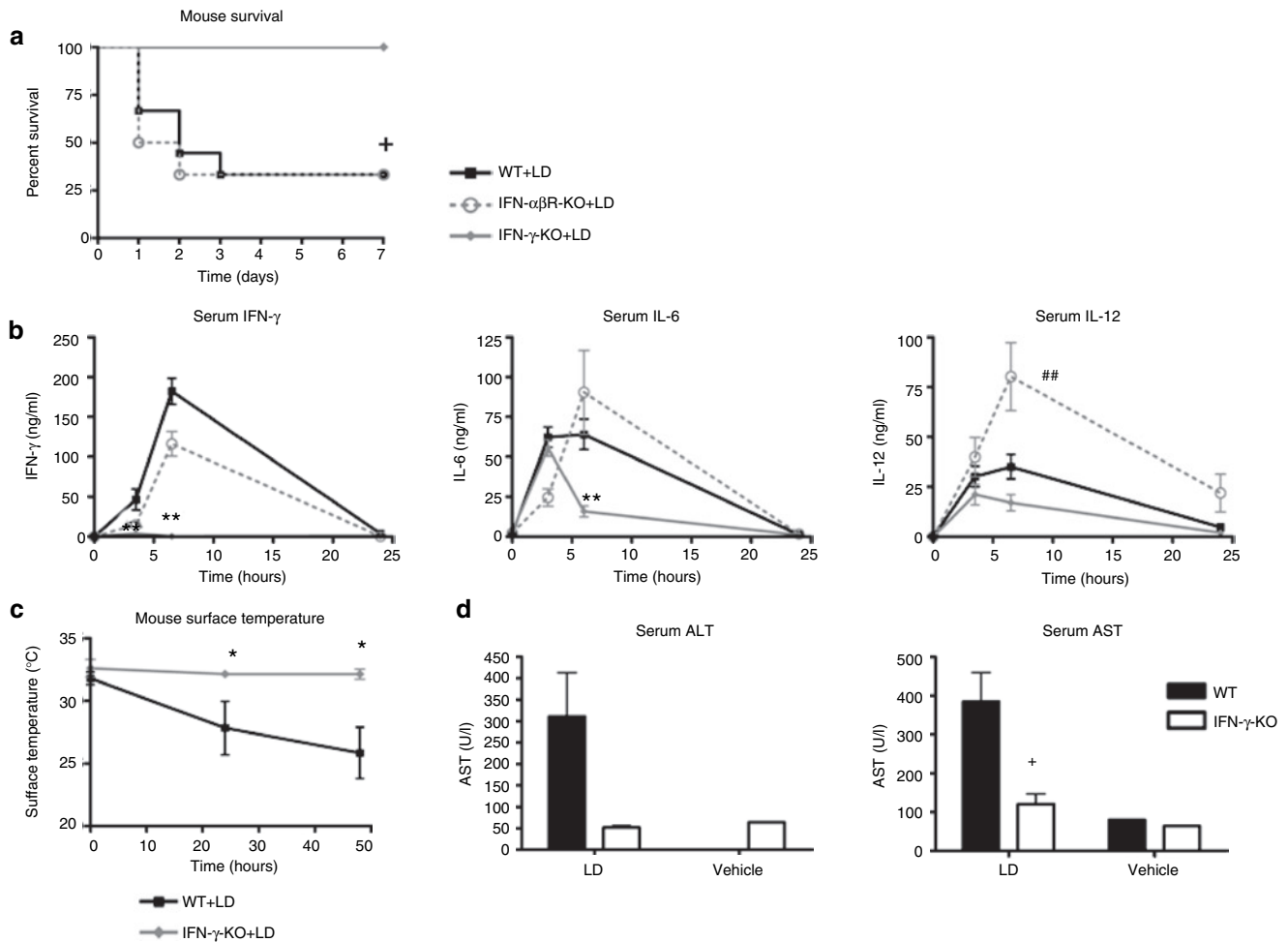


Figure 3 IFN- γ played a key role in the innate immune response to liposome:DNA (LD) vector complex: experiments using genetic knockouts. **(a)** There was significant mortality in wild-type (WT) and IFN- $\alpha\beta$ R-KO mice following injection of LD, whereas all IFN- γ -KO mice survived. Graph shows survival fractions (Kaplan–Meier method), $^+P = 0.0487$ by Logrank test. **(b)** Serum IFN- γ and IL-6 levels were significantly reduced in IFN- γ mice versus WT mice, and there was a trend toward reduced IL-12, after injection of LD. IL-6 and IL-12 levels were similar in IFN- $\alpha\beta$ R-KO mice and WT mice, whereas IL-12 levels were significantly higher in IFN- $\alpha\beta$ R-KO mice, after injection of LD. For all graphs $P \leq 0.0387$ for 0- to 6-hour time points by two-way analysis of variance (ANOVA), Bonferroni post-test $^{**}P < 0.01$ for IFN- γ -KO versus WT. $^{##}P < 0.01$ for IFN- $\alpha\beta$ R-KO versus WT. **(c)** Surface body temperature dropped significantly in WT mice injected with LD, whereas IFN- γ -KO mice maintained normal temperature. $P = 0.0033$ by two-way ANOVA for 0- to 48-hour time points, Bonferroni post-test $^*P < 0.05$ for IFN- γ -KO versus WT. **(d)** There was trend toward reduced serum ALT, and AST values were significantly lower in IFN- γ -KO mice versus WT mice at 24 hours after injection of LD. ALT and AST remained at background levels in mice injected with 5% dextrose vehicle. $^*P < 0.05$ by *t*-test. **(b–d)** Graphs show mean \pm SE. ALT, alanine aminotransferase; AST, aspartate aminotransferase; IFN, interferon; IL, interleukin; KO, knockout.

CD11c⁺ cells contributed to cytokine production in response to *in vivo* liposome:DNA vector administration

We next examined the consequence of depleting CD11c⁺ or NK1.1⁺ cells *in vivo*. To deplete CD11c⁺ cells, we employed CD11c-DTR mice, which express the diphtheria toxin receptor under the control of the CD11c promoter. Diphtheria toxin was administered to CD11c-DTR or WT control mice *i.p.* 18 hours before injection of liposome:DNA. To deplete NK cells, WT mice were administered an NK1.1-depletional antibody or mouse-IgG2a isotype control *i.p.* 18 hours before injection of liposome:DNA. Depletion of CD11c⁺ and NK1.1⁺ cells was confirmed by antibody staining and fluorescence-activated cell sorting analysis of spleen cells (data not shown).

Depletion of CD11c⁺ cells inhibited the production of IFN- γ and IL-12 to a substantial degree and there was a trend

toward reduced IL-6 production (Figure 6a). NK1.1⁺ cell depletion had only a minor effect on IFN- γ and IL-6 production, and IL-12 production was unaffected (Figure 6b). Cytokines remained at background levels in control mice administered 5% dextrose vehicle, regardless of CD11c⁺ or NK1.1⁺ depletion (data not shown). These data demonstrate that CD11c⁺ cells played a role in the systemic inflammatory cytokine response to liposome:DNA.

DISCUSSION

Liposome:DNA vectors elicit a strong innate immune response, which is problematic for nonviral gene therapy. Following injection of the liposome:DNA vector into WT mice, we observed elevations in proinflammatory cytokines accompanied by signs of SIRS including hypothermia, serum enzyme changes indicating organ

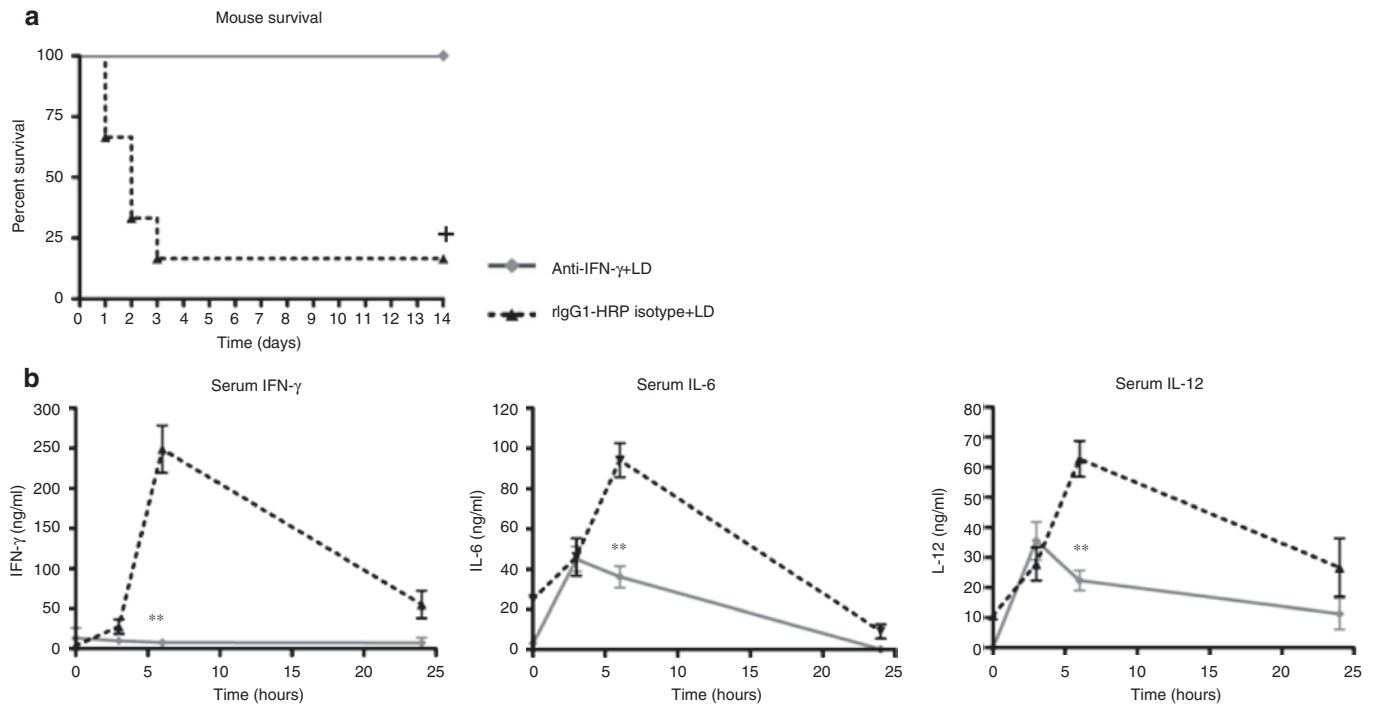


Figure 4 IFN- γ played a key role in the innate immune response to liposome:DNA (LD) vector: experiments using a neutralizing antibody. **(a)** 100% of wild-type (WT) mice survived when administered an IFN- γ -neutralizing antibody (anti-IFN- γ) before the injection of a high-dose of LD vector (50 μ g plasmid DNA), in contrast to counterparts administered rat-IgG1 anti-horseradish peroxidase (rlgG1-HRP) isotype control. Graph shows survival fractions (Kaplan–Meier method). $^+P = 0.0043$ by Logrank test. **(b)** Serum IFN- γ , IL-6, and IL-12 cytokine levels were lower in WT mice that received an IFN- γ -neutralizing antibody versus rlgG1-HRP isotype, before injection of LD. Graphs show mean \pm SE. $P \leq 0.01$ by two-way analysis of variance for 0- to 6-hour time points, Bonferroni post-test $^{**}P \leq 0.01$. IFN, interferon; IL, interleukin.

damage and death (Figures 1 and 2, Table 1). These observations are consistent with prior studies.¹⁵

Prior reports indicate that the innate immune response to liposome:DNA is partially, but not entirely, dependent on CpG residues in plasmid DNA, which activate TLR9 (refs. 4,5,7,14,15,36–40). This report demonstrates that a separate IRF3-dependent pathway also contributes to this response. We provide direct evidence that IRF3 is activated by liposome:DNA vector *in vitro*, independent of TLR9. IRF3 serine-396 became phosphorylated in WT and TLR9-KO DCs treated with liposome:DNA *in vitro* (Figure 1a).

In future studies, it will be important to determine whether activation of IRF3 by liposome:DNA involves DNA-dependent activator of IRF, or RNA polymerase III, as these proteins play a role in the innate immune response to cytosolic DNA.^{29–32} Furthermore, these reports indicate that there are additional unidentified cytosolic DNA receptors, which play a redundant role with DNA-dependent activator of IRF and RNA polymerase III in sensing cytosolic DNA *in vivo*. It will be important to elucidate these receptors in future studies.

The TLR9 and IRF3 pathways cooperate to induce an innate immune response and SIRS *in vivo*. Serum cytokines were substantially elevated in WT mice injected with liposome:DNA vector, whereas the cytokine levels were lower in TLR9-KO, IRF3-KO and TLR9-IRF3-DKO mice (Figure 1b). Furthermore, WT mice injected with liposome:DNA exhibited signs of SIRS, including hypothermia, elevations in serum enzymes indicating organ damage and mortality (Figure 1 and 2, Table 1). In contrast, all the TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice treated

with liposome:DNA survived. Hypothermia was attenuated in these mice and there was a trend toward reduced ALT and AST levels. Collectively, these data indicate that both the TLR9 and IRF3 pathways must be activated for liposome:DNA to induce a maximal proinflammatory response and SIRS. Prior reports indicate that the innate immune response can compromise transgene expression.^{5,37,42,43} We observed increased transgene expression in IRF3-KO and TLR9-IRF3-DKO mice, relative to WT mice, after administration of liposome:DNA (Supplementary Figure S4). Thus, inhibiting IRF3 might be a useful strategy to reduce the innate immune response, prevent SIRS and increase transgene expression in nonviral gene therapy.

Type I IFNs (IFN- α and IFN- β) are induced by IRFs and play an important role in host defense against viruses.⁴⁴ However, IFN- $\alpha\beta$ R-KO mice exhibited similar mortality, similar IFN- γ and IL-6, and increased IL-12 levels relative to WT mice, following injection of liposome:DNA (Figure 3a,b). These data indicate that type I IFN signaling is not critical to the innate immune response to liposome:DNA, or the associated mortality. Of relevance to this result, the Tyro3, Axl, and Mer (TAM) receptors form a complex with the IFN- $\alpha\beta$ receptor and act as negative regulators of the TLR response.⁴⁵ We speculate that TAM ligands might be produced during the host response to liposome:DNA and activate TAM receptors. This might explain the increased IL-12 cytokine production in IFN- $\alpha\beta$ R-KO mice, as the TAM inhibitory pathway should also be abrogated in these mice.

IFN- γ plays a critical role in the innate immune response to liposome:DNA. Mice lacking IFN- γ had reduced levels of

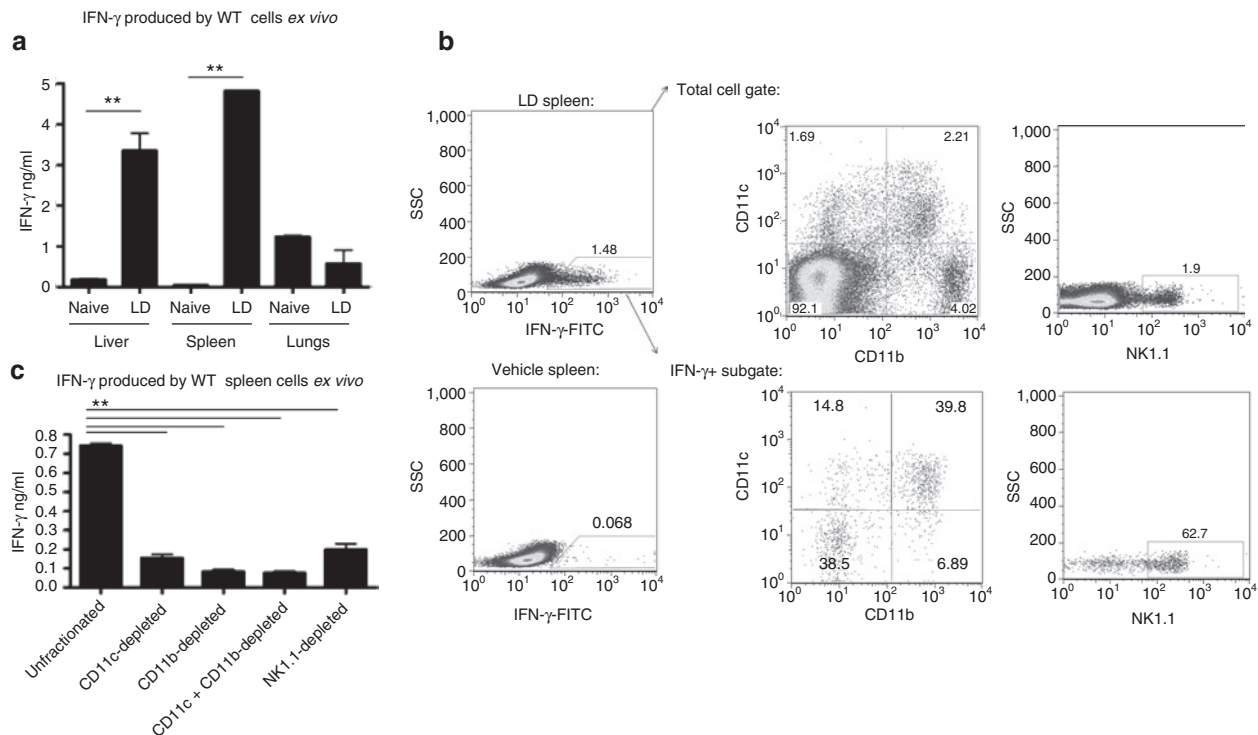


Figure 5 Liposome:DNA (LD) vector induced IFN- γ production by CD11c⁺CD11b⁺ and NK1.1⁺ spleen cells. **(a)** IFN- γ was produced *ex vivo* by liver and spleen cells from wild-type (WT) mice injected with LD. In contrast, lung cells from these mice failed to produce more IFN- γ than naive counterparts. $P < 0.01$ by one-way analysis of variance (ANOVA), Bonferroni post-test $**P < 0.01$. **(b)** Left panels: intracellular cytokine staining revealed an IFN- γ ⁺ population in spleen cells from WT mice injected with LD (top). This IFN- γ ⁺ population was not present in mice that received 5% dextrose vehicle (bottom). Center and right panels: the IFN- γ ⁺ subgate predominantly contained CD11c⁺CD11b⁺ and NK1.1⁺ cells (39.8 and 62.7% respectively, bottom panels), whereas these cell types were rare in the total spleen cell gate (2.21 and 1.9% respectively, top panels). **(c)** Depletion of CD11c, CD11b, CD11c+CD11b, or NK1.1 populations abrogated IFN- γ production *ex vivo* by spleen cells from mice injected with LD. $P < 0.0001$ by one-way ANOVA, Bonferroni post-test $**P < 0.01$ unfractionated versus CD11c-depleted, CD11b-depleted, CD11c+CD11b-depleted, and NK1.1-depleted. **(a and c)** Graphs show mean \pm SE.

proinflammatory cytokines, reduced serum ALT and AST and did not exhibit mortality following the injection of liposome:DNA (Figures 3 and 4). These data imply that inhibiting IFN- γ might be a useful strategy to reduce the innate immune response and prevent SIRS in nonviral gene therapy.

Interestingly, the effect of IFN- γ on the production of other proinflammatory cytokines did not become evident until the 6-hour time point (Figures 3 and 4). IFN- γ is known to signal in a positive feedback loop with IL-12, each cytokine inducing the other.⁴⁶ We speculate that the serum proinflammatory cytokines are initially elevated as a direct consequence of TLR9 and IRF3 activation, then IFN- γ and IL-12 act in a positive feedback loop to maintain and increase cytokine production.

We demonstrated that IFN- γ is produced by spleen and liver cells derived from mice injected with liposome:DNA (Figure 5a). Our experiments indicate that splenic CD11c⁺CD11b⁺, and NK1.1⁺ cells produce IFN- γ (Figure 5b). *Ex vivo*, depletion of either subset reduced IFN- γ to near background levels (Figure 5c). *In vivo*, depletion of CD11c⁺ cells substantially reduced the levels of proinflammatory cytokines in mice treated with liposome:DNA, whereas depletion of NK cells caused only a minor reduction in cytokine levels (Figure 6). We speculate that an interaction between DCs and NK1.1⁺ cells is required to elicit the full levels of IFN- γ that we observed. Perhaps either (i) DC-mediated IFN- γ

production drives NK cell IFN- γ production, or (ii) DCs produce another molecule (possibly IL-12), which drives both DC and NK-mediated IFN- γ production. Our data imply that inhibiting CD11c⁺ cells may be a viable strategy to prevent cytokine production in nonviral gene therapy. Further investigation is required to elucidate the full interplay of CD11c⁺ and NK1.1⁺ cells and the role of IL-12 in this system.

The CD11c⁺CD11b⁺ subset in the spleen represents the myeloid DC lineage. The authors of a prior study concluded that macrophages contribute to the cytokine response induced by liposome:DNA, as treating these mice with gadolinium chloride inhibited cytokine production in response to this vector.¹² However, prior reports show clodronate liposomes, an agent with very similar properties to gadolinium chloride, deplete certain splenic DC populations as well as macrophages.^{47,48} Hence, the relative roles of DCs and macrophages in the innate immune response to liposome:DNA requires further investigation in future studies.

Because liposome:DNA induced clinical symptoms of SIRS, this may be an appropriate model for further research into the mechanisms of SIRS and sepsis. Commonly used mouse models of sepsis include cecal ligation and puncture, administration of large doses of LPS, and administration of CpG to D-galactosamine-sensitized mice. We propose that administering liposome:plasmid

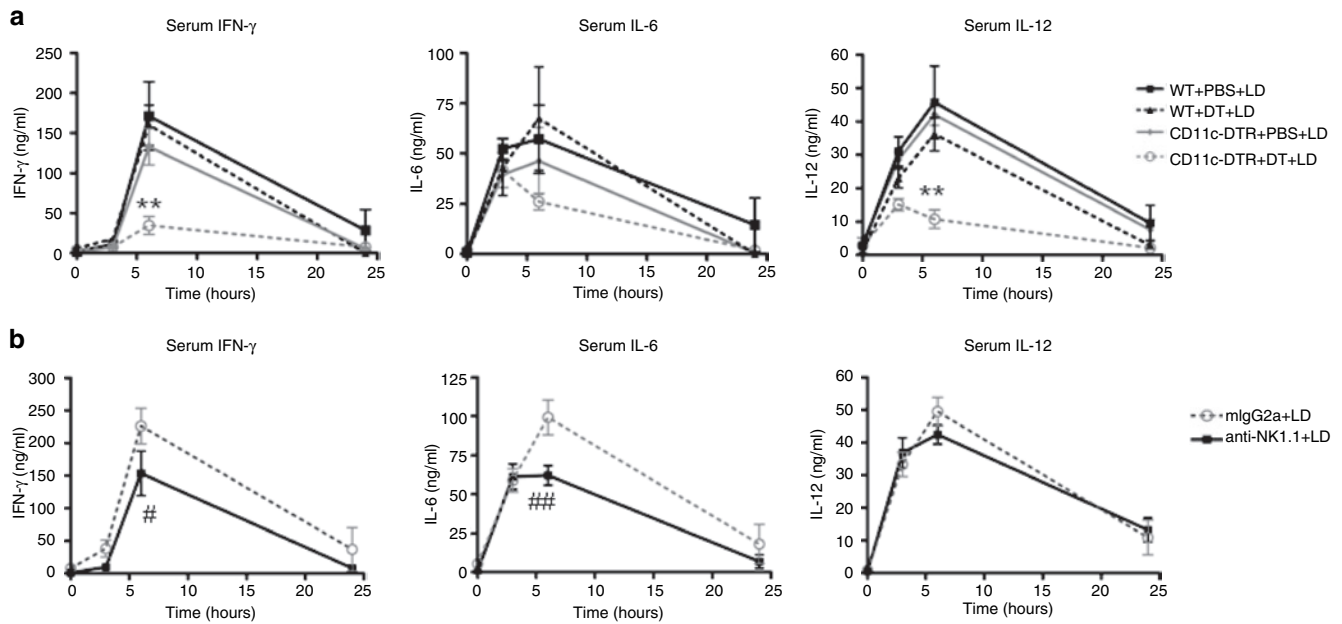


Figure 6 CD11c⁺ cells played a major role in proinflammatory cytokine production *in vivo*. **(a)** Depletion of dendritic cells in CD11c-DTR mice *via* diphtheria toxin (DT) treatment reduced IFN- γ and IL-12 production and there was a trend toward reduced IL-6 production, in comparison to control mice [CD11c-DTR mice treated with phosphate-buffered saline (PBS) and wild-type (WT) mice treated with DT or PBS], following injection of LD. For IFN- γ and IL-12 graphs $P \leq 0.0055$ by two-way analysis of variance (ANOVA) for 0- to 6-hour time points, Bonferroni post-test $^{**}P < 0.01$ for CD11c-DTR+DT versus CD11c-DTR+PBS, WT+DT, and WT+PBS. **(b)** Depletion of NK1.1⁺ cells in WT mice *via* administration of NK1.1 depletion antibody (anti-NK1.1) reduced IFN- γ and IL-6 production to a small degree and did not affect IL-12 production, in comparison to control mice [administered mouse-IgG2a (mlgG2a) isotype control], following injection of LD vector. For IFN- γ and IL-6, $P \leq 0.04$ by two-way ANOVA for 0- to 6-hour time points, (IL-12 = ns), Bonferroni post-test $^*P < 0.05$ and $^{**}P < 0.001$ for anti-NK1.1 versus mlgG2a. Graphs show mean \pm SE. IFN, interferon; IL, interleukin; LD, liposome:DNA.

DNA to mice may be an appropriate model of sepsis, and should be investigated in more detail in future studies. It will also be interesting to determine the role of cytosolic DNA and IRF3 in other models of sepsis in future studies.

This report investigates how the TLR9 and IRF3 pathways synergize during the *in vivo* host response to foreign DNA. We also present evidence that DNA-induced IRF3 activation can play a crucial role in SIRS. Our results imply that the IRF3 pathway represents a novel target for inhibition in the quest to create safer gene therapy approaches for clinical application. Similar strategies may prove useful in other forms of SIRS, including sepsis.

MATERIALS AND METHODS

Liposome:DNA preparation. pGL4.13 plasmid DNA was prepared with an endotoxin-free maxiprep kit (QIAGEN, Valencia, CA). To make the liposomes, DOTAP methyl-sulfate (Avanti polar Lipids, Alabaster, AL) and cholesterol (Sigma-Aldrich, St Louis, MO) were mixed in a 1:1 molar ratio in chloroform:methanol (3:1). The solvent was evaporated and then the lipid film was hydrated with 5% dextrose and extruded twice through 0.2 μ m Anotop-10 filters (Whatman, Piscataway, NJ). Plasmid DNA diluted in 5% dextrose was added to the liposomes drop-wise (1:14 weight ratio). A volume of 250 μ l (containing 50 μ g plasmid DNA) was injected into the tail vein of each mouse. E-Toxate reagent (Sigma-Aldrich) was used to quantify endotoxin. LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich) was used for control experiments.

Animals. All mice used in the study were on a C57BL/6 background. WT and IFN- γ -KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR9-KO mice and IFN- α / β -KO mice were a gift from Dr Iwasaki (Yale University, USA). IRF3-KO mice were a gift from

Dr Medzhitov (Yale University, USA). IRF3-KO mice were used with the kind permission of Dr Taniguchi (University of Tokyo, Japan). These strains were intercrossed to generate the TLR9-IRF3-DKO mice. All mice were housed under specific pathogen-free conditions. The Yale University Institutional Animal Care and Use Committee approved the use of animals in this study.

Depletional and neutralizing antibodies. To deplete NK cells, mice were injected *i.p.* with 500 μ g NK1.1 depletional antibody (clone PK136) or mIgG2a isotype control (Bio X Cell, West Lebanon, NH). To neutralize IFN- γ , mice were injected *i.p.* with 2 mg anti-IFN- γ (clone R4-6A2) or rIgG1-HRP isotype control (Bio X Cell).

Serum preparation, ELISA, and blood chemistry. Blood was collected by retro-orbital bleed and spun down in BD microtainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ). Cytokine levels were analyzed by enzyme-linked immunosorbent assay (ELISA) (Becton Dickinson and PBL Biomedical Laboratories, Piscataway, NJ). The rIgG1-HRP isotype control created an artifact in these ELISA assays, which use HRP chemistry. To correct for this, the ELISAs were repeated without the detection antibody and this background signal was subtracted. ALT and AST were measured with Infinity GPT/GOT reagents (Thermo Scientific, Waltham, MA). Blood from mice utilized for creatine phosphokinase, amylase, ALT, and AST was collected after killing by carbon dioxide asphyxiation before cessation of cardiac function by cardiac puncture and assays were performed by Antech Diagnostics (Irvine, CA).

Primary cell culture. Spleen cells were dissociated from the capsule by mastication between glass slides. Lungs and livers were chopped up and digested with collagenase A (Sigma-Aldrich). Red blood cells were lysed and the cells were filtered as previously described.^{49,50} For intracellular cytokine staining, 2×10^6 cells were seeded in each well of a 96-well plate

in growth media (RPMI, 10% fetal calf serum, 1% penicillin/streptomycin) with 1 μ l/ml GolgiPlug (Becton Dickinson). At 4 hours, the cells were collected, incubated with FcBlock (eBioscience, San Diego, CA), and then stained with antibodies to cell surface markers (eBioscience). The cells were fixed and permeabilized, then stained with IFN- γ -FITC or isotype control (eBioscience) on ice for 30 seconds. For the depletion study, cells were stained with phycoerythrin (PE)-labeled antibodies to CD11c, CD11b, CD19, B220, CD3, NK1.1, and CD1d-tetramer (eBioscience). PE-labeled cells were depleted with the EasySep PE-selection kit (StemCell, Vancouver, BC). A volume of 5×10^5 cells were cultured in growth media for 18 hours before analysis of supernatant cytokine levels by ELISA. Spleen DCs were purified using the EasySep mouse CD11c-PE selection kit (StemCell). DCs (1×10^6) were cultured in growth media in a 96-well plate with 25 μ l liposome:DNA (containing 5 μ g DNA).

Western blots. Protein was extracted in 50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1% Nonidet-p40, 5 mmol/l orthovanadate, 1% deoxycholate, pH 8.0 + Complete EDTA-free protease inhibitor (Roche, Indianapolis, IN) for 20 seconds on ice. Ten micrograms of each protein extract was mixed with loading dye and incubated at 95 °C for 10 seconds, then separated on a 12% NuPage Novex Bis-Tris Mini Gel (Invitrogen, Carlsbad, CA). The proteins were transferred onto an Immobilon-P PVDF membrane (Millipore, Billerica, MA), then incubated with primary antibodies against phosphorylated IRF3 serine-396 (Cell Signaling Technology, Danvers, MA), total IRF3 (Abcam, Cambridge, MA) or Actin 1-19 (Santa Cruz, Santa Cruz, CA) and secondary antibodies (eBioscience) in 5% milk, Tris-buffered saline + 0.05% tween-20. Luminol chemiluminescent reagent (Santa Cruz) was used to detect signal.

Luciferase transgene expression. For *in vivo* detection of luciferase activity, mice were injected with 150 mg D-luciferin/kg body weight (AnaSpec, Fremont, CA) i.p. 5 minutes before imaging with a Xenogen IVIS 50 Bioluminescent imaging system (Caliper Life Sciences, Hopkinton, MA). For the biochemical assay, mice were killed and their lungs were harvested, homogenized, and spun down; supernatants were collected and the Luciferase assay system (Promega, Madison, WI) was used according to the manufacturer's instructions. Luciferase values were normalized to protein levels, determined with the Pierce BCA protein assay (Thermo Scientific), according to the manufacturer's instructions.

Statistical analysis. All experiments were performed on at least three separate occasions with $n \geq 3$ /experiment. Graphs show representative or pooled data from these experiments. For comparison of one variable between two groups, we employed a two-tailed *t*-test, assuming unequal variance between groups. For comparison of one variable between three or more groups, we employed a one-way analysis of variance. For comparison of two or more groups over time, we employed a two-way repeated measures analysis of variance. A Bonferroni post-test was performed to assess pair-wise differences. $P \leq 0.05$ was considered significant, ≤ 0.01 highly significant.

SUPPLEMENTARY MATERIAL

Figure S1. Further characterization of cytokine production in wild-type, TLR9-KO, IRF3-KO, and TLR9-IRF3-DKO mice injected with liposome:DNA.

Figure S2. Plasmid DNA was required to elicit the serum cytokine response, and endotoxin contamination was not a factor.

Figure S3. Wild-type mice became lymphopenic after liposome:DNA administration.

Figure S4. IRF3 reduced luciferase transgene expression in the lungs.

ACKNOWLEDGMENTS

This work was supported by NIH R01AI064660, R01AG028082, K02AG033049, and AHA 0940006N (D.R.G.); NIH T32 HL007778-

12(fellowship support for W.E.W.), and NIH 2P30DK034989 (Yale Liver Center pilot feasibility grant awarded to W.E.W.). We thank Anushree Shirali, Heather Stout-Delgado, Hua Shen, Wei Du, and Lauren Cohn for valuable discussions. We thank Maria Volkova, April Kalinowski, Ravi Singh, Miriam Alonso, and Jonathan Alderman for their assistance with experimental techniques. We thank Dr Flavell for use of the IVIS imaging system. We thank Akiko Iwasaki for the TLR9-KO and IFN- α 3R-KO mice and Ruslan Medzhitov for the IRF3-KO mice.

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