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*J Immunol* 2013; 191:2104-2114; Prepublished online 5 August 2013;
doi: 10.4049/jimmunol.1300341
http://www.jimmunol.org/content/191/5/2104

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/08/06/jimmunol.1300341.DC1

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Overexpression of Membrane-Bound Fas Ligand (CD95L) Exacerbates Autoimmune Disease and Renal Pathology in Pristane-Induced Lupus

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Loss-of-function mutations in the Fas death receptor or its ligand result in a lymphoproliferative syndrome and exacerbate clinical disease in most lupus-prone strains of mice. One exception is mice injected with 2,6,10,14-tetramethylpentadecane (TMPD), a hydrocarbon oil commonly known as pristane, which induces systemic lupus erythematosus–like disease. Although Fas/Fas ligand (FasL) interactions have been strongly implicated in the activation-induced cell death of both lymphocytes and other APCs, FasL can also trigger the production of proinflammatory cytokines. FasL is a transmembrane protein with a matrix metalloproteinase cleavage site in the ectodomain. Matrix metalloproteinase cleavage inactivates membrane-bound FasL and releases a soluble form reported to have both antagonist and agonist activity. To better understand the impact of FasL cleavage on both the proapoptotic and proinflammatory activity of FasL, its cleavage site was deleted through targeted mutation to produce the deleted cleavage site (ΔCS) mouse line. ΔCS mice express higher levels of membrane-bound FasL than do wild-type mice and fail to release soluble FasL. To determine to what extent FasL promotes inflammation in lupus mice, TMPD-injected FasL-deficient and ΔCS BALB/c mice were compared with control TMPD-injected BALB/c mice. We found that FasL deficiency significantly reduced the early inflammatory exudate induced by TMPD injection. In contrast, ΔCS mice developed a markedly exacerbated disease profile associated with a higher frequency of splenic neutrophils and macrophages, a profound change in anti-nuclear Ab specificity, and markedly increased proteinuria and kidney pathology compared with controls. These results demonstrate that FasL promotes inflammation in TMPD-induced autoimmunity, and its cleavage limits FasL proinflammatory activity. The Journal of Immunology, 2013, 191: 2104–2114.

Fas ligand (FasL, CD95L) was initially identified as a potent proapoptotic type II transmembrane protein belonging to the TNF gene family (1), and it is predominantly expressed by CD4⁺, CD8⁺, NKT, and NK cytotoxic effector cells (2). FasL-mediated cytotoxicity plays a key role in limiting the expansion and function of Fas receptor (CD95, TNFRSF6)–positive target populations such as activated T cells, B cells, macrophages, and dendritic cells (3–5). Additionally, as with other TNF family members, FasL can also trigger the production of IL-1β and other proinflammatory cytokines and chemokines, especially in macrophages, neutrophils, and other cells of the innate immune system (6, 7). It follows that FasL is a dangerous molecule and strict regulation of its activity is a necessity. FasL expression is controlled at a number of levels, including transcription, vesicular compartmentalization, and cleavage. The latter depends on the activity of matrix metalloproteases (MMPs) that recognize a cleavage site located in the extracellular region of FasL between the transmembrane and the trimerization domains (8–10). FasL cleavage releases a soluble isoform whose function is somewhat controversial. Several studies point to a loss of function of the cleavage product whereas data from our own studies and those of others indicate that soluble FasL can serve as an antagonist of the membrane-bound molecule (11–14).

Exactly what circumstances promote apoptosis and/or the release of proinflammatory cytokines is unclear, but the functional outcome of Fas engagement may reflect the relative levels of the membrane-bound and soluble forms and/or inherent properties of the Fas⁺ target populations. To better understand the significance of FasL cleavage in a physiologically relevant system, we made a gene-targeted mouse line in which the FasL MMP recognition site has been mutated to render FasL resistant to MMP-mediated cleavage (15). We refer to these mice as deleted cleavage site (ΔCS) FasL mice. Although it might be anticipated that the failure
to appropriately cleave FasL would perturb normal lymphocyte homeostasis, unmanipulated ΔCS mice do not exhibit any apparent immune phenotype (data not shown), and they therefore resemble a similar gene-targeted line described by others (16). However, FasL is also expressed at sites of immune privilege, such as the eye, where it has been reported to block both angiogenesis (migration of Fas+) endothelial cells) and the influx of Fas+ proinflammatory cells, thereby protecting the eye from immune-mediated damage (17, 18). Apparently, FasL expression and function in the eye is maintained in a remarkably delicate balance between the full-length and cleaved isoforms, as we have recently found that ΔCS mice develop markedly exacerbated pathology in both spontaneous and induced murine models of glaucoma (15).

FasL/Fas interactions have a profound impact on self-tolerance and autoimmune development. Failure to express either Fas or FasL leads to the production of autoantibodies in numerous mouse strains and is associated with accelerated clinical disease in almost all systemic lupus erythematosus (SLE)–prone murine lines (19). A reported exception appears to be C57BL/6 (B6) mice injected with 2,6,10,14-tetramethylpentadecane (TMPD), commonly referred to as pristane (20). TMPD-injected mice develop a chronic inflammatory response that eventually presents as an SLE-like autoimmune disease normally associated with autoantibodies reactive to a panel of RNA- and DNA-containing antigens. Somewhat unexpectedly, TMPD-injected B6/pr and B6/gld mice, which harbor loss-of-function mutations in Fas and FasL, respectively, were reported to make significantly less autoantibody specific for common RNA-associated autoantigens than did TMPD-injected B6 mice (20). These results implicate FasL/Fas interactions in TMPD-induced autoimmune reactions. Moreover, the disease-promoting activity of FasL also emerged from a clinical study in which a single nucleotide polymorphism (rs1076611 C > T) at the FasL promoter, associated with increased expression of FasL, was found to be a risk factor for SLE in an African American cohort (21). To further explore the contribution of FasL in SLE, we have now evaluated the effect of FasL deficiency, as well as FasL overexpression, on TMPD-injected BALB/c mice.

Materials and Methods

Mice

Wild-type BALB/c and C57BL/6 mice as well as B6lpr/lpr and B6/gld/gld mice, which harbor loss-of-function mutations in Fas and FasL, respectively, were reported to make significantly less autoantibody specific for common RNA-associated autoantigens than did TMPD-injected B6 mice (20). These results implicate FasL/Fas interactions in TMPD-induced autoimmune reactions. Moreover, the disease-promoting activity of FasL also emerged from a clinical study in which a single nucleotide polymorphism in the FasL promoter, associated with increased expression of FasL, was identified as a risk factor for SLE in an African American cohort (21). To further explore the contribution of FasL in SLE, we have now evaluated the effect of FasL deficiency, as well as FasL overexpression, on TMPD-injected BALB/c mice.

Flow cytometry

The following conjugated anti-mouse Abs were used: anti-CD19 (1D3), anti-IL-6 (1A8), anti-CD11b (M1/70), anti-CD3 (17A2), anti-CD95L (F4-80), anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-CD45R (RA3-6B2) (eBiosciences); and anti–TCR-β (H57-597), anti-CD11c (3.9), and anti–Ly-6C (ER-MP20) (Serotec). Cells were incubated in CD16/32 (Fc Block; BD Biosciences) prior to staining. Cell death was determined by flow cytometry using 7-aminoactinomycin D (7-AAD; BD Pharmingen). The sorting strategy was designed to increase purity by exclusion of B and T cells. Sorted cells were directly harvested into RLT buffer containing 2-ME for subsequent processing with the RNAeasy Mini kit (Qiagen). Each RNA sample was adjusted to contain the same quantity by using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). RNA was then hybridized and quantified with the NanoString nCounter analysis system (NanoString Technologies, Seattle, WA) per the manufacturer’s protocol.

Cytokine assays

RNA was isolated from total spleen or total peritoneal lavage cells 14 d after TMPD injection with the Qiagen RNAeasy Mini kit. The iScript cDNA synthesis kit (Bio-Rad) was used for reverse transcription. Quantitative RT-PCR was performed using SYBR Green PCR Master Mix (Bio-Rad) with the following primer pairs: virus inhibitory protein, endoplasmic reticulum–associated, IFN-inducible (viperin), sense, 5′-AACCCCGCGTGTGCTAGTT-CACA TA-3′, antisense, 5′-AACCGAGCTTGGTGAACGAA-3′; β-actin, sense, 5′-TGGCATAGAGCTTTTCAGGA-3′, antisense, 5′-TTGACAA-TGGCATTGTAACAA-3′. The quantitative PCR was performed on a CFX96 real-time PCR detection system (Bio-Rad). Viperin expression is presented relative to β-actin expression. For purified cell populations, cells were sorted based on the gating strategy employed in Fig. 1 A either on a BD FACSVerse DV-1 cell sorter or a BD FACS Aria cell sorter (both BD Biosciences). The sorting strategy was designed to increase purity by exclusion of B and T cells. Sorted cells were directly harvested into RLT buffer containing 2-ME for subsequent processing with the RNAeasy Mini kit (Qiagen). Each RNA sample was adjusted to contain the same quantity by using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). RNA was then hybridized and quantified with the NanoString nCounter analysis system (NanoString Technologies, Seattle, WA), per the manufacturer’s protocol.

Gene expression analysis

The following conjugated anti-mouse Abs were used: anti-CD19 (1D3), anti-IL-6 (1A8), anti-CD11b (M1/70), anti-CD3 (17A2), anti-CD95L (F4-80), anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-CD45R (RA3-6B2) (eBiosciences); and anti–TCR-β (H57-597), anti-CD11c (3.9), and anti–Ly-6C (ER-MP20) (Serotec). Cells were incubated in CD16/32 (Fc Block; BD Biosciences) prior to staining. Cell death was determined by flow cytometry using 7-aminoactinomycin D (7-AAD; BD Pharmingen). The sorting strategy was designed to increase purity by exclusion of B and T cells. Sorted cells were directly harvested into RLT buffer containing 2-ME for subsequent processing with the RNAeasy Mini kit (Qiagen). Each RNA sample was adjusted to contain the same quantity by using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). RNA was then hybridized and quantified with the NanoString nCounter analysis system (NanoString Technologies, Seattle, WA) per the manufacturer’s protocol. The following expression data were first normalized to an internal positive control set, secondly to an internal negative control set, and then to six housekeeping genes, that is, GAPDH, β-glucuronidase, hypoxanthine phosphoribosyltransferase 1, tubulin β, phosphoglycerate kinase 1, and clathrin H chain 1. All values were scaled relative to the minimum value (x − min(x)) and a pseudocount of 1 was added to each value. This operation sets the smallest value in the dataset to 1. All values were log-transformed and a heat map was generated using the gplots package within the open source R software environment.

Ab and autoantibody titers

Anti-nuclear Abs (ANAs) were detected by immunofluorescence on Hep-2 slides (Antibodies, Inc.) with serum diluted at 1:100 using anti-mouse IgG (H+L) DyLight 488–conjugated F(ab)2 (Jackson ImmunoResearch) in Vectashield antifade mounting medium (Vector Laboratories). Images were captured on a Nikon E600 at 200× magnification and processed in Adobe Photoshop. Smi (Sm) Ag was detected by ELISA using SmB′D′Ag-coated wells (Immunovision) and a goat anti-mouse total IgG (Sigma-Aldrich) with tetramethylbenzidine substrate (Dako) as detecting reagents. The anti-Sm Ag–reactive mAb Y12 was used as a standard. Total serum IgG1, IgG2a, and IgG2b were measured by ELISA using the following coating Abs: goat anti-mouse anti-IgG2a (SouthernBiotech), goat anti-mouse IgG2b (Jackson ImmunoResearch), and goat anti-mouse IgG1 (Jackson ImmunoResearch). Goat–anti-mouse IgG HRP (Sigma-Aldrich) was used as detection Ab and the ELISA was developed with tetramethylbenzidine substrate (Dako). IgG1, IgG2b (BD Pharmingen), and IgG2a (BioLegend) were used as standards.

Autoantibody array

Autoantibody reactivity was further examined using an autoantigen proteomic array containing 88 autoantigens and 10 control proteins (23).
Serum samples, diluted 1:100, were detected with Cy3-labeled anti-mouse IgG and Cy5-labeled anti-mouse IgM (Jackson ImmunoResearch). Net fluorescence intensity was normalized using anti-mouse IgG or IgM. Results were plotted using GraphPad Prism software (GraphPad Software, San Diego, CA). The autoantigen heatmap was generated using GenePattern software from the Broad Institute.

Histology

Kidneys were fixed in paraformaldehyde, embedded in paraffin, and sections were stained with H&E. H&E-stained kidney sections were scored in a blinded fashion to determine a glomerular and interstitial inflammation score as described (24). Briefly, a mean glomerular score was calculated for each mouse by grading injury in 50 glomeruli. Glomeruli were scored as follows: 0, normal; 1, mesangial expansion; 2, endocapillary proliferation; 3, capillaritis or necrotic changes; 4, crescents. The interstitial score was determined by examining 50 high-power fields and scoring the interstitial inflammation on a scale from 0 to 4 as absent or involving <25, 25–50, or >50% of the interstitium. For immunofluorescence, kidneys were fixed in 0.7% paraformaldehyde-lysine-periodate solution and frozen in OCT (TissueTek) after dehydration in 30% sucrose solution. For the detection of glomerular immune complexes, kidney sections were stained with DyLight 488–labeled goat anti-mouse IgG (minimal x-reactivity; BioLegend). Using the NIS-Elements imaging software BR3.10 (Nikon), 20–30 randomly picked glomeruli were circled and analyzed for the mean fluorescence intensity (MFI) emitted in the green channel over the circled areas.

Spleen tissue was snap-frozen in OCT and cryostat sections were fixed in acetone, blocked with 10% rat serum, and stained with biotinylated anti–Moma-1 (Abcam) and FITC-labeled anti–Ly-6G. Streptavidin-Alexa Fluor 555 (Invitrogen) was used as a secondary reagent. Nuclei were counterstained with DAPI (Invitrogen). Images were captured on a Nikon E600 (kidney immunofluorescence) or on an Olympus IX 70 inverted light microscope (spleen immunofluorescence).

Statistical analysis

Experiments are reported as the means ± SEM. Statistical comparisons were made using a Student t test GraphPad Prism software (GraphPad Software) unless otherwise indicated in the figure legends. A p value <0.05 was considered to be statistically significant.

Results

Fas/FasL interactions promote the TMPD-induced inflammatory response

Numerous studies have documented the rapid influx of both neutrophils and monocytes into the peritoneum following TMPD injection (25, 26). To carefully examine the potential role of FasL in the early events of TMPD-induced inflammation, wild-type (WT), ΔCS, FasL-deficient, and gld mice were injected with TMPD and the cell subsets that accumulated in the peritoneum 14 d later were characterized by flow cytometry. Consistent with previous reports, in WT mice TMPD injection led to loss of the resident CD11b+ F4/80+ macrophages (Fig. 1A, R0) and to a rapid and prolonged extravasation of inflammatory monocytes, identified as CD11b+ Ly6Cint and Ly6Ghi (Fig. 1A, R2), into the peritoneal cavity (25). Approximately the same number of cells accumulated in the peritoneum of WT and ΔCS mice (Fig. 1B). In contrast, mice that failed to express FasL (FasL+/−) or expressed a loss-of-function point mutation (gld) had significantly fewer CD11b+ cells, particularly the inflammatory monocytes (R1) and neutrophil (R2) subsets (Fig. 1B). Furthermore, the peritoneal exudate from the FasL-deficient mice included a substantial number of CD11bhi Ly6CloLy6Glo cells (R3) found only in small number in TMPD-treated WT or ΔCS mice. The differences in number and type of exudate cells that accumulated in the FasL-sufficient and FasL-deficient mice demonstrate that FasL plays a major role in promoting the early stages of the TMPD-induced inflammatory response.

**FIGURE 1.** Fas/FasL interactions promote TMPD-induced peritoneal inflammation. (A) Phenotype of peritoneal cavity washout cells from untreated mice, as well as day 14 PECs from TMPD-injected mice, obtained from the indicated genotypes. Top panel depicts all peritoneal cells gated on live/singlet events stained with a combination of F4/80 and CD11b. The lower panel depicts the CD11b+ cells stained for Ly6C and Ly6G. (B) Total number of cells collected from the peritoneum of untreated BALB/c mice (open bars) or TMPD-treated BALB/c, ΔCS, FasL+/−, or gld mice (filled bars), as well as cell numbers in the CD11b+, R0, R1, R2, and R3 gates and total number of CD8+ and CD4+ T cells (±SEM, n = 4). *p < 0.05.
**Fas and FasL expression by TMPD-induced cells**

To better understand how FasL could be regulating peritoneal inflammation in this model, it was important to determine which subpopulations expressed high levels of Fas and were thereby likely to be targeted by FasL effector populations. To avoid any confounding effects of autofluorescence, we compared cell populations collected from un.injected BALB/c mice (R0), TMPD-injected BALB/c mice (R1 and R2), and TMPD-injected FasL−/− mice (R3) to the corresponding populations obtained from Fas-deficient lpr mice. The R3-gated cells, found predominantly in the Fas-deficient TMPD-injected mice, resembled the mature R0-gated resting peritoneal macrophages found in untreated WT mice; both groups included cells that express F4/80 and relatively high levels of Fas (Fig. 2A). The inflammatory monocytes (R1) obtained from the TMPD-injected BALB/c and ΔCS mice expressed lower levels of Fas, whereas the neutrophils (R2), and especially the Ly6CLy6Ghi subset (R2#) found in the TMPD-injected BALB/c and ΔCS mice, expressed high levels of Fas (Fig. 2B).

If Fas+ exudate cells are induced to undergo apoptosis by a FasL+ effector population, there should be more cell death in the TMPD-injected ΔCS mice than in the TMPD-injected BALB/c mice. To test this premise, we further analyzed the day 14 exudates for percentage of dead cells by determining the number of 7-AAD+ cells in the peritoneal cavity of all mice. We were able to detect exceedingly low amounts of FasL on the FasL+ effector population, there should be more cell death in the TMPD-injected ΔCS mice, whereas the neutrophils (R2), and especially the Ly6CLy6Ghi subset (R2*) found in the TMPD-injected BALB/c and ΔCS mice, expressed high levels of Fas (Fig. 2B).

We also analyzed the peritoneal exudate cells for FasL expression. In contrast to Fas expression, FasL expression is tightly regulated and often difficult to detect. Moreover, the point mutation expressed by gld cells is recognized by most FasL-specific Abs. To accurately detect subtle changes in FasL expression, we took advantage of our Fas−/− strain, as it does not express FasL protein and is therefore an appropriate negative staining control (15). We were able to detect exceedingly low amounts of FasL on the R1 and R2 subsets (Supplemental Fig. 1A). In addition to the influx of monocytes and neutrophils, the peritoneal cavity of all TMPD-injected mice contained a small, but substantially elevated, number of CD8+ and CD4+ T cells (Fig. 1B). We could not detect any difference in FasL expression on the CD8+ cells. However, a small subset of the CD4+ cells, found only in the TMPD-injected ΔCS mice, consistently expressed significantly higher levels of FasL than did the CD4+ cells from any of the other mouse strains (Fig. 3A). Because the FasL+CD4+ subset was not found in TMPD-injected BALB/c mice, we conclude that FasL expression in these cells is regulated by MMP cleavage and might account for the higher numbers of 7-AAD+ cells in the ΔCS exudate. However, we cannot rule out the potential contribution of the R1 and R2 subsets, or even other FasL+ cell types, not present in the exudate.

**ΔCS T cells induce BMDCs to make IL-1β**

From previous studies, we know that FasL induces resting peritoneal (R0) cells to rapidly produce IL-1β and other neutrophil-attracting chemokines and then undergo apoptosis (4). FasL can also directly induce neutrophils to produce IL-1β (27). Therefore, cell subsets from TMPD-injected mice include populations that are very sensitive to FasL and respond by producing inflammatory mediators. To determine whether BALB/c ΔCS T cells express more FasL than do BALB/c T cells, we compared OVA-specific T cells activated in vitro under conditions that favor the development of FasL+ T cells. From previous studies, we know that FasL is expressed at higher levels upon secondary stimulation than after the first stimulation, and that the restimulated WT T cells undergo activation-induced cell death in vitro whereas Fas-deficient lpr/lpr cells do not (28). Therefore, to best study viable T cells, TCR transgenic DO11 lpr and DO11 lpr ΔCS cells were stimulated with OVA peptide and APCs, expanded in culture for 5 d, and then allowed to revert to a resting phenotype. The rested cells were then restimulated with plated-bound anti-CD3, and FasL expression was determined by FACS. Both populations expressed higher levels of FasL than did resting cells, and the DO11 lpr ΔCS cells expressed more FasL than did the DO11 lpr cells, presumably because FasL was not cleaved from the surface (Fig. 3B).

To determine whether the level of FasL expressed by activated T cells was sufficient to induce inflammatory cytokine production, the rested DO11 cells were also cultured for 20 h with LPS-primed BMDCs in the presence of OVA peptide. Culture supernatants were collected and tested for IL-1β by ELISA. The DO11 lpr ΔCS T cells induced more BMDC-derived IL-1β than did the DO11 lpr T cells (Fig. 3C). Furthermore, IL-1β production was dependent on the presence of OVA peptide and expression of FasL by the BMDCs (Fig. 3D). These data show that ΔCS T cells express higher levels of functional FasL and that the level of FasL expressed by activated T cells is sufficient to drive myeloid cells to make IL-1β through an Ag- and Fas-dependent mechanism.

If T cells are a relevant source of FasL in TMPD-injected mice, then TMPD-injected BALB/c Rag2−/− mice should develop...
a suboptimal response. In fact, the total number of CD11b+ cells was markedly decreased in the Rag2−/− mice, and the Rag2−/− mice retained a population of CD11b+Ly6C− cells (Supplemental Fig. 1B). These data are consistent with the notion that T cell expression of FasL contributes to the TMPD-induced inflammatory response in the peritoneum, although the potential contribution of other T cell cytokines and/or FasL+ cells, for instance neutrophils (29), cannot be excluded.

**∆CS mice display increased cytokine production in response to TMPD**

To explore the effect of the ∆CS mutation on the production of cytokines and chemokines in TMPD-injected mice, peritoneal lavage fluid at 14 d after inoculation was assayed for proinflammatory cytokine and chemokine content using a Luminex array. Although most cytokines, including IL-1β and IL-1α, were detected at comparable levels in ∆CS mice and BALB/c samples, higher levels of IL-6, IL-9, and MCP-1 were detected in the peritoneal fluid of the ∆CS mice compared with those of the BALB/c mice (Supplemental Fig. 1C).

**Monocytes and neutrophils from day 14 TMPD-injected mice express a strong IFN signature and high levels of IL-1β and IL-18**

To elucidate the immune pathways activated by TMPD challenge, we used a NanoString code set, designed to quantify IFN-stimulated genes (ISGs) and proinflammatory cytokines/chemokines (30). We began by comparing the resident macrophage population isolated from untreated mice (R0) to the major macrophage-lineage populations identified in the peritoneum of TMPD-injected BALB/c mice (R1, R3). We found that the inflammatory monocytes (R1) expressed very high levels of ISGs such as IFN regulatory factor 7, viperin, IFIT1, IFIT2, CXCL10, and CCR2, consistent with the high levels of type I IFN previously reported for TMPD-treated mice (25). In contrast, both the resident peritoneal macrophages obtained from untreated mice and the low number of Ly6Chigh R3 macrophages isolated from the TMPD-treated BALB/c mice exhibited a less active phenotype (Fig. 4A, Supplemental Table 1). We also compared uninduced neutrophils (isolated from normal bone marrow) to peritoneal neutrophils isolated from the TMPD mice (R2). The morphology of these cells is shown in Fig. 4B. Remarkably, the difference between uninduced neutrophils and TMPD-induced “inflammatory” neutrophils with regard to ISGs and inflammatory cytokine production was even greater than the difference between resident peritoneal macrophages and inflammatory monocytes.

Of note, certain ISGs were differentially regulated in monocytes and neutrophils. For example, IFI204 was more highly induced in monocytes than in neutrophils, whereas IFIT1 was more highly expressed in neutrophils. This is in agreement with published results showing that expression of IFI204 distinguishes the monocytic lineage from the granulocytic lineage (31). In contrast, viperin was uniformly upregulated in both TMPD-induced subsets.

The expression profile for the IL-1 family proinflammatory cytokines also revealed clearly distinguishable patterns. IL-18 appeared to be constitutively expressed in the macrophage lineage cells but was dramatically upregulated in the TMPD-induced neutrophils compared with resting bone marrow–purified neutrophils. IL-18, similar to IL-1β, exists as a proform in the cytosol and has to be processed by caspase-mediated cleavage to gain biological activity and be secreted. We have previously shown that FasL induces Fas+ BMDCs to transcribe and produce both IL-1β and IL-18 (32). If FasL contributes to IL-18 production in ∆CS mice, then IL-18 levels in TMPD-injected mice should correlate with the potential capacity to make functional FasL. To address this issue, we screened the peritoneal fluid collected from day 14 TMPD-treated mice. In fact, the ∆CS samples contained significantly higher levels of IL-18 protein, and the FasL-deficient

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**FIGURE 3.** ∆CS T cells express higher levels of FasL than do non-∆CS T cells and induce more IL-1β by Fas+ target cells. (A) FasL expression of CD4+ T cells collected from untreated or day 14 TMPD-injected mice of the indicated genotypes (left, representative contour plots; right, percentage of cells in the indicated gate). (B) Histograms for FasL expression on unstimulated (shaded histogram) and anti-CD3-stimulated DO11 lpr T cells (line) or DO11 lpr ∆CS T cells (broken line). (C) Increasing numbers of DO11 lpr T cells (gray bars) or DO11 lpr ∆CS T cells (black bars) were cocultured for 20 h in the presence of OVA-peptide with BALB/c WT BMDCs, and IL-1β present in the supernatant was determined by ELISA. White bars depict control coculture conditions in the absence of OVA peptide and presence of DO11 lpr T cells (−OVA) or in the complete absence of T cells (−T cells). (D) DO11 lpr ∆CS T cells were cocultured for 20 h in the presence of OVA peptide together with BALB/c WT (open bars) or BALB/cOVA BMDCs (filled bars), and IL-1β present in the supernatant was determined by ELISA. Cell-free membrane-bound FasL-expressing microvesicles (mFasLVes) and poly (deoxyadenylic-thymidylic) acid (dAdT) were used as controls (as described previously in Ref. 32). *p < 0.05.
samples contained significantly lower levels of IL-18 when compared with the BALB/c samples (Fig. 4C). These data indicate that TMPD-induced IL-18 production is at least partially due to a process involving Fas/FasL interactions. Moreover, in the total peritoneal sample, IL-1β expression showed nearly a 50-fold increase over the untreated control cells, but IL-1α transcripts were not detectably upregulated. Of the three major CD11b⁺ cell fractions, IL-1β message was most highly upregulated in neutrophils (R2), somewhat upregulated in monocytes (R1), and barely changed in the F4/80⁺ fraction (R3) compared with their unstimulated counterparts. These data demonstrate that both monocytes and neutrophils produce significant amounts of proinflammatory cytokines following TMPD injection.

In contrast to a previous report (33), we did not detect mRNA for type I IFNs in the sorted inflammatory monocytes (or in any of the other exudate subsets). However, TMPD did not induce the up-regulation of ISGs in mice lacking a type I IFN receptor (data not shown), confirming an important role for type I IFNs in this process involving Fas/FasL interactions. Moreover, in the total peritoneal sample, IL-1β expression showed nearly a 50-fold increase over the untreated control cells, but IL-1α transcripts were not detectably upregulated. Of the three major CD11b⁺ cell fractions, IL-1β message was most highly upregulated in neutrophils (R2), somewhat upregulated in monocytes (R1), and barely changed in the F4/80⁺ fraction (R3) compared with their unstimulated counterparts. These data demonstrate that both monocytes and neutrophils produce significant amounts of proinflammatory cytokines following TMPD injection.

Having established an IFN signature in the TMPD-injected mice, we decided to compare day 14 spleen cells from WT and ΔCS, and FasL⁻/⁻ mice for relative expression of one of the highly expressed ISGs, viperin. Viperin plays an important role in TLR signal transduction (35) and the development of SLE-like disease in TMPD-injected mice is TLR-dependent. Importantly, at this time point, the FasL⁻/⁻ mice expressed significantly less viperin than did the FasL⁺/⁺ mice (Fig. 4D). These results demonstrate that FasL, either directly or indirectly (perhaps by increasing the number of dead cells and cell debris), promotes the induction of ISGs.

Moreover, to our knowledge this dataset characterizes for the first time the gene expression profile of all the major cell populations likely to be important in the proinflammatory feed-forward loops instigated by TMPD. Thus, considering the overall impact of FasL overexpression on the early stages of TMPD-induced inflammation, it seemed likely that FasL would also contribute to the long-term outcomes of TMPD challenge.

**Overexpression of membrane-bound FasL leads to more severe splenomegaly and neutrophilic infiltrates**

To determine the effect of FasL overexpression on disease progression, we went on to compare WT and ΔCS mice 6 mo after TMPD injection. As anticipated from chronic immune activation, there was a significant increase in spleen weight in TMPD-treated BALB/c mice compared with age-matched controls. Of note, the spleen weight of the TMPD-treated ΔCS mice was even further increased (Fig. 5), whereas spleens from aged matched, untreated ΔCS mice were comparable in weight to untreated WT mice (data not shown). Spleen weight was mirrored by a significant increase in the number and percentage of splenic CD11b⁺ cells recovered from TMPD-injected ΔCS mice and BALB/c mice. Normally very few neutrophils are detected in the spleens of unmanipulated BALB/c mice. Histopathological examination of spleen sections confirmed the increased numbers of neutrophils in ΔCS mice (Supplemental Fig. 2A). Thus, overexpression of FasL contributes to the long-term effects of TMPD injection.

**The autoantibody repertoire induced by TMPD is skewed by overexpression of membrane-bound FasL**

The ΔCS mutation had additional effects on the B cell compartment. Although there was considerable variability in the total IgG isotype titers, the overall ratio of IgG1/IgG2a serum isotype was higher in the ΔCS compared with WT mice, even though total IgG titers were comparably elevated in both strains (Fig. 6A). This
may reflect elevated levels of Th2-associated cytokines such as IL-9 (Supplemental Fig. 1C).

There was also an effect on the autoantibody specificity, as can be seen from the immunofluorescence staining patterns of HEp2 cells. TMPD-injected BALB/c mice frequently make autoantibodies associated with TLR7-driven responses that commonly present as a speckled nuclear pattern typical of SmB/D or spliceosome reactivity. In contrast, most TMPD-injected ΔCS mice exhibited a predominantly cytoplasmic staining pattern, or an atypical nuclear pattern seen infrequently among the TMPD-injected BALB/c mice (Fig. 6B). The absence of Sm-reactive Abs in the TMPD-treated ΔCS mice was confirmed by an Sm Ag ELISA (Fig. 6C). Untreated ΔCS mice were followed for up to 12 mo of age and were never seen to develop spontaneous ANAs (data not shown).

To further evaluate the autoantibody repertoire, representative serum samples were screened on an autoantigen array (23). The array data recapitulated the overall change in ANA pattern apparent from the HEp2 screen. Certain autoantigens were preferentially targeted in ΔCS mice, whereas others were predominantly targeted in the WT mice. The change in Sm Ag specificity (Fig. 6C) was confirmed by loss of small nuclear ribonucleoprotein (U1-snRNP-A, snRNP-BB, U1-snRNP-C) reactivity as assessed by the array (Fig. 6D, Supplemental Fig. 3). Of note, key autoantigens associated with kidney disease, such as C1q (36), were preferentially targeted in ΔCS mice. TMPD also promoted the production of autoantibodies reactive with neutrophil-associated autoantigens. Intriguingly, BALB/c anti-neutrophil autoantibodies were almost exclusively directed against an Ag stored in the azurophilic granules of activated neutrophils, the lipid transfer protein bactericidal permeability/increasing protein (37). In contrast, ΔCS autoantibodies did not target bactericidal permeability/increasing protein, but reacted preferentially with a neutrophilic

FIGURE 5. TMPD induces significantly greater splenomegaly and neutrophil accumulation in ΔCS mice. Spleen weight, total number of splenocytes, and percentage total of the indicated subsets from untreated BALB/c mice or TMPD-injected mice 6 mo after treatment are shown. Each dot represents an individual mouse (untreated BALB/c mice, n = 7; TMPD BALB/c mice, n = 13; TMPD ΔCS mice, n = 13). Line indicates the mean. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 6. Ab and autoantibody differences between TMPD-treated ΔCS and WT mice. (A) Total serum levels of IgG1, IgG2a, and IgG2b in mice 6 mo after TMPD and the calculated IgG2a/IgG1 ratio for BALB/c and ΔCS mice (BALB TMPD mice, n = 9; TMPD ΔCS mice, n = 7). Each dot represents an individual mouse. Original magnification ×200. (B) Representative images of HEp2 ANA staining patterns from untreated and TMPD-treated BALB/c and ΔCS mice. (C) SmB/D autoantibody titers of BALB/c and ΔCS mice 6 mo after TMPD treatment. (D) MFI (mean ± SEM; TMPD BALB/c mice, n = 4; TMPD ΔCS mice, n = 6) for IgG and IgM autoantibodies against selected autoantigens as quantified by autoantigen array. **p < 0.01.
protein stored in the very same granules, myeloperoxidase. The two strains also differed as far as histone reactivity; WT mice had a stronger reactivity to total histones and the H2a subtype, whereas histone H3 was predominantly targeted by the ΔCS mice. These data demonstrate that the ΔCS mutation dramatically reshapes the autoantibody repertoire of TMPD-treated mice and that Fas/FasL interactions skew the type of ensuing autoimmune response.

FasL overexpression leads to more severe renal disease
TMPD-injected BALB/c mice develop early signs of renal disease at ∼6 mo of age and therefore provide a useful model for assessing the effects of the ΔCS mutation on SLE-associated glomerulonephritis. Importantly, urine samples collected from the ΔCS mice at 6 mo of age contained significantly higher levels of albumin than did samples collected from the BALB/c mice (p < 0.01) (Fig. 7A). As a further measure of renal disease, kidneys obtained from BALB/c and ΔCS mice were subjected to histopathological analysis to evaluate glomerular and interstitial pathology. Consistent with the proteinuria data, the TMPD-treated ΔCS mice had greater interstitial and glomerular disease scores than did the TMPD-treated BALB/c mice (Fig. 7B). Frozen sections of kidneys were also stained with a fluorescent anti-IgG Ab to assess the extent of glomerular immune complex deposition. The ΔCS mice had more extensive glomerular IgG deposition (Fig. 7C, Supplemental Fig. 2B), perhaps due to autoantibodies reactive with C1q and/or collagen type IV or other renal components, and such deposits most likely promoted renal pathology. Hence, the noncleavable mutation in FasL expressed in the ΔCS mice leads to worsened autoimmune kidney disease.

Discussion
TMPD-injected BALB/c mice routinely develop a form or autoimmune disease that resembles other genetically programmed models of SLE. They make similar autoantibodies, develop glomerular nephritis, and disease progression is regulated by endosomal TLRs (25, 38). In humans, environmental exposure to hydrocarbons is considered a potential risk factor for the development of autoimmune diseases (39, 40). Moreover, hydrocarbons are widely used to potentiate vaccines, as in the case of the adjuvant MF59, where the active ingredient squalene is structurally similar to the active ingredient in TMPD. Therefore, elucidating mechanisms by which hydrocarbons promote immune activation, inflammation, and autoimmunity is highly relevant. Previous reports have documented, first, the capacity of TMPD to induce the transcription of both Fas and FasL in primary peritoneal macrophages and in the murine thymoma cell line BW5147 (41), and second, the loss of autoantibodies reactive with RNA-associated autoantigens in FasL-deficient TMPD-injected mice (20). Moreover, certain SLE patients have lower levels of potentially antagonistic soluble FasL in their serum than do healthy controls (42). We therefore decided to further explore the potential contribution of FasL and FasL cleavage to TMPD-induced inflammation.

Fas and FasL deficiencies have been repeatedly associated with loss of B cell tolerance, lymphoid hypertrophy, and SLE-like disease in numerous autoimmune-prone and nonautoimmune-prone mouse strains, as well as in patients that inherit loss-of-function mutations in either the Fas or FasL genes (1, 7, 43). Presumably autoreactivity in all these instances reflects the absence of FasL-induced apoptosis of autoreactive lymphocytes and/or a relevant autoantigen-presenting APC. However, consistent with the correlation between a gain-of-function FasL variant and increased risk for SLE in an African American cohort (21), we now demonstrate that FasL is a two-edged sword in that FasL can also promote both the early inflammatory response as well as the subsequent autoimmune disease triggered by TMPD inoculation. Moreover, FasL overexpression can further exacerbate renal pathology in these mice.

There are a number of possible mechanisms whereby failure to appropriately cleave FasL, and thereby extend the expression time of the full-length molecule, may promote autoimmunity. One possibility is that greater FasL expression leads to more extensive cell death and the release of endogenous danger signals and/or self-Ags. Increased autoantigen load could also promote the upregulation of IFN-inducible genes through TLR-dependent pathways. TMPD had previously been shown to induce cell death, especially in peritoneal B cells and dendritic cells, that appeared to be at least partly attributable to FasL-mediated apoptosis (41). Importantly, resident peritoneal macrophages in untreated WT mice express high levels of Fas, and i.p. injection of FasL+ tumor lines or FasL-expressing microvesicles can lead to the death of the resident Fas+ macrophages within a matter of hours (4). Therefore, it is not surprising that this same resident macrophage population, essentially the R0 population, disappears by day 14 in TMPD-injected BALB/c and ΔCS mice, but persists in the FasL-deficient strains. Moreover, 14 d after TMPD injection, the neutrophil R2 cells express high levels of Fas, and the extent of neutrophil death is increased in ΔCS mice and decreased in FasL-deficient mice compared with controls. These data are consistent with the notion that FasL directly contributes to TMPD-induced cell death.

However, FasL engagement of resident peritoneal macrophages not only induces apoptosis, but also induces these cells to more...
rapidly transcribe and secrete a number of proinflammatory cytokines/chemokines, including IL-1β, MIP2, MIP1α, MCP-1, and KC, cytokines known to recruit myeloid cells to the peritoneal cavity (4). Proinflammatory cytokine production in response to FasL engagement has been demonstrated for many other cell types as well (27, 32, 44, 45). Therefore, a second mechanism whereby FasL promotes inflammation could be by inducing cytokine production.

Intriguingly, we found that peritoneal fluid collected from the day 14 ACS mice contained higher levels of MCP-1, IL-6, and IL-9 than did fluid collected from the day 14 BALB/c mice. MCP-1 is known to recruit monocytes to sites of inflammation and is likely to contribute to the peritoneal inflammation elicited by TMPD. IL-6 is also considered a proinflammatory cytokine, produced by macrophages and dendritic cells during an inflammatory response and can be directly induced by Fas in fibroblasts (44). It can stimulate the production of neutrophils in the bone marrow, support the growth of B cells, and is known to play a key role in the TMPD response (46). The role of IL-9 in this context is less clear, but interestingly IL-1 has been shown to promote Th2 production more than did fluid collected from the day 14 BALB/c mice. MCP-1 is also considered a proinflammatory cytokine produced by myeloid cells to produce more IL-1β than did non-ΔCS–activated T cells. Among its pleiotropic effects, IL-1 can also drive T cells toward the Th17 lineage (51), and Th17 cells are a prominent effector population in SLE (52, 53). Future studies will examine whether IL-1β contributes to preferential induction of Th17 cells in ΔCS mice.

FasL can also induce the cleavage of IL-18 (32) and, quite remarkably, we found that the IL-18 protein level in peritoneal fluid collected from day 14 TMPD-injected mice was significantly higher in the ΔCS compared with the BALB/c group and significantly lower in the Fasl−/− group. Elevated levels of IL-18 in the Fas(+/+) R2 neutrophils may well be a source of the IL-18 that accumulates in the peritoneal fluid. IL-18 is a pleiotropic proinflammatory cytokine initially identified as an IFN-γ-inducing cytokine (54), but depending on the cytokine milieu it can augment both Th1 and Th2 responses (55). Furthermore, IL-18 is increased in patients with SLE (56), and IL-18 injections worsened autoimmune glomerulonephritis in the MRL-Fas(h)/lupus mouse model (57). Additionally, FasL itself is induced by IL-18 (58), and therefore increased levels of IL-18 may preferentially drive a deleterious feed-forward loop in the ΔCS mice, promoting further FasL-induced apoptosis, inflammation, and tissue damage.

Reeves and colleagues (25) have shown that neutrophil recruitment to the peritoneal cavity is at least partially dependent on IL-1α, whereas the recruitment of inflammatory monocytes depends on MyD88 and type I IFN, presumably via TLR7. However, they found that TLR7 deficiency does not lead to a complete reduction of inflammatory monocytes as does MyD88 deficiency. Intriguingly, Fas activation has been reported to induce production of the proinflammatory chemokines CXCL1/KC and CXCL2/MIP2 through a MyD88–dependent, IL-1/caspase–independent pathway (59), and this might account for the TLR–independent effect of MyD88 in monocyte recruitment.

To better define the properties of the infiltrating monocytes and neutrophils in TMPD mice at the day 14 time point, we used NanoString-based gene expression profiling to compare the TMPD-elicited populations to their uninduced counterparts with regard to a panel of ISGs and proinflammatory cytokines. The TMPD-induced inflammatory monocytes (R1 gate) and especially the R2 neutrophils (R2 gate) dramatically upregulated IL-1β, CXCL10, TNF-α, and ISGs. Thus, we propose the designation of “inflammatory neutrophils” for the R2 population, as they most likely represent a major source of the cytokines and chemokines responsible for disease progression.

One of the TMPD-induced ISGs, viperin, is rapidly induced in response to an extensive range of viral infections, and depending on the circumstances can be downstream of TLR3, TLR4, TLR7, or TLR9 engagement. It is also induced by the retinoic acid–inducible gene I–like receptor and stimulator of IFN genes signaling pathways. Importantly, viperin is a critical mediator of TLR7- and TLR9-dependent production of type I IFNs and thus contributes to host defense against viral infections (35). Considering the connection between TLR7 and TLR9 and the response to nucleic acid–associated autoantigens, viperin is also likely to play a pivotal role in the activation of both dendritic cell and autoreactive B cell subsets. Viperin also associates with cell membranes and can disrupt lipid rafts. Additionally, it can be found in the endoplasmic reticulum and in lipid droplet–like vesicles in neutrophils (60). Therefore, viperin could further modulate cell death as well as neutrophil and macrophage function in autoimmune disease. Finally, viperin has been found to promote the differentiation of Th2 cells (61), and the slight increase in viperin expression may contribute to the shift in the IgG2a/IgG1 serum titers of the TMPD-injected ΔCS mice. Thus, viperin warrants further investigation in the context of autoimmune disease.

Owing to the prominent expression of viperin in the TMPD-activated peritoneal cell populations, revealed by the microarray data, we went on to examine viperin expression in the TMPD-stimulated spleen cells from the ΔCS and the Fasl−/− strains. Viperin expression was modestly increased in the TMPD-treated ΔCS mice over TMPD-treated BALB/c mice. More remarkably, we found that viperin expression was not detectably upregulated in the Fasl−/− mice, at least at this time point, and was significantly lower in the gld mice compared with WT levels. From prior studies, we know that gld mice, expressing a point mutation in FasL, can make low levels of functional protein whereas FasL is completely missing in the Fasl−/− mice. Overall, these data clearly demonstrate that FasL contributes to the activation of a number of major ISGs, most likely by increasing the amount of autoantigen debris and ensuing TLR-dependent responses.

Despite the pronounced IFN signature, in contrast to a previous report (25), our NanoString array did not show an increase in either IFN-α or IFN-β RNA levels in either the total peritoneal washout or the sorted populations from the TMPD-activated mice. Type I IFN is clearly a critical factor in TMPD SLE, as TMPD-injected mice that lacked a type I IFN receptor did not upregulate ISG expression (L. Bossaller, unpublished observations), failed to produce the common SLE autoantibody reactivity, and did not develop proteinuria or histological evidence of renal disease (62). Further studies are required to identify the major IFN-producing cell types in TMPD-stimulated mice.

Based on the differences in cell phenotypes and cytokine levels between the ΔCS and BALB/c groups detected at day 14, we went on to examine the long-term effects of FasL overexpression in TMPD-injected mice. By 6 mo after injection, the spleens of the ΔCS mice were almost 50% larger than the spleens of the BALB/c mice and contained a significantly greater number of neutrophils. SLE patients frequently express a neutrophil signature, and neutrophils can contribute to the disease process through
a variety of mechanisms that include proinflammatory cytokine production, release of elastase, myeloperoxidase, and various cathepsins than can cause vascular damage, tissue injury, and glomerular destruction (63). Additionally, neutrophil extracellular traps, released by IFN-primered and then immune complex-activated neutrophils, have been proposed as a key source of autoantigen in the context of SLE (64). Therefore, the discovery of increased numbers of neutrophils in the ACS mice, as well as the concomitant upregulation of Fas expression on activated neutrophils, is likely to be a highly relevant outcome of FasL overexpression.

Based on the previous report from Reeves and colleagues (25), we were particularly interested in examining the effect of FasL overexpression on autoantibody repertoire selection. A careful comparison of the autoantibodies elicited in the BALB/c and ACS mice revealed intriguing differences in specificity, first apparent in the ANA staining patterns. Interestingly, C1q is known to bind apoptotic cells and apoptotic membrane blebs, and it thereby aids in the timely removal of apoptotic debris. Thus, in the presence of anti-C1q Abs, apoptotic cells can no longer be efficiently removed. These apoptotic cells can then progress to secondary necrosis or other forms of more proinflammatory cell death and differential release of autoantigens. It is tempting to speculate that anti-C1q Abs could also alter the removal of dying Fas’ neutrophils in a way that they might expose specific neutrophil-associated autoantigens.

Collagen type IV is also targeted by autoantibodies in SLE (65) and is a major constituent of the glomerular basement membrane. In this study, we found that there were higher titers of autoantibodies to collagen type IV in the ACS mice. Interestingly, exacerbation of Goodpasture’s syndrome, a rare autoimmune disease associated with autoantibodies to type IV collagen, has also been linked to a case of accidental hydrocarbon exposure (66). Thus, associated with autoantibodies to type IV collagen, has also been reported to fix complement more effectively than IgG1. However, the in-vivo relevance of specific ISGs.

Most importantly, the ACS mice developed more severe renal disease, evident from both the histological scores and the extent of proteinuria. This outcome was somewhat unexpected, based on the shift in IgG2a/IgG1 serum concentrations, as IgG2a is thought to fix complement more effectively than IgG1. However, the increased renal disease in the ACS mice may reflect the presence of autoantibody specificities that bind to glomerular components such as type IV collagen directly or may be due to deposition of C1q-immune complexes. Because renal IL-18 levels correlate with disease activity in MRL-Fas−/− lupus mice (67), it is likely that FasL-expressing effector cells can directly target kidney cells and promote renal IL-18 cytokine production, contributing to further local tissue damage in ACS mice. Treatment of New Zealand Black/White F1 mice with anti-FasL Ab has been shown to prevent lupus nephritis (68), and agonistic anti-Fas Abs were found to induce glomerular cell apoptosis in vivo (69). Furthermore, 50–70% of the renal tubule cells appear to express Fas on the cell surface. These studies are in line with a critical role of Fas signaling in mediating organ pathology in experimental lupus erythematosus. Considering the numerous cell types that can express either Fas and/or FasL, it is likely that the ACS mutation contributes to disease progression through multiple pathways. Therefore, it is not surprising that modest yet chronic overexpression of FasL may not reveal critical effects early in the disease process and still promote quite dramatic changes in end-stage disease.

In summary, our studies using both loss-of-function and gain-of-function FasL mutant mice reveal a critical FasL/Fas signaling axis in the well-characterized TMPD model of systemic autoimmunity. These data establish that not only loss of Fas signaling but also increased Fas signaling can contribute to SLE, and they point to the potential therapeutic application of Fas/FasL antagonists.

Acknowledgments

We thank Glennice Ryan for assistance with the Luminesx analysis, Zhaozhao Jiang and Suzann Carpenter for assistance in the nanostring analysis, and Ted Giehl and Marc Barnard for excellent technical help in cell sorting, Lisa Waggoner for outstanding technical support, and Lino Teichman, Mark Shlomchik, Evelyn Kurt-Jones, and Krishna Moody for advice and discussion.

Disclosures

The authors have no financial conflicts of interest.

References


Fas gene overexpression exacerbates murine SLE. Immunity 7: R1296–R1303.


Supplemental Table 1: Nanostring raw data for the mRNA expression values used to generate the heatmap in Figure 4A before normalization as described in the materials and methods section.

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Supplementary Figure Legends

(Lukas Bossaller et al., Overexpression of Membrane-bound Fas Ligand (CD95L) Exacerbates Autoimmune Disease and Renal Pathology in Pristane-induced Lupus)

Supplementary Figure 1A: Relative levels of Fas-L expression by monocyte and neutrophil subsets in TMPD-injected mice. Histograms for FasL expression on peritoneal monocytes (R1) and neutrophils (R2) from day 14 TMPD-treated mice. Cells obtained from FasL−/− mice (shaded histogram) served as controls.

Supplementary Figure 1B: Rag2 deficiency ameliorates the TMPD-induced early inflammatory responses. Cell number (+/- SEM n=3) and phenotype of PWCs (white bars) and PECs (left bars) collected from BALB/c or Rag2−/− day 14 TMPD-treated mice.

Supplementary Figure 1C: Increased peritoneal cavity cytokine secretion in ΔCS TMPD-treated mice. Cytokine concentration of peritoneal wash fluids collected from untreated mice (white bars) or day 14 TMPD-treated mice (black bars) (+/- SEM n=4).
Supplementary Figure 2: TMPD induces significantly greater neutrophil accumulation in spleens of ΔCS mice and glomerular IgG deposition is increased in kidneys from ΔCS mice. (A) Immunofluorescent stain of spleen sections for MOMA-1+ macrophages (red) and Ly6G positive neutrophils (green) from untreated BALB/c mice or TMPD-injected mice 6 months post-treatment. (B) Immunofluorescence images depict glomerular IgG deposition (green channel) and nuclei (blue channel, DAPI) for representative kidney sections from untreated BALB/c mice or TMPD-injected mice 6 months post-treatment.

Supplementary Figure 3: Distinct autoantibody profile in ΔCS mice compared to BALB/c mice. (A) Hierarchical clustered heatmap in which each row represents one autoantigen and each column represents an individual mouse. The mean value for each row was determined and relative serum concentrations depicted on a scale from dark red (3-fold higher than the mean) and dark blue (2.5-fold lower than the mean). Y-axis clustering identifies three groups. Group A autoantigens are preferentially targeted in ΔCS mice and group B autoantigens are preferentially targeted in BALB/c mice. Group C defines autoantigens with a mixed distribution in both groups. (B) Images of HEp2 ANA staining patterns TMPD-treated BALB/c and ΔCS mice of sera used for the autoantibody array in (B).
Supplemental Figure 1
Supplemental Figure 3
### Supplemental Table 1: Nanostring raw data for the mRNA expression values used to generate the heatmap in Figure 4A before normalization as described in the materials and methods section.

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