TLR7-dependent and FcγR-independent production of type I interferon in experimental mouse lupus

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Increased type I interferon (IFN-I) production and IFN-stimulated gene (ISG) expression are linked to the pathogenesis of systemic lupus erythematosus (SLE). Although the mechanisms responsible for dysregulated IFN-I production in SLE remain unclear, autoantibody-mediated uptake of endogenous nucleic acids is thought to play a role. 2,6,10,14-tetramethylpentadecane (TMPD; also known as pristane) induces a lupus-like disease in mice characterized by immune complex nephritis with autoantibodies to DNA and ribonucleoproteins. We recently reported that TMPD also causes increased ISG expression and that the development of the lupus is completely dependent on IFN-I signaling (Nacionales, D.C., K.M. Kelly-Scumpia, P.Y. Lee, J.S. Weinstein, R. Lyons, E. Sobel, M. Satoh, and W.H. Reeves. 2007. *Arthritis Rheum.* 56:3770–3783). We show that TMPD elicits IFN-I production, monocyte recruitment, and autoantibody production exclusively through a Toll-like receptor (TLR) 7– and myeloid differentiation factor 88 (MyD88)–dependent pathway. In vitro studies revealed that TMPD augments the effect of TLR7 ligands but does not directly activate TLR7 itself. The effects of TMPD were amplified by the Y-linked autoimmune acceleration cluster, which carries a duplication of the *TLR7* gene. In contrast, deficiency of Fcγ receptors (FcγRs) did not affect the production of IFN-I. Collectively, the data demonstrate that TMPD-stimulated IFN-I production requires TLR7/MyD88 signaling and is independent of autoantibody-mediated uptake of ribonucleoproteins by FcγRs.

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Abbreviations used: ago2, argonaute 2; ANA, antinuclear antibody; clo-lip, clodronate-containing liposomes; ds, double stranded; IC, immune complex; IFN-I, type I IFN; IPS-1, IFN-b promoter stimulator 1; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; MCP, monocyte chemoattractant protein; Mda5, melanoma differentiation-associated gene 5; MFI, mean fluorescence intensity; Mx1, myxoma response protein 1; MyD88, myeloid differentiation factor 88; PDC, plasmacytoid DC; PEC, peritoneal exudate cell; RIG-I, retinoic acid–inducible gene I; RT-PCR, real-time quantitative PCR; SLE, systemic lupus erythematosus; snRNP, small nuclear ribonucleoprotein; ss, single stranded; TBK-1, TANK-binding kinase 1; TLR, Toll-like receptor; TMPD, 2,6,10,14-tetramethylpentadecane; TRIF, Toll/IL-1 receptor domain–containing adaptor inducing IFN-b; Yaa, Y-linked autoimmune accelerating.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of antibodies against an array of self-antigens such as double-stranded (ds) DNA and components of small nuclear ribonucleoproteins (snRNPs), including the Sm/RNP antigens (U1, U2, U4-6, and U5 snRNPs), Ro/SS-A antigens (Y RNAs), and other antigens (1). Recent evidence strongly suggests that type I IFNs (IFN-Is), a family of antiviral cytokines, are integral to the pathogenesis of SLE. Elevated serum levels of IFN-I and overexpression of IFN-stimulated genes (ISGs) in the peripheral blood of SLE patients have been demonstrated by several groups (2–4). This "IFN signature" is associated with more active disease and the presence of autoantibodies against dsDNA and the Sm/RNP and Ro/SS-A antigens (5, 6).

The etiology of excess IFN-I in SLE is incompletely understood. Research on innate immunity has led to the identification of several pathways mediating IFN-I production in mammalian cells. Toll-like receptor (TLR) 3, a sensor for viral dsRNA, and TLR4, the receptor for LPS, both stimulate IFN-I secretion through

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Toll/IL-1 receptor domain–containing adaptor inducing IFN-β (TRIF) (7). In contrast, TLR7/8 and TLR9 mediate IFN-I production via myeloid differentiation factor 88 (MyD88) in response to single-stranded (ss) RNA and unmethylated CpG DNA, respectively (8–10). In addition, cytoplasmic receptors that recognize intracellular nucleic acids and induce IFN-I have been described recently. Retinoic acid–inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) recognize cytoplasmic RNA and trigger IFN-I by activating IFN-β promoter stimulator 1 (IPS-1; also known as MAVS, VISA, and CARDIF) and IFN regulatory factor (IRF) 3 (11–14). Cytoplasmic DNA binds to a newly described cytoplasmic sensor and triggers IFN-I production via a pathway requiring TANK-binding kinase 1 (TBK-1) and IRF3 (15, 16).

It has been hypothesized that nucleic acids from dying cells may act as ligands for TLR7/8 and TLR9 to trigger IFN-I production in SLE. Immune complexes (ICs) formed by autoantibodies to DNA and snRNPs help to transport these "endogenous ligands" to endosomes where TLR7, 8, and 9 are normally found (17). Activation of these TLRs then induces the production of IFN-I by plasmacytoid DCs (PDCs). This hypothesis is supported by numerous in vitro studies (18, 19). However, therapeutic administration of recombinant IFN-α can directly trigger the production of anti-dsDNA antibodies (20), and in several mouse model of lupus, IFN-I production is required for the induction of autoantibodies (21–23), suggesting that IFN-I dysregulation may occur upstream of autoantibody development. Therefore, it remains controversial whether nucleic acid–containing ICs in SLE initiate IFN-I production or act to perpetuate a positive feedback loop of IFN production initiated by another factor, such as a viral infection.

Experimental lupus induced by the hydrocarbon oil 2,6,10,14-tetramethylpentadecane (TMPD; also known as pristane) displays many key immunological and clinical features of human SLE, including the presence of the IFN signature and lupus autoantibodies such as anti-dsDNA, -Sm, and -RNP (24–26). Importantly, IFN-I play an essential role in this model, as the development of glomerulonephritis and production of autoantibodies (anti-Sm/RNP, -dsDNA, and -Su) are abolished in IFN-I receptor–deficient ($IFNAR^{-/-}$) mice (22). Unexpectedly, a population of $Ly6C^{hi}$ immature monocytes that accumulates in the peritoneal cavity after TMPD treatment, rather than DCs, is the major source of the excess IFN-I seen in this model (27). The persistent influx of $Ly6C^{hi}$ monocytes and production of IFN-I occur within 2 wk of TMPD treatment, long before the appearance of autoantibodies against snRNPs and dsDNA (3–5 mo), indicating that the initial wave of IFN-I production may be independent of the presence of RNA-containing ICs. In this study, we aimed to elucidate the mechanism of IFN-I production in TMPD-induced lupus.

RESULTS

TMPD-induced IFN-I production requires MyD88

To identify the mechanism of IFN-I induction by TMPD, we first analyzed the effect of TMPD on mice with deficiency of the adaptor molecules TRIF or MyD88. TRIF is required to trigger IFN-I production by TLR3 and TLR4 (7), whereas MyD88 mediates TLR7/8 and TLR9 signaling (8–10). We have previously shown that within 2 wk of TMPD treatment, an accumulation of IFN-I–producing $CD11b^{+}Ly6C^{hi}$ monocytes can be detected in the peritoneal cavity in wild-type mice concurrent with increased IFN-I production and ISG expression (27). Compared with wild-type mice, the total number of peritoneal exudate cells (PECs) was significantly reduced in $MyD88^{-/-}$ mice after TMPD treatment (Fig. 1 A).

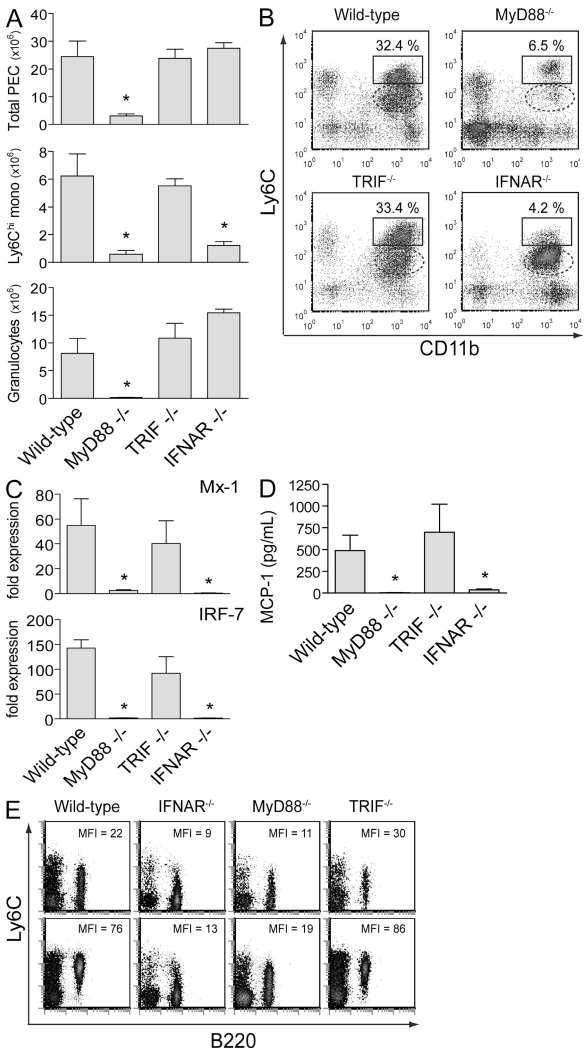

Figure 1. TMPD-induced IFN-I production requires MyD88. (A) Comparison of the number of total PECs, $Ly6C^{hi}$ monocytes, and granulocytes 2 wk after TMPD treatment in wild-type ($n = 5$), $MyD88^{-/-}$ ($n = 6$), $TRIF^{-/-}$ ($n = 4$), and $IFNAR^{-/-}$ mice ($n = 4$). (B) Flow cytometry of peritoneal cells (box indicates $Ly6C^{hi}$ monocytes and dashed oval indicates granulocytes). (C) RT-PCR analysis of *Mx1* and *IRF7* expression in PECs (normalized to peritoneal cells from an untreated wild-type mouse). (D) ELISA quantification of MCP-1 in the peritoneal lavage fluid of TMPD-treated mice. (E) Flow cytometry analysis of Sca-1 expression on peripheral blood mononuclear cells. Mean fluorescence intensity (MFI) of Sca-1 on $B220^{+}$ cells is shown. Each bar represents the mean, and error bars indicate SE. Data are representative of two or more independent experiments. *, $P < 0.05$ using the Student's *t* test.

Both Ly6C^{hi} monocytes and granulocytes (defined as CD11b^{+} Ly6G^{+}Ly6C^{mid}) were decreased by >90% (Fig. 1, A and B). Importantly, we found that IFN-I induction by TMPD was completely dependent on MyD88, because elevated expression of the ISGs *myxoma response protein 1* (*Mx1*) and *IRF7* in PECs was abolished in MyD88$^{-/-}$ mice, as also seen in IFNAR-deficient mice (Fig. 1 C). The levels of the IFN-inducible chemokine monocyte chemoattractant protein 1 (MCP-1; also known as CCL2) in the peritoneal lavage fluid were also reduced in the absence of MyD88 (Fig. 1 D). In contrast, TRIF deficiency did not affect the accumulation of PEC populations or the increased expression of ISGs (Fig. 1, A–D). Although we were unable to detect significant changes in serum IFN-α/β levels by ELISA, IFN-I secretion was required for the response to TMPD, as the up-regulation of ISGs and recruitment of Ly6C^{hi} monocytes were abolished in IFNAR$^{-/-}$ mice (Fig. 1, A–D). The absence of IFN-I signaling, however, did not affect the influx of granulocytes (Fig. 1, A and B).

The increase in IFN-I after TMPD treatment is not limited to the peritoneal cavity, as the IFN signature is also detectable in the peripheral blood (22). We found that surface expression of the IFN-inducible gene Sca-1 (Ly6A/E) on B cells was dramatically up-regulated in wild-type mice treated with TMPD (Fig. 1 E). Although Sca-1 is naturally expressed by certain lymphocyte subsets and hematopoietic stem cells (28), TMPD induced Sca-1 expression in virtually all B cells in wild-type, but not IFNAR$^{-/-}$, mice (Fig. 1 E). Increased Sca-1 expression was also evident on CD8^{+} and CD4^{+} T cells (unpublished data). Similar to the pattern of ISG expression in PECs, the up-regulation of Sca-1 was reduced in MyD88$^{-/-}$ but not TRIF$^{-/-}$ mice (Fig. 1 E), further supporting an essential role of MyD88 in IFN-I production in this model.

To address whether the cytoplasmic nucleic acid sensors also contribute to TMPD-induced IFN-I production, we tested the effect of TMPD on IPS-1$^{-/-}$ and TNF$^{-/-}$TBK1$^{-/-}$ mice (TBK-1$^{-/-}$ is embryonically lethal unless crossed with mice deficient of TNF-α [29]). IPS-1 is a required adaptor for intracellular viral RNA detection via RIG-I and MDA5 (12), whereas TBK-1 is required for cytoplasmic DNA-induced IFN-I secretion (30). The expression of *Mx1* and *IRF7* in PECs was comparable in wild-type, IPS-1$^{-/-}$, TNF$^{-/-}$TBK-1$^{-/-}$, and TNF$^{-/-}$ mice (Fig. 2), suggesting that the intracellular nucleic acid–sensing pathways are not required for IFN-I production in this model. The patterns of peritoneal cell influx and Sca-1 expression on peripheral blood lymphocytes were also similar among these strains (unpublished data). Collectively, our data indicate that TMPD-elicited IFN-I production was strictly MyD88 dependent.

TMPD-induced IFN-I production is TLR7 dependent, IC independent

Because MyD88 mediates IFN-I induction by TLR7 and TLR9, we next investigated which of these innate receptors is responsible for the effect of TMPD. 2 wk after TMPD treatment, the number of PECs in TLR9$^{-/-}$ mice was reduced by ~20% compared with TLR7$^{-/-}$ and wild-type controls. The numbers of Ly6C^{hi} monocytes and granulocytes were also reduced in the absence of TLR9, whereas the pattern of the cellular influx remained similar to wild-type animals (Fig. 3, A and B).

Interestingly, TLR7$^{-/-}$ mice exhibited a specific reduction of Ly6C^{hi} monocytes in the peritoneal cavity (Fig. 3 A). Despite the decrease in these immature monocytes, total PEC counts in TLR7$^{-/-}$ mice were comparable to wild-type controls because of a significant increase in the number of granulocytes (Fig. 3, A and B). An accumulation of CD11b^{+}Ly6C^{-} residential macrophages was also evident in the absence of TLR7 (Fig. 3 B). These patterns are strikingly similar to those observed in IFNAR$^{-/-}$ mice (Fig. 1, A–D). Indeed, the increased *Mx1* and *IRF7* expression and MCP-1 production were abrogated in TLR7$^{-/-}$ mice (Fig. 3 C). Up-regulation of Sca-1 expression on B cells was also reduced in these mice after TMPD treatment (Fig. 3 D), recapitulating the findings in MyD88$^{-/-}$ and IFNAR$^{-/-}$ mice. On the contrary, ISG expression in PECs and surface expression of Sca-1 were similar in TLR9$^{-/-}$ mice compared with wild-type controls. These findings suggest that although TLR9 may contribute to the inflammatory response, IFN-I induction by TMPD was mediated primarily by TLR7.

To further define the role of TLR7 in the inflammatory response to TMPD, we compared the expression of various cytokines and chemokines in wild-type and TLR7$^{-/-}$ animals using a PCR array. After TMPD treatment, peritoneal cells from wild-type mice displayed dramatically higher

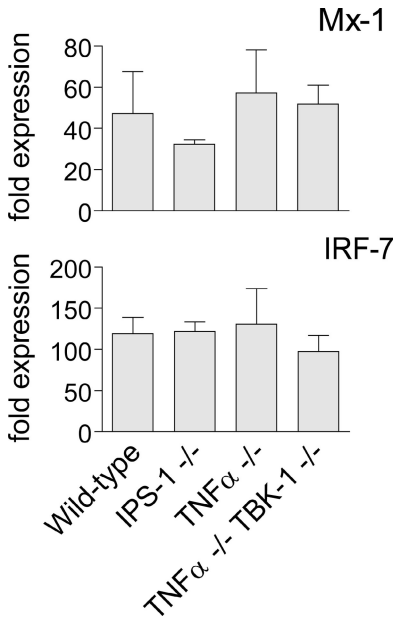

Figure 2. Cytoplasmic nucleic acid sensors do not contribute to IFN-I production. RT-PCR analysis of *Mx1* and *IRF7* expression in PECs from wild-type (n = 5), IPS-1$^{-/-}$ (n = 5), TNF$^{-/-}$ (n = 2), and TNF$^{-/-}$TBK-1$^{-/-}$ (n = 4) animals (normalized to peritoneal cells from an untreated wild-type animal). Each bar represents the mean, and error bars indicate SE. Data are representative of two independent experiments.

expression of several IFN-stimulated chemokines, including *CCL2*, *CCL12*, *CCL7*, and *CXCL10*, in comparison with TLR7$^{-/-}$ mice (Table S1, available at http://www.jem.org/cgi/content/full/jem.20080462/DC1). This pattern of chemoattractant production is similar to the chemokine signature recently described in SLE patients (31). These observations support the role of TLR7 as the primary mediator of IFN-I production in the TMPD lupus model. Interestingly, consistent with the increased number of peritoneal granulocytes in TMPD-treated TLR7$^{-/-}$ mice (Fig. 3 A), the neutrophil chemoattractant *CXCL5* was up-regulated in the absence of TLR7, whereas the expression of other inflammatory mediators was comparable between the groups (Table S1).

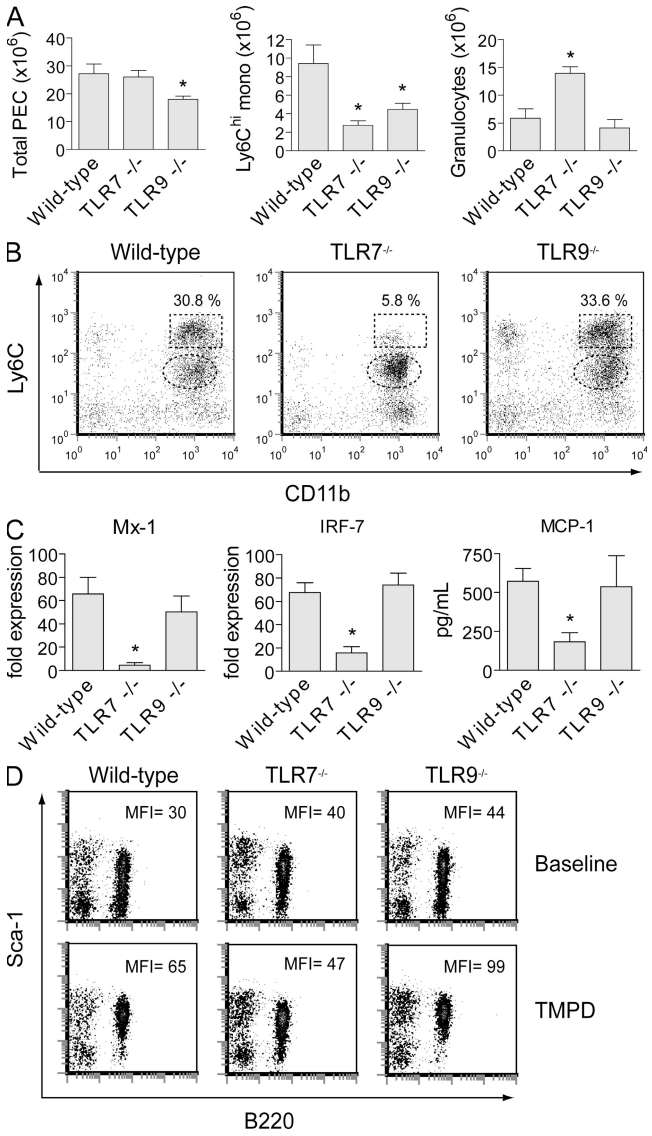

Figure 3. IFN-I production induced by TMPD is TLR7 dependent. (A) Comparison of the number of total PECs, Ly6C^{hi} monocytes, and granulocytes 2 wk after TMPD treatment in wild-type ($n = 5$), TLR7$^{-/-}$ ($n = 5$), and TLR9$^{-/-}$ ($n = 5$) mice. (B) Flow cytometry analysis of peritoneal cells (box indicates Ly6C^{hi} monocytes and dashed oval indicates granulocytes). (C) RT-PCR analysis of *Mx1* and *IRF7* expression in PECs (normalized to peritoneal cells from an untreated wild-type animal) and ELISA quantification of MCP-1 in the peritoneal lavage fluid. (D) Flow cytometry of Sca-1 surface expression on peripheral blood mononuclear cells. MFI of Sca-1 on B220$^+$ cells is shown. Each bar in A and C represents the mean ($n > 4$ per group), and error bars indicate SE. Data are representative of two independent experiments. *, $P < 0.05$ using the Student's *t* test.

Several studies have demonstrated that self-RNA present in ICs may act as an endogenous TLR7 ligand causing the production of IFN-I (18, 19). FcγRs have been reported to play an essential role in this process by enhancing the internalization of ICs, thereby allowing RNA to interact with TLR7 in endosomes (32). Deficiency of either TLR7 or FcγR γ chain, an integral component of FcγRI and FcγRIII, renders mouse DCs unable to produce IFN-I in response to lupus ICs (33). Because TMPD induces IFN-I production long before the onset of lupus autoantibodies and ICs (27), we examined the potential role of FcγR in the IFN-I response to TMPD. Surface expression of FcγRI (CD64) and FcγRII/III (CD32/CD16) in TMPD-induced PECs was prominent on Ly6C^{hi} monocytes (Fig. 4 A). FcγRs were also expressed by a fraction of granulocytes, whereas lymphocytes and DCs in the peritoneal cavity displayed little surface expression of these receptors (Fig. 4 A).

We next analyzed the effects of TMPD on FcγR γ chain–deficient (FcγR$^{-/-}$) mice. The accumulation of Ly6C^{hi} monocytes and up-regulation of ISG expression were comparable in FcγR$^{-/-}$ and wild-type mice (Fig. 4, B and C). Elevated surface expression of Sca-1 on peripheral blood lymphocytes was also similar between the groups (unpublished data). Collectively, these findings suggest that TMPD elicits IFN-I production through a TLR7-dependent but FcγR-independent pathway.

Ly6C^{hi} monocytes express high levels of TLR7

Next, we examined the distribution of *TLR7* expression in the inflammatory infiltrate induced by TMPD. PECs from mice treated with TMPD 2 wk earlier were sorted into four distinct populations based on surface marker phenotype: Ly6C^{hi} monocytes (CD11b$^+$Ly6C^{hi}; ∼30% of PECs), granulocytes (CD11b$^+$Ly6G$^+$; ∼30% of PECs), DCs (CD11c$^+$; ∼2% of PECs), and a negative fraction containing mainly B and T lymphocytes. As reported previously (27), the DC fraction consisted of >80% CD11b$^+$ myeloid DCs, and few PDCs (CD11c$^+$CD11b$^-$B220$^+$) were present.

Quantitative PCR revealed that Ly6C^{hi} monocytes expressed higher levels of *TLR7* than other peritoneal cell subsets (Fig. 5 A). Prominent expression of the chemokine receptor CCR2 is consistent with their immature monocytic phenotype, as reported previously (34). In striking contrast, elevated expression of *TLR7* was not a feature of Ly6C^{hi} monocytes in the spleen or bone marrow. Although *TLR7* expression also was found on other PEC subsets, DCs displayed greater expression of *TLR3* and *TLR9*, whereas *TLR4* transcripts were predominantly found in granulocytes (Fig. 5 A).

In line with these findings, when peritoneal Ly6C^{hi} monocytes were depleted by i.p. injection of clodronate-containing

liposomes (clo-lip), the expression of *TLR7* by PECs was greatly reduced, whereas the levels of *TLR9* transcripts remained unaffected (Fig. 5 B). We further analyzed the response of Ly6C^{hi} monocytes to various TLR ligands in vitro. Sorted Ly6C^{hi} monocytes secreted large amounts of MCP-1 and IL-6 when co-cultured with the synthetic TLR7 ligand R848 (Fig. 5 C). They also responded to the TLR4 ligand LPS, albeit less strongly even at the highest dose of LPS tested (10 μg/ml). Consistent with their low levels of TLR3 and TLR9 expression, Ly6C^{hi} monocytes exhibited weak responses to poly I:C and CpG DNA (Fig. 5 C). In contrast, isolated granulocytes did not produce measurable amounts of MCP-1 or IL-6 in response to any TLR ligands used in this study (unpublished data). Hence, Ly6C^{hi} monocytes recruited to the peritoneal cavity after TMPD treatment expressed high levels of *TLR7* and actively responded to TLR7 ligands.

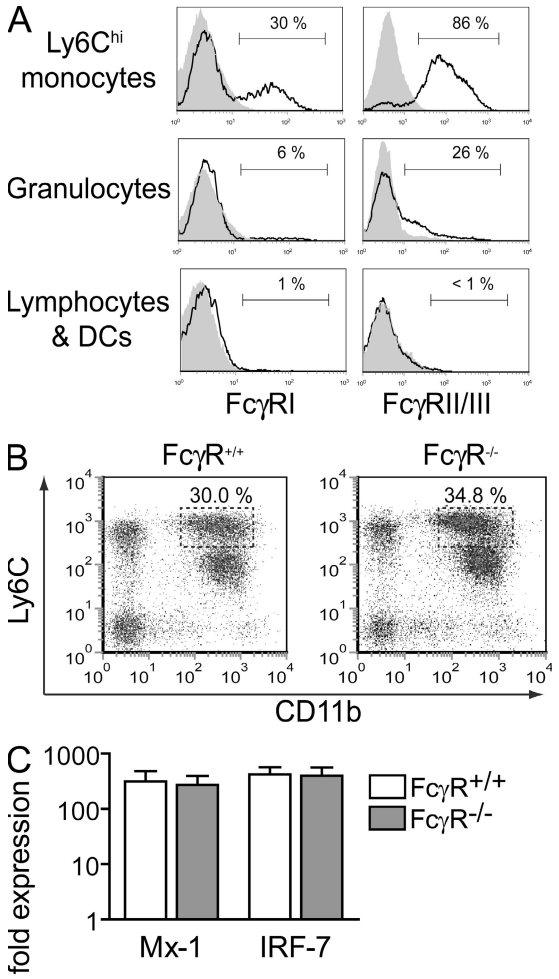

Figure 4. FcγRI and FcγRIII are dispensable for IFN-I induction by TMPD. (A) Flow cytometry analysis of FcγRI (CD64) and FcγRII/III (CD32/CD16) in PEC populations in TMPD-treated wild-type mice (open histograms). Shaded histograms represent staining with isotype control antibodies. (B) Peritoneal cell influx and (C) ISG expression in wild-type mice and FcγRI/III$^{-/-}$ (γ chain–deficient) mice (n = 3 per group) 2 wk after TPMD treatment. Dashed boxes in B indicate Ly6C^{hi} monocytes. Each bar in C represents the mean, and error bars indicate SE. Data are representative of two independent experiments.

The Y-linked autoimmune accelerating (Yaa) locus amplifies the effects of TMPD

The Yaa locus is essential for the spontaneous development of autoantibodies and glomerulonephritis in the BXSB model of mouse lupus (35). Yaa also accelerates disease onset in other lupus-prone strains (36, 37). Interestingly, *TLR7* is among the 17 genes found in the Yaa locus (38, 39). Because *TLR7* is normally located on the X chromosome in both humans and mice, males possess one copy of the gene, whereas a similar gene dosage is achieved in females through X inactivation. Male mice with the Yaa locus, however, have two functional copies of the gene (38, 39). A recent study showed that the *TLR7* gene duplication alone was responsible for the autoimmune features associated with the Yaa mutation (40).

Because TMPD induces IFN-I production via TLR7, we asked whether the effects of the adjuvant oil are more pronounced in the presence of the Yaa locus. Compared with female controls (designated TLR7$^{+/+}$), BXSB × B6 male mice carrying the Yaa mutation (TLR7$^{+/Yaa}$) exhibited greater accumulation of Ly6C^{hi} monocytes in the peritoneal cavity 2 wk after TMPD treatment (Fig. 6 A). Importantly, increasing the gene dosage of TLR7 was associated with significantly higher production of IFN-I, assessed by measuring ISG expression (Fig. 6 B). In contrast, the IFN-I response to TMPD was similar between males and females in wild-type strains, including C57BL/6, BALB/c, and 129Sv (unpublished data).

We further examined the long-term effect of TMPD treatment in the BXSB model. Consistent with previous studies (35), BXSB males died prematurely beginning at 5 mo of age (Fig. 6 C). Mortality was significantly accelerated when a single dose of TMPD was administered i.p. at 8–10 wk of age. 60% of TMPD-treated animals succumbed by 5 mo of age compared with 20% in the control group. Although 5 out of 10 PBS-treated animals survived until 7 mo, only 1 out of 10 in the TMPD-treated group remained. Thus, not only was the induction of IFN-I accentuated by the Yaa mutation, TMPD treatment also hastened disease progression in the BXSB model of lupus.

TLR7 is essential for the development of autoantibodies against RNA-associated antigens

A recent study using MRL-*lpr* mice showed that TLR7 is required for the development of anti-Sm antibodies (41). To assess whether TLR7 is also involved in autoantibody production in the TMPD model of lupus, we compared the long-term response to TMPD in wild-type BALB/c and BALB/c. TLR7$^{-/-}$ mice. No mortality was found in either group and only mild proteinuria was detected by 24 wk after treatment (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080462/DC1). Comparable to previous observations (42), BALB/c mice displayed significant hypergammaglobulinemia 24 wk after TMPD treatment (Fig. 7 A). The increase in serum IgG was reduced by 50% in TLR7$^{-/-}$ mice,

whereas IgM levels were similar between the groups. Consistent with the prominent role of IFN-I in IgG2a isotype switch (43), a profound reduction of IgG2a was found in TLR7$^{-/-}$ mice (Fig. 7 B). IgG1 and IgG2b levels were also mildly reduced in the absence of TLR7, whereas IgG3 was slightly increased.

As early as 12 wk after TMPD treatment, 8 out of 12 wild-type mice developed detectable serum levels of antinuclear antibodies (ANAs) and anti-nRNP/Sm autoantibodies (Fig. 7, C and D). In contrast, only one animal in the TLR7$^{-/-}$ group showed a low titer ANA and none developed autoantibodies to nRNP/Sm (Fig. 7, C and D). Similar to IFNAR$^{-/-}$ animals (22), most TLR7$^{-/-}$ mice exhibited low levels of ANAs of unknown specificity by 24 wk after treatment, but their titers remained significantly lower than BALB/c controls (Fig. 7 C). A single TLR7$^{-/-}$ animal with a high ANA titer (1:1,280) produced autoantibodies against DNA/chromatin (unpublished data). In contrast, autoantibodies against nRNP-Sm remained undetectable in all TLR7$^{-/-}$ mice at this time point (Fig. 7 D). We further confirmed the autoantibody profile by immunoprecipitation using nuclear extracts from ^{35}S-labeled K562 cells. Consistent with the findings by ELISA, autoantibodies against various components of nRNP/Sm (A-G) were found in wild-type but not TLR7$^{-/-}$ mice (Fig. 7 E).

Immunoprecipitation studies also revealed that autoantibodies against the Su antigen, which develops in ~20% of lupus patients (26) and ~50% of TMPD-treated BALB/c mice (26), were not induced in TLR7$^{-/-}$ mice (Fig. 8 A). A recent study identified the Su antigen as an RNA-binding component of the microRNA pathway known as argonaute 2 (ago2) (44). ELISA using recombinant ago2 confirmed that the development of autoantibodies against Su/ago2 was TLR7 dependent (Fig. 8 B), suggesting that the autoimmune response to microRNA-associated antigens is also mediated by TLR7. Collectively, in addition to its role in mediating IFN-I production, TLR7 is essential for the generation of autoantibodies against RNA-associated antigens (nRNP/Sm and Su/ago2) in TMPD-induced lupus.

TMPD enhances TLR7 stimulation in vitro

To further investigate the link between TMPD treatment and TLR7 activation, we studied the effect of TMPD in vitro. Although TMPD has been used for decades, mechanistic studies on its cellular effects have been limited by the hydrocarbon's poor immiscibility in aqueous solutions. We found that this problem could be circumvented by first mixing TMPD with fetal bovine serum before the addition of culture medium (unpublished data). Unlike stimulation with R848, a TLR7 ligand that elicits IL-6 production and co-stimulatory molecule up-regulation in mouse J774 macrophages, the addition of TMPD in vitro did not induce these responses (Fig. 9 A and not depicted). TMPD solubilized in nonphysiological solvents such as ethanol, DMSO, mannide monooleate, or

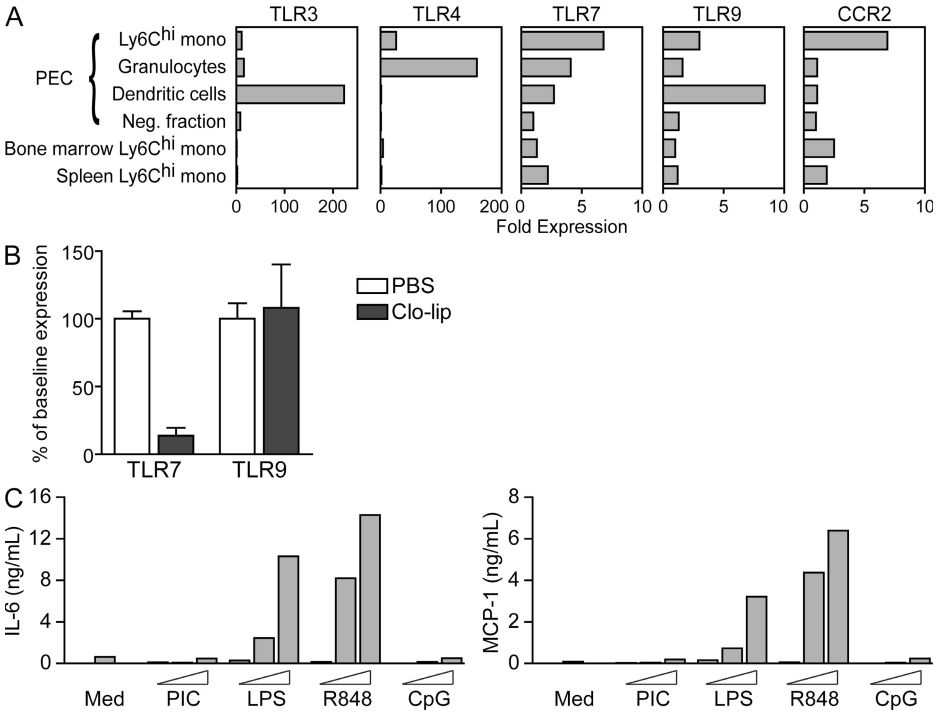

Figure 5. Ly6C^{hi} monocytes express high levels of TLR7. (A) RT-PCR analysis of TLR expression in sorted PEC populations, splenic Ly6C^{hi} monocytes, and bone marrow monocyte precursors from wild-type TMPD-treated mice. (B) RT-PCR analysis of TLR7 and TLR9 expression in PECs from TMPD-treated mice 48 h after i.p. injection of PBS or clo-lip. Each bar represents the mean, and error bars indicate SE. (C) ELISA for MCP-1 and IL-6 produced by sorted peritoneal Ly6C^{hi} monocytes (5×10^4 cells/well) 24 h after TLR ligand stimulation. Wedges denote increasing concentrations of LPS, R848, and CpG DNA (100 ng/ml, 1 μg/ml, and 10 μg/ml), and poly I:C (200 ng/ml, 2 μg/ml, and 20 μg/ml). Data are representative of two independent experiments.

β-cyclodextrin was also ineffective in triggering TLR7 activation (Fig. 9 A), suggesting that TMPD itself is not a ligand for TLR7.

However, exposure to TMPD dramatically enhanced the response to subsequent stimulation with TLR7 ligands. R848-induced IL-6 production and co-stimulatory molecule up-regulation were augmented in J774 cells pretreated overnight with TMPD (Fig. 9 B). Similar enhancement of chemokine and cytokine production was found in bone marrow–derived macrophages stimulated with R848 but not the TLR9 ligand ODN 2395 (Fig. 9 C). Importantly, these observations were specific to TMPD, as treatment with other hydrocarbon oils that do not induce lupus, such as medicinal mineral oil and squalene, did not enhance stimulation by R848 (Fig. 9 D). We further examined whether TMPD augments the response to TLR7 ligands by altering the expression or location of TLR7. TLR7 remained exclusively intracellular regardless of TMPD treatment, and its expression levels were not affected at the protein or mRNA level (Fig. 9 E). Moreover, TMPD treatment did not enhance endocytosis of FITC-dextran or phagocytosis of latex beads or apoptotic cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20080462/DC1), suggesting that accelerated uptake of ligands is also unlikely to explain our findings. Collectively, these data indicate that TMPD is not a direct ligand for TLR7 but instead acts to enhance the response to TLR7 stimulation.

DISCUSSION

Recent studies strongly suggest a link between elevated IFN-I production and the pathogenesis of SLE. More than half of SLE patients display increased expression of ISGs, often in association with active disease and autoantibodies against snRNPs and DNA, as well as renal involvement and endothelial dysfunction (2–6, 45). However, the exact cause of IFN-I dysregulation in lupus remains controversial, and it is unclear whether IFN-I overproduction promotes autoantibody production or vice versa.

The TMPD model of lupus recapitulates many features of human SLE, including glomerulonephritis, arthritis, and autoantibodies against dsDNA and snRNPs (25, 26). Moreover, like SLE, TMPD-induced lupus is more severe in females than males (46). We recently found that TMPD-treated mice exhibit the IFN signature and that their lupus is dependent on IFN-I signaling (22). The major IFN-I–producing cells in TMPD lupus are Ly6C^{hi} monocytes rather than PDCs (27). In this study, we show that TMPD triggers IFN-I production via the TLR7–MyD88 pathway. Our data also exclude a major role of other pathways of IFN-I production, including TLR9, TLR3–TLR4–TRIF, RIG-I–Mda5–IPS-1, and DAI–TBK1. Although there was a strict requirement for TLR7 and MyD88, expression of ISGs and recruitment of Ly6C^{hi} monocytes in response to TMPD was unexpectedly independent of FcγRs.

The innate sensors TLR7 and TLR9 have been implicated in SLE because of their ability to recognize endogenous nucleic acids and trigger IFN-I production (47). Mammalian nucleic acids are generally weak TLR ligands because of their inability to reach the endosomal compartment where TLR7 and TLR9 are localized. When they form ICs with lupus autoantibodies (anti-Sm/RNP or anti-dsDNA), endogenous nucleic acids may be delivered more efficiently to endosomes because of uptake by FcγRs, stimulating IFN-I production by PDCs (17, 47). This model for IFN-I induction in human SLE is supported by numerous in vitro studies (19, 32, 48). Although FcγRIIa mediates the activation of human PDCs, ICs trigger IFN-I production by mouse PDCs in a TLR7- and FcγRI/III-dependent manner (33).

However, it is not known whether the development of antiribonucleoprotein autoantibodies is a cause of IFN-I dysregulation or a consequence of it. Therapeutic use of IFN-α can induce many features of SLE, including anti-dsDNA antibodies, suggesting that IFN-I dysregulation occurs upstream of IC formation. Consistent with that view, IFN-I up-regulation in the TMPD model occurs within the first 2 wk of treatment, more than 2 mo before the onset of lupus autoantibodies (27). FcγRI and FcγRIII were not required for the IFN-I

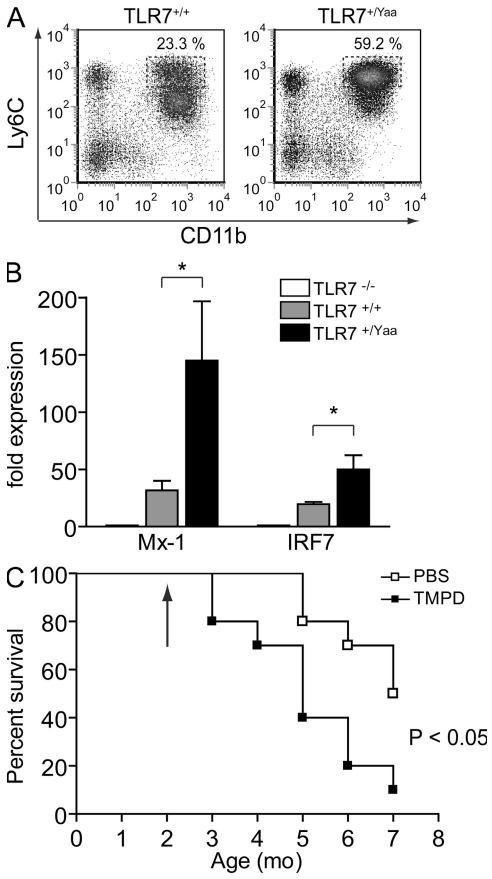

Figure 6. Yaa mutation amplifies the effects of TMPD. (A) Flow cytometry of peritoneal cells and (B) RT-PCR analysis of ISG expression in BXSB × B6 female (TLR7$^{+/+}$) and male (TLR7$^{+/Yaa}$) mice. Dashed boxes in A indicate Ly6C^{hi} monocytes. Each bar in B represents the mean, and error bars indicate SE. Data are representative of two independent experiments. *, $P < 0.05$ using the Student's *t* test. (C) Survival curve for male BXSB mice after PBS or TMPD treatment (arrow denotes treatment at 8–10 wk of age).

response to TMPD because it was unaffected in γ chain–deficient mice. Moreover, a previous study also showed that the absence of FcγRI/III or FcγRIIb does not affect anti-Sm/RNP autoantibody production in TMPD-treated mice (49). Although IC formation and FcγRs are not required to initiate IFN-I production, we cannot exclude the possibility that they amplify IFN-I secretion and accelerate disease progression subsequent to the development of autoantibodies.

A pathogenic role of TLR7 has been described in several mouse models of SLE. In MRL-*lpr* mice, TLR7 ligands accelerate the onset of glomerulonephritis, whereas deletion of TLR7 abrogates the development of anti-Sm autoantibodies and lessens the severity of kidney disease (41, 50). Lupus in MRL-*lpr* mice has been reported to be ameliorated, not exacerbated, by IFN-I (51), and the *lpr* defect prevents induction of TMPD lupus (52).

Dual engagement of TLR7 and the B cell receptor can directly activate autoreactive B cells in the AM14 model, and TLR7 is also required for the spontaneous production of autoantibodies against ssRNA in 564Igi transgenic mice (53, 54). The connection between TLR7 and the generation of RNA-associated autoantibodies is further illustrated by the recent demonstration of a duplication of the TLR7 gene in Yaa mice. The presence of the Yaa cluster was sufficient to induce production of RNA-associated autoantibodies in C57BL/6 FcγIIB$^{-/-}$ and C57BL6.Sle1 mice, two autoimmune strains that normally lack these antibody specificities (38, 39). TLR7, and not the other 16 genes affected by the Yaa mutation, is responsible for the autoimmune pathology (40). However, increased IFN-I production has not been reported in these models. Our findings indicate that TLR7 also plays an essential role in TMPD lupus. Similar to the MRL-*lpr* model (41), TLR7 is required for the generation of anti-nRNP/Sm autoantibodies in TMPD-treated mice. Importantly, the IFN signature in TMPD-treated mice, which is established within 2 wk of treatment (long before the appearance of anti-nRNP autoantibodies), was abolished in the absence of TLR7. In contrast, the effects of TMPD were amplified in the presence of the Yaa locus. Therefore, this study provides evidence for direct in vivo involvement of TLR7 in the induction of IFN-I, even in the absence of autoantibodies and ICs.

We have previously shown that Ly6C^{hi} monocytes are a major source of IFN-I in the TMPD model (27). Depletion of monocytes but not DCs reduced IFN-I production and ISG expression. In this study, we found that Ly6C^{hi} monocytes also express higher levels of TLR7 and display a greater response in vitro to R848 than to other TLR ligands. Although TLR7 is normally found on monocytes and macrophages, its expression on peritoneal Ly6C^{hi} monocytes in TMPD-treated mice was several fold higher than on splenic

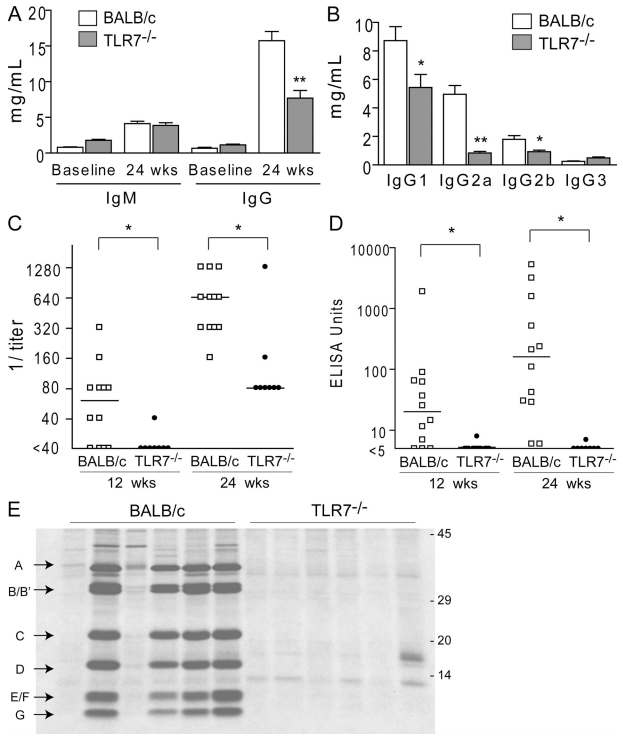

Figure 7. TLR7 promotes hypergammaglobulinemia and mediates the development of anti-nRNP/Sm autoantibodies. (A) Total IgM and IgG and (B) IgG subclass levels in BALB/c.TLR7$^{-/-}$ (n = 8) and wild-type BALB/c (n = 12) mice before and 24 wk after TMPD treatment. Bars represent the mean, and error bars indicate SE. *, $P < 0.05$; and **, $P < 0.001$ using the unpaired t test. (C) Fluorescent ANA titers (titration emulation) and (D) anti-nRNP/Sm IgG levels (antigen-capture ELISA) at 12 and 24 wk after TMPD treatment. Horizontal lines indicate medians. *, $P < 0.05$ using the Mann-Whitney U test. (E) Immunoprecipitation of serum autoantibodies (n = 6 per group) using nuclear extracts from ^{35}S-labeled K562 cells (12.5% polyacrylamide gel). Arrows indicate components of nRNP/Sm, and numerical values denote the molecular mass (kD).

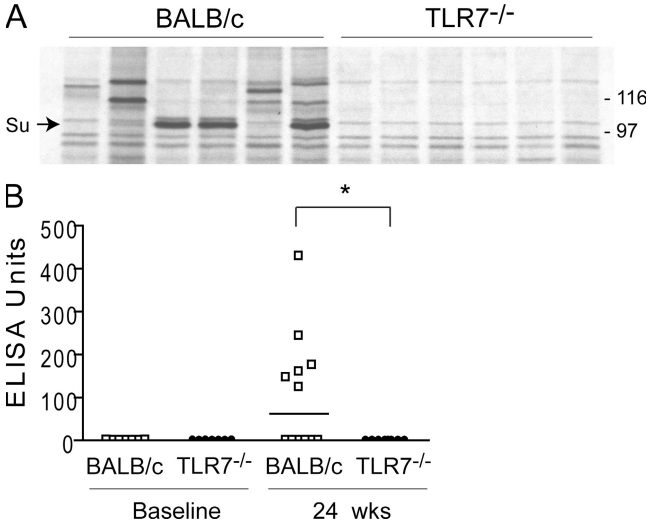

Figure 8. TLR7 is required for the development of anti-Su/ago2 autoantibodies. (A) Immunoprecipitation of serum autoantibodies 24 wk after TMPD treatment (8% polyacrylamide gel; n = 6 per group). Arrows indicate the 100-kD Su antigen, and numerical values denote the molecular mass (kD). (B) Anti-Su/ago levels at baseline and 24 wk after TMPD treatment measured by ELISA using recombinant ago2 protein. Horizontal lines indicate medians. *, $P < 0.05$ using the Mann-Whitney U test.

or bone marrow monocytes. In contrast, DCs in the peritoneal exudate displayed lower levels of TLR7 and more prominent TLR3 and TLR9 expression. The high expression level of TLR7 by Ly6C^{hi} monocytes may be of critical importance in the pathogenesis of TMPD lupus, as a recent study demonstrated that increased gene dosage of TLR7 is sufficient to trigger anti-RNA antibodies and glomerulonephritis in C57BL/6 mice (40). Interestingly, the recruitment of Ly6C^{hi} monocytes to the peritoneum seems to be partially dependent on IFN-I, as seen in TLR7$^{-/-}$, MyD88$^{-/-}$, and IFNAR$^{-/-}$ mice. TLR7 signaling also induces the expression of several IFN-stimulated chemokines (*CCL2*, *CCL7*, and *CCL12*), suggesting that the mechanism may involve enhanced production of monocyte chemoattractants, creating an amplification loop of Ly6C^{hi} monocyte recruitment and IFN-I production. The recruitment of DCs and granulocytes, on the other hand, was not dependent on IFN-I or TLR7.

The mechanism linking TMPD to the activation of TLR7 has been partially elucidated by our studies. The hydrocarbon structure of TMPD is distinct from known TLR7 ligands, including ssRNA, R848, loxoribine, and other guanosine analogues. Indeed, our in vitro studies showed that TMPD did not activate TLR7 directly but instead augmented the inflammatory response to TLR7 ligands such as R848. The ability of hydrocarbon oils to enhance TLR7 stimulation in vitro seems related to their ability to induce lupus-like disease in vivo. Unlike TMPD, squalene and medicinal mineral oil were ineffective in augmenting the response to R848. Mice treated with squalene and mineral oil do not display the IFN signature and few develop lupus autoantibodies (27, 55). TMPD appears to enhance activation via the TLR7 pathway in at least two ways: (a) by augmenting the recruitment of Ly6C^{hi} monocytes, which express high levels of TLR7, and (b) by enhancing the intrinsic responsiveness of TLR7 to its ligands. Additional studies will be needed to elucidate whether TMPD causes increased uptake of apoptotic/necrotic material, enhancing the recognition of TLR7 ligands, or if TMPD interacts with components of the TLR7 signaling pathway, augmenting the response to receptor–ligand interactions.

Although the underlying mechanisms are distinct, the pathological consequences of excess TLR7 activation are shared by TMPD-induced lupus and the Yaa model. An important unanswered question that encompasses both models is the nature of the exogenous or endogenous ligands responsible for activating the TLR7 pathway. It is possible that chronic TMPD-stimulated inflammation provides a persistent source of autoantigens from apoptotic cells and that endogenous TLR7 ligands such as the U1 RNA component of the Sm/RNP antigen (56–58) trigger the first wave of IFN-I production. Downstream signaling events may elicit further IFN-I production (59) and *TLR7* expression (60), culminating in a positive feedback cycle that promotes autoimmunity by persistently activating TLR7. It remains to be verified experimentally whether or not RNA-associated autoantigens from apoptotic cells are key mediators of Yaa- and TMPD-induced lupus in vivo.

It is noteworthy that besides activating IFN-I production via TLR7, TMPD also induced the recruitment of granulocytes via an MyD88-dependent but TLR7-independent pathway. The number of peritoneal granulocytes actually increased in the absence of IFN-I production, as seen in TLR7$^{-/-}$ and IFNAR$^{-/-}$ mice. MyD88 is used in the signaling pathways of other cytokines (IL-1 and IL-18) and TLRs (except TLR3), which are potential mediators of granulocyte recruitment in this model.

Finally, our findings may shed light on other pathology induced by TMPD. The development of plasmacytomas after i.p. injection of TMPD in BALB/cAnPt mice was first described more than three decades ago (61). Subsequently TMPD was used to enhance monoclonal antibody production by hybridomas (62). How TMPD elicits these effects is incompletely understood, although IL-6 has been implicated. Interestingly,

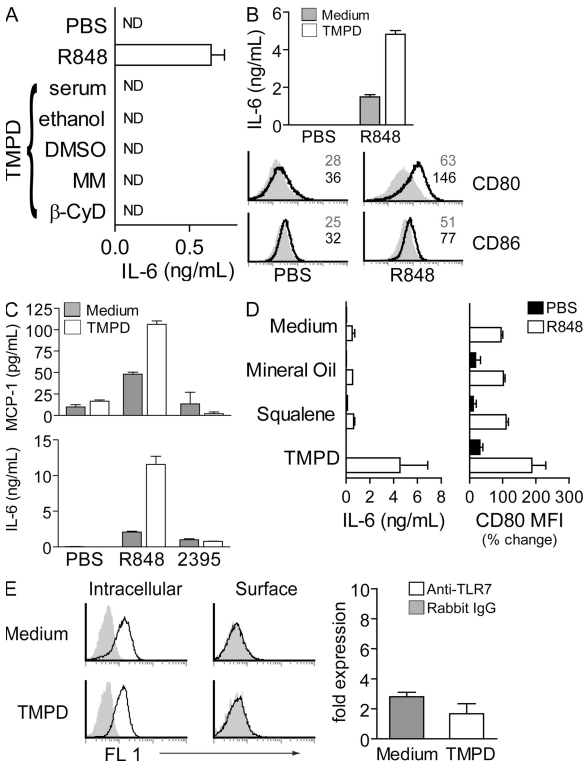

Figure 9. TMPD enhances TLR7 stimulation in vitro. (A) ELISA for IL-6 production in J774 cells cultured in the presence of 1 μg/ml R848, 1 μg/ml TMPD incorporated in serum, or 300 μM TMPD solubilized in ethanol, DMSO, mannide monooleate (MM), or β-cyclodextrin (β-CyD). ND, not detectable. (B) ELISA (IL-6 and MCP-1) and flow cytometric analysis (CD80 and CD86) in J774 cells or (C) bone marrow-derived macrophages cultured for 20 h with or without TMPD and stimulated with PBS, 1 μg/ml R848, or 2 μg/ml ODN 2395 for 24 h. MFI values are provided. Shaded histograms represent J774 cells cultured in medium alone, whereas open histograms represent cells treated with TMPD. (D) Comparison of IL-6 production and CD80 expression (MFI) in J774 cells cultured with various hydrocarbon oils and stimulated with PBS or R848. (E) Flow cytometry and RT-PCR analysis of TLR7 expression in J774 cells cultured with or without TMPD for 20 h. Each bar represents the mean, and error bars indicate SE. Data are representative of three or more independent experiments.

although TLR7 can trigger B cell activation and antibody production (53), IFN-I plays an important role in antibody class switching and promotes plasma cell differentiation in the presence of IL-6 (63). Whether TLR7 activation and IFN-I production are involved in the pathogenesis of plasmacytomas and enhancement of antibody production by hybridomas warrants further investigation.

MATERIALS AND METHODS

Mice. MyD88$^{-/-}$, TRIF$^{-/-}$, TLR7$^{-/-}$, TLR9$^{-/-}$, and IFNAR$^{-/-}$ mice (backcrossed >7 generations to the C57BL/6 background), and IPS-1$^{-/-}$, TNF$^{-/-}$, TNF$^{-/-}$TBK1$^{-/-}$ mice (on a mixed 129Sv/B6 background) have all been described previously (7, 8, 29, 64–66). Wild-type C57BL/6 and heterozygous littermates were used as controls. BALB/c.TLR7$^{-/-}$ (backcrossed >8 generations to the BALB/c background) and wild-type BALB/c mice were used for long-terms studies of autoantibody production. Animals were bred and maintained in a specific pathogen-free facility of the Research Institute for Microbial Diseases, Osaka University. C57BL/6 wild-type and FcγRI/III$^{-/-}$ mice (Taconic) and BXSB mice (The Jackson Laboratory) were maintained in a specific pathogen-free facility at the University of Florida. BXSB × B6 F1 mice were generated by breeding BXSB males with C57BL/6 females. 12–16-wk-old animals received a single i.p. injection of 0.5 ml TMPD (Sigma-Aldrich). Blood samples were obtained before TMPD treatment and weekly thereafter. Peritoneal cells, spleen, and blood were harvested 2 wk after treatment. Monocyte depletion was performed by i.p. injection of 200 μl clo-lip, as previously described (67). These studies were approved by the University of Florida Institutional Animal Care and Use Committee and the Osaka University Animal Care and Use Committee.

Real-time quantitative PCR (RT-PCR). RT-PCR was performed as previously described (24). In brief, total RNA was extracted from 10^6 peritoneal cells using TRIzol reagent (Invitrogen), and cDNA was synthesized using the Superscript II First-Strand Synthesis kit (Invitrogen) according to the manufacturer's protocol. SYBR green RT-PCR analysis was performed using a thermocycler (Opticon II; MJ Research). Amplification conditions were as follows: 95°C for 10 min, followed by 45 cycles of 94°C for 15 s, 60°C for 25 s, and 72°C for 25 s. After the final extension (72°C for 10 min), a melting-curve analysis was performed to ensure specificity of the products. Primers used in this study are listed in Table S2 (available at http://www.jem.org/cgi/content/full/jem.20080462/DC1). Cytokine/chemokine PCR array (Superarray) analysis was performed using a sequence detector (ABI 7700; Applied Biosystems) according to the manufacturer's protocols.

Flow cytometry and cell sorting. The following conjugated antibodies were used: anti–CD11b-PE, anti–CD8-allophycocyanin (APC), anti–CD4-FITC, anti–CD11c-PE, anti–B220-PerCPCy5.5, anti–Sca-1–PE, anti–CD64-PE, anti–CD32/16-PE (all from BD), anti–Ly6C-FITC, anti–Ly6C-biotin, and avidin-APC (all from eBioscience). Before surface staining, peritoneal or peripheral blood cells were incubated with anti–mouse CD16/32 (Fc block; BD) for 10 min. Cells were then stained with an optimized amount of primary antibody or the appropriate isotype control for 10 min at room temperature before washing and resuspending in PBS supplemented with 0.1% BSA. Intracellular staining for TLR7 was performed as previously described (50) using rabbit anti–mouse TLR7 or rabbit IgG isotype control (eBioscience) and goat anti–rabbit IgG-FITC (SouthernBiotech). 50,000 events per sample were acquired using a FACSCalibur (BD) and analyzed with FCS Express 3 software (De Novo Software). Cell sorting was performed using a flow cytometer (FACSDiva; BD). Peritoneal, splenic, and bone marrow Ly6C^{hi} monocytes (CD11b$^+$Ly6C^{hi}), peritoneal DCs (CD11c$^+$), and granulocytes (CD11b$^+$Ly6G$^+$) were sorted to >90% purity for cell culture or RNA isolation.

TLR stimulation. Sorted cells resuspended in complete DMEM (containing 10% FCS, 10 mmol/liter Hepes, glutamine, and penicillin/streptomycin plus 10 U/ml heparin) were seeded on 96-well cell-culture plates (5×10^4 cells/well). Cells were stimulated with the doses indicated in the figures of peptidoglycan, poly I:C, R848, CpG ODN2395 (InvivoGen), or LPS (from *Salmonella typhimurium*; Sigma-Aldrich) and were incubated at 37°C in a 5% CO_2 atmosphere for 24 h before collecting the supernatant. MCP-1 and IL-6 ELISAs (BD) were performed according to the manufacturer's instructions. Optical density was converted to concentration using standard curves based on recombinant cytokines analyzed by a four-parameter logistic equation (Softmax Pro 3.1 software; MDS Analytical Technologies).

Cell culture with TMPD. 1 ml TMPD, mineral oil or squalene was added to 9 ml of fetal bovine serum in a 15-ml polypropylene tube and was rotated for 48 h at 4°C. The surface layer of unincorporated hydrocarbon oil was removed by aspiration at the end of the incubation. The amount of TMPD incorporated using this method was ~1 μg/ml, as determined by gas chromatography/mass spectroscopy (not depicted; Analytical Toxicology Core, University of Florida). J774 cells or bone marrow–derived macrophages were seeded on 24-well plates (5×10^5 cells/well) and cultured overnight in complete DMEM containing 10% FCS with or without hydrocarbon oils. For subsequent stimulation, cells were washed with PBS, and fresh complete medium was added before the addition of TLR ligands. Incorporation of TMPD in DMSO and β-cyclodextrin (Sigma-Aldrich) has been described previously (68). TMPD (10% vol/vol) also was added to ethanol or mannide monooleate (5% in PBS; Sigma-Aldrich). Solvent alone was used as a control, and a range of TMPD concentrations (3–300 μM) was tested. Endocytosis was quantified by uptake of 5 μg/ml FITC-dextran (Sigma-Aldrich), and phagocytosis by internalization of FITC-labeled microbeads (10:1 beads/cells ratio; Invitrogen) or tetramethylindodicarbocyanine perchlorate (DiD)–labeled apoptotic BW5147 cells (10:1 apoptotic cell/ target cell ratio) after overnight incubation of J774 cells in complete medium with or without TMPD. Apoptosis of BW5147 cells was induced by heat shock in a 45°C water bath for 10 min. After 4 h of incubation at 37°C, apoptotic cells (>80% annexin V positive; not depicted) were labeled with the fluorescent dye DiD (Invitrogen). J774 Cells were washed and incubated with the fluorescent substrates for 30 min at 37°C (in PBS with 0.5% BSA), washed three times, and analyzed by flow cytometry. ELISA, flow cytometry, and RT-PCR were performed as described. Bone marrow–derived macrophages were generated from BALB/c mice as previously described (8).

Autoantibody analysis. Serum ANAs in BALB/c.TLR7$^{-/-}$ and wild-type BALB/c mice were determined 12 and 24 wk after TMPD by indirect immunofluorescence using HEp-2 cells (Innova). Sera were diluted 1:40, and titers were determined using a titration emulation system (Image Titer; Rhigene, Inc.). Immunoprecipitation and antigen-capture ELISA to detect serum autoantibodies against nRNP/Sm were performed as previously described (26, 69). Determination of anti-Su/ago2 by ELISA has also been previously described (44). Recombinant ago2 protein was a gift from E. Chan and K. Ikeda (University of Florida, Gainesville, FL).

Statistical analysis. For quantitative variables, differences between groups were analyzed by the unpaired Student's *t* test. Survival curves were analyzed using the log-rank test. ANA titers and autoantibody levels were compared using the Mann-Whitney U test. Data are presented as means ± SD. All tests were two-sided, and $P < 0.05$ was considered significant. Statistical analyses were performed using Prism 4.0 software (GraphPad Software, Inc.).

Online supplemental material. Table S1 provides PCR array analysis of cytokine/chemokine expression in PECs from wild-type and TLR7$^{-/-}$ mice. Table S2 provides the sequence of all PCR primers used in this study. Fig. S1 shows the levels of proteinuria in BALB/c and TLR7$^{-/-}$ mice 24 wk after TMPD treatment. Fig. S2 shows the effect of TMPD on endocytosis of FITC-dextran and phagocytosis of FITC-coated latex beads or apoptotic lymphocytes in J774 cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080462/DC1.

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