Toll-like receptor–independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation

Yasutaka Okabe,[1] Kohki Kawane,[1,2] Shizuo Akira,[3] Tadatsugu Taniguchi,[4] and Shigekazu Nagata[1,2,5]

[1]Department of Genetics, Osaka University Medical School, [2]Laboratory of Genetics, Integrated Biology Laboratories, Graduate School of Frontier Biosciences, and [3]Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871
[4]Department of Immunology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan
[5]Solution Oriented Research for Science and Technology, Japan Science and Technology Corporation, Osaka 565-0871, Japan

Deoxyribonuclease (DNase) II in macrophages cleaves the DNA of engulfed apoptotic cells and of nuclei expelled from erythroid precursor cells. *DNase II*–deficient mouse embryos accumulate undigested DNA in macrophages, and die in feto because of the activation of the *interferon β* (*IFNβ*) gene. Here, we found that the F4/80-positive macrophages in *DNase II*$^{-/-}$ fetal liver specifically produce a set of cytokines such as IFNβ, TNFα, and CXCL10. Whereas, IFN-inducible genes (2′5′-oligo(A) synthetase, IRF7, and ISG15) were expressed not only in macrophages but also in other F4/80-negative cells. When *DNase II*$^{-/-}$ macrophages or embryonal fibroblasts engulfed apoptotic cells, they expressed the *IFNβ* and *CXCL10* genes. The ablation of Toll-like receptor (TLR) 3 and 9, or their adaptor molecules (MyD88 and TRIF), had no effect on the lethality of the *DNase II*$^{-/-}$ mice. These results indicate that there is a TLR-independent sensing mechanism to activate the innate immunity for the endogenous DNA escaping lysosomal degradation.

CORRESPONDENCE
Shigekazu Nagata:
nagata@genetic.med.osaka-u.ac.jp

Abbreviations used: IRF3, IFN regulatory factor 3; MACS, magnetic-activated cell sorting; MEF, mouse embryonal fibroblast; TLR, Toll-like receptor; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β.

DNA is actively degraded in various mammalian developmental processes (1). It is degraded in the programmed cell death that occurs during embryogenesis; in definitive erythropoiesis in the fetal liver, spleen, and bone marrow; and in lens cell differentiation in the eye. DNase II, a lysosomal enzyme, is expressed in various types of cells, with prominent expression in macrophages (2). Macrophages engulf the apoptotic dying cells that are generated during programmed cell death and digest DNA by DNase II. Macrophages also engulf and digest the nuclei expelled from erythroid precursor cells during erythropoiesis (3).

DNase II$^{-/-}$ mouse embryos accumulate undigested DNA in macrophages, which are present in various tissues, such as the thymus, kidney, interdigit, spleen, and liver (4–6). The development of lymphocytes and erythrocytes is severely impaired in *DNase II*$^{-/-}$ embryos that die in feto (4, 5). We recently found that the *IFNβ* gene is strongly activated in the *DNase II*$^{-/-}$ fetal liver and thymus (5). When *DNase II*$^{-/-}$ mice were crossed to mice deficient in the IFN type I receptor (*IFN-IR*) gene, the *DNase II*$^{-/-}$*IFN-IR*$^{-/-}$ embryos developed normally to birth, indicating that IFNβ, which is produced by the macrophages carrying undigested DNA, has a lethal effect on the development of mouse embryos (7).

Type I IFNs, to which IFNβ belongs, play a central role in antiviral innate immunity, and are used to treat human patients with C-type hepatitis and leukemia (8). The effects of IFNs are the result of their direct inhibitory action against viral replication in the infected cells, and to their pleiotropic immunomodulating activity on NK cells, macrophages, and T lymphocytes (9). Most cells produce IFNβ when they are infected with a DNA or RNA virus. Expression of the *IFNβ* gene is also induced in macrophages and dendritic cells by bacterial components such as LPS and nonmethylated CpG DNA (10, 11). The signal transduction leading to the *IFNβ* gene expression by viral and bacterial components has been extensively studied (12–14). According to these studies, viral and bacterial components bind to Toll-like receptors (TLRs) expressed on mac-

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rophages and dendritic cells, which leads to the recruitment of specific adaptor molecules to the receptors. Specifically, TLR3, TLR4, TLR7, and TLR9 function as signaling receptors for double-stranded RNA, LPS, viral single-stranded RNA, and CpG DNA, respectively. An adaptor called MyD88 (myeloid differentiation primary response gene 88) transduces the signal from TLR7 or TLR9 to activate the *IFNβ* gene. TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN-β), another adaptor, is recruited to TLR3 and TLR4, which leads to *IFNβ* gene expression through the activation of a transcription factor called IFN regulatory factor 3 (IRF3).

In this report, we show that macrophages can produce IFNβ and CXCL10 (chemokine, CXC motif, ligand) when they cannot degrade DNA of the engulfed apoptotic cells. We crossed the lethal *DNase II*$^{-/-}$ embryos to mouse strains deficient in genes for TLRs or their adaptors, and found that the TLR system has little role in the induction of the *IFNβ*, *CXCL10,* and *TNFα* genes by mammalian DNA accumulated in the lysosomes of macrophages. These results imply that there is a novel TLR-independent mechanism that recognizes the undigested endogenous DNA to activate the innate immune system.

RESULTS

Constitutive expression of a set of genes in the macrophages of *DNase II*$^{-/-}$ fetal liver

The fetal livers of *DNase II*$^{-/-}$ embryos contain many F4/80-positive macrophages that carry undigested DNA, and constitutively express IFNβ and γ, and IFN-inducible genes. To examine which genes were primarily activated in these macrophages, macrophages of the fetal liver were isolated by magnetic-activated cell sorting (MACS) using an antibody against the macrophage-specific, F4/80 antigen. The F4/80-positive cells accounted for <1% (0.3%) of the fetal liver cells from both wild-type and *DNase II*$^{-/-}$ embryos (Fig. 1 A and not depicted). This proportion increased to 6–10% after sorting by the MACS procedure, whereas almost no F4/80-positive cells (<0.03%) were found in the F4/80-negative fraction. The enrichment of F4/80-positive macrophages by the MACS procedure was confirmed by quantifying the F4/80 mRNA level by real-time PCR. That is, the F4/80 mRNA level in the sorted fraction was about sixfold that in the same amount of RNA from unsorted fetal liver cells. In contrast, its level was negligible in the cells of the F4/80-negative fraction (Fig. 1 B).

The mRNA level of IFNβ, IFNγ, TNFα, and IFN-inducible genes (Fig., 1 B, *CXCL10*, *2′5′ oligo(A) synthetase* [*OAS*], *IRF7*, and *IFN-stimulated gene 15* [*ISG15*]) was then quantified by real-time PCR. As shown in Fig. 1 B, the mRNA for IFNβ, TNFα, and CXCL10 was exclusively found in the F4/80-positive fraction from the *DNase II*$^{-/-}$ fetal liver, suggesting that these genes could have been directly activated in macrophages carrying undigested DNA. Among the genes activated in the macrophages of *DNase II*$^{-/-}$ fetal liver, the absolute level of CXCL10 mRNA was ~10–100 times that of the IFNβ or TNFα mRNAs. In contrast to these genes, the IFNγ, OAS, IRF7, and ISG15 mRNAs—the expression of which was also strongly activated in *DNase II*$^{-/-}$ fetal liver—were found not only in the F4/80-positive but also in the F4/80-negative fractions. These results indicate that these genes could be activated secondarily by cytokines and chemokines produced by the *DNase II*$^{-/-}$ macrophages.

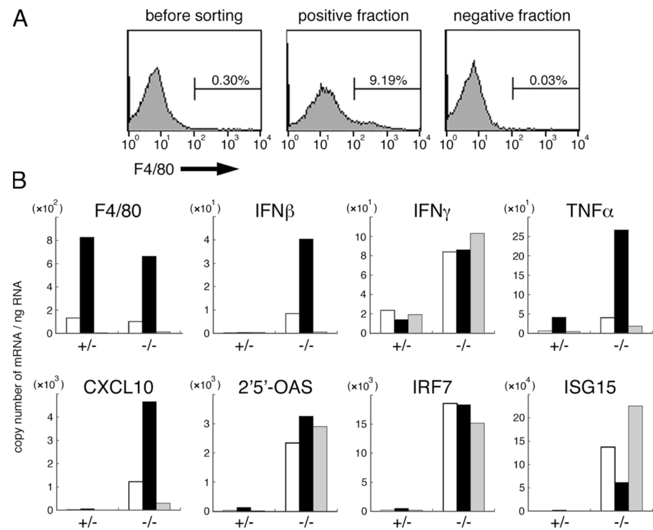

Figure 1. Constitutive expression of *IFNβ* and IFN-inducible genes by F4/80-positive cells in the *DNase II*$^{-/-}$ fetal liver. (A) The fetal liver cells from wild-type E14.5 mouse embryos were sorted by MACS using a biotinylated anti-F4/80 mAb. The cells retained on (positive fraction) and passing through (negative fraction) the column were collected. Aliquots of each fraction, as well as the cells before sorting were stained with PI and Alexa 488–conjugated streptavidin, and analyzed by flow cytometry. The staining profiles for F4/80 in the PI-negative fraction are shown. (B) RNA was prepared from *DNase II*$^{+/-}$ (+/−) or *DNase II*$^{-/-}$ (−/−) fetal liver cells before sorting (open bar), the population enriched for F4/80-positive cells (closed bar), and the F4/80-negative cells (gray bar). The mRNA level for F4/80 antigen (GenBank/EMBL/DDBJ accession no. X93328), IFNβ (NM_010510), IFNγ (NM_008337), TNFα (NM_013693), CXCL10 (M33266), 2′5′-OAS (BC013715), IRF7 (NM_016850), and ISG15 (NM_015783) was quantified by real-time PCR, and expressed as copy number per nanogram of total RNA.

Activation of the *CXCL10* and *IFNβ* genes in phagocytes engulfing apoptotic cells

Macrophages engulf apoptotic cells and the nuclei expelled from erythroid precursor cells, and degrade their DNA using DNase II in lysosomes (1). To confirm that the undigested DNA accumulated in macrophages could activate the genes, W3/Ildm cells, which do not undergo apoptotic DNA fragmentation (15), were induced to undergo apoptosis with Fas ligand, and then added to fetal liver macrophages. As found previously (5), the primary macrophages from wild-type or *DNase II*$^{-/-}$ embryos efficiently engulfed the apoptotic cells; however, the DNA of the engulfed apoptotic cells was efficiently degraded in the wild-type, but not the *DNase II*$^{-/-}$, macrophages. The effect of undigested DNA on the gene expression in macrophages was then quantified by real-time PCR for CXCL10 mRNA. As shown in Fig. 2, the

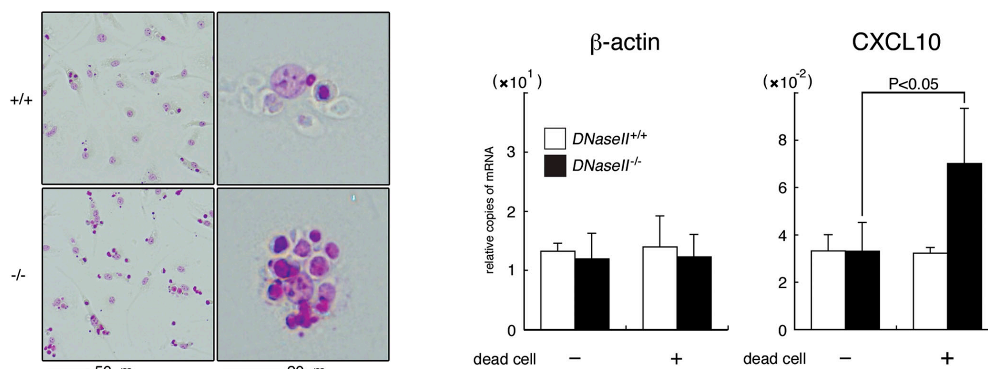

Figure 2. In vitro activation of the *CXCL10* gene in macrophages engulfing apoptotic cells. Apoptotic W3/Ildm cells were added to primary macrophages from the *DNase II*$^{+/+}$ or *DNase II*$^{-/-}$ fetal liver at a 10:1 ratio, incubated at 37°C for 2 h, and stained with Feulgen. (left panel, left micrograph) Original magnification, 200; bar, 50 μm. A single macrophage carrying apoptotic cells is also shown at a higher magnification in the right micrograph. Original magnification, 400; bar, 20 μm. In the right panels, macrophages from *DNase II*$^{+/+}$ (open bar) or *DNase II*$^{-/-}$ (closed bar) fetal liver were cultured with or without apoptotic W3/Ildm cells for 6 h. The β-actin and CXCL10 mRNA levels were determined by real-time PCR, and normalized to the expression level of F4/80 mRNA. Experiments were performed three times, and the mean values are shown with standard deviation (error bars).

CXCL10 mRNA level in the *DNase II*$^{-/-}$ macrophages engulfing apoptotic cells was about twice that in the wild-type macrophages. The increase of CXCL10 mRNA was specific, because no induction of the β-actin gene was observed in the *DNase II*$^{-/-}$ macrophages engulfing apoptotic cells.

Mouse embryonal fibroblasts (MEF) transformed with $\alpha_v\beta_3$ integrin efficiently engulf apoptotic cells in the presence of milk fat globule EGF factor 8 (MFG-E8) (16). To confirm that the *CXCL10* gene could be activated by mammalian DNA, wild-type and *DNase II*$^{-/-}$ MEF expressing $\alpha_v\beta_3$ integrin were established. As expected, both the wild-type and *DNase II*$^{-/-}$ MEF efficiently engulfed apoptotic thymocytes in the presence of MFG-E8; but, condensed undigested nuclear DNA was found only in the *DNase II*$^{-/-}$ MEF (Fig. 3 A). The CXCL10 mRNA level was similar between the wild-type and *DNase II*$^{-/-}$ MEF when they were incubated without apoptotic cells (Fig. 3 B). However, its level increased 4.4-fold when the *DNase II*$^{-/-}$ MEF were incubated with apoptotic cells. When chloroquine, which inhibits the acidification of lysosomes (17), was added to the engulfment assay, the DNA of the engulfed thymocytes remained undigested even in the wild-type MEF, and this was accompanied by activation of the *CXCL10* gene. These results confirmed that undigested chromosomal DNA in the lysosomes of phagocytes could activate the *CXCL10* gene. Similarly, the IFNβ mRNA, detected as a 368-bp RT-PCR product, was found when *DNase II*$^{-/-}$ but not wild-type MEF was incubated with apoptotic thymocytes (Fig. 3 C). To further confirm that the deficiency of the *DNase II* gene (whereby there is no DNA degradation in lysosomes), is responsible for the activation of *CXCL10* gene, the *DNase II* gene was introduced into *DNase II*$^{-/-}$ MEF. As shown in Fig. 3 D, the stable transformants reexpressing DNase II did not accumulate undigested DNA in lysosomes, and the expression level of CXCL10 was reduced to that observed with the wild-type MEF.

No involvement of a Toll-like receptor system in the *IFNβ* gene activation in *DNase II*$^{-/-}$ embryos

Bacterial DNA activates the innate immune system via TLR9 in a MyD88-dependent manner (12, 13), whereas double-stranded RNA such as poly(I)(C), activates the *IFNβ* gene via the TLR3–TRIF pathway. To examine whether the endogenous DNA that escapes lysosomal degradation uses any of these TLRs and adaptors to activate the *IFNβ* gene, *DNase II*$^{+/-}$*TLR9*$^{-/-}$, *DNase II*$^{+/-}$*TLR3*$^{-/-}$, *DNase II*$^{+/-}$*MyD88*$^{-/-}$, and *DNase II*$^{+/-}$*TRIF*$^{-/-}$ mice were intercrossed. The genotype analysis of their neonatal offspring did not reveal double-deficient mice lacking any of these genes with the *DNase II* gene, which is in a sharp contrast to the result with *DNase II*$^{-/-}$*IRF-IR*$^{-/-}$ embryos (Table I). Embryos that carry the corresponding genotypes could be found at a normal Mendelian ratio at embryonic day (E)12.5, indicating that the double-deficient mice died at the late stage of embryogenesis like *DNase II*$^{-/-}$ embryos. Real-time PCR analysis of RNA from

Table I. No effect of the null mutation of *TLR9*, *TLR3*, *MyD88*, or *TRIF* on the lethality of *DNase II*$^{-/-}$ embryos

Genotypes	*DNase II*$^{+/+}$	*DNase II*$^{+/-}$	*DNase II*$^{-/-}$
+/+	29	50	0
TLR9$^{-/-}$	42	65	0
TLR3$^{-/-}$	14	29	0
MyD88$^{-/-}$	14	37	0
TRIF$^{-/-}$	35	73	0
MyD88$^{-/-}$ *TRIF*$^{-/-}$	6	12	0
IFN-IR$^{-/-}$	19	36	23

Mouse parents carrying the *DNase II*$^{+/-}$*TLR9*$^{-/-}$, *DNase II*$^{+/-}$*TLR3*$^{-/-}$, *DNase II*$^{+/-}$*MyD88*$^{-/-}$, *DNase II*$^{+/-}$*TRIF*$^{-/-}$, *DNase II*$^{+/-}$*MyD88*$^{-/-}$*TRIF*$^{-/-}$genotypes were intercrossed, and the genotype for the *DNase II* gene in their offspring was determined by PCR. The number of offspring carrying the indicated genotype is shown. Results of the intercross between *DNase II*$^{+/-}$*IFN-IR*$^{-/-}$ are from Yoshida et al. (7).

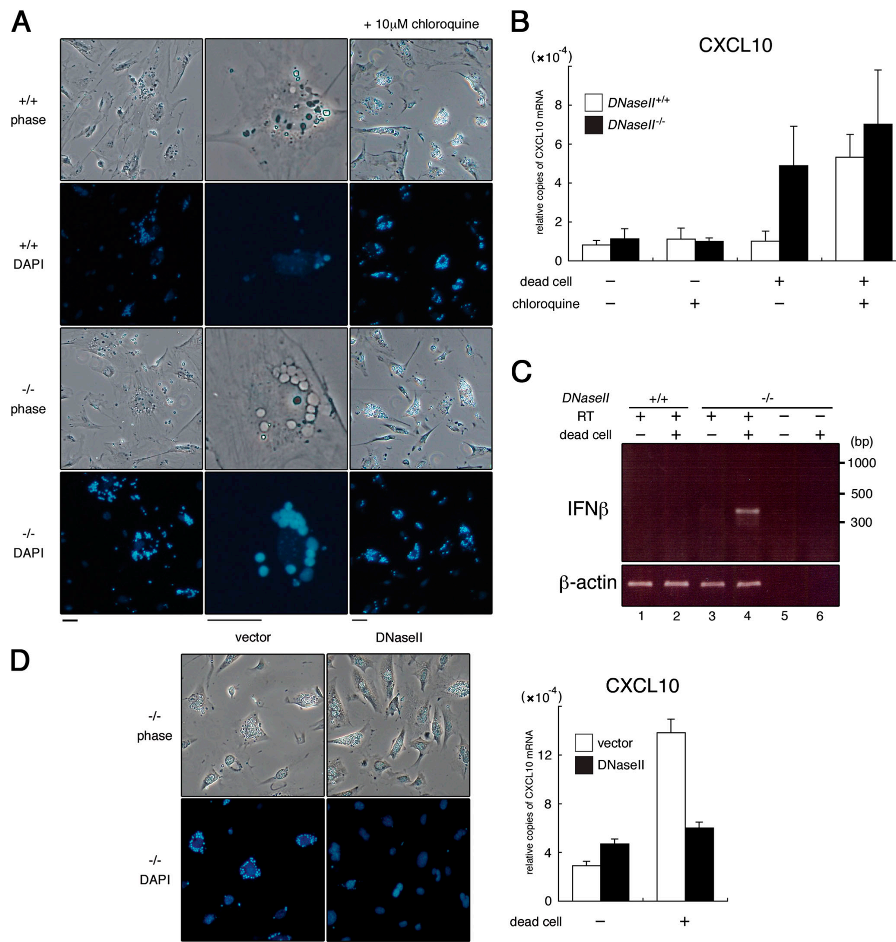

Figure 3. In vitro activation of the *CXCL10* and *IFNβ* genes in embryonal fibroblasts engulfing apoptotic cells. (A) Accumulation of the undigested DNA in *DNase II*$^{-/-}$ MEF. *DNase II*$^{+/+}$ or *DNase II*$^{-/-}$ MEF were transformed to express $\alpha_v\beta_3$-integrin. Apoptotic *CAD*$^{-/-}$ thymocytes were added to the MEF at 125:1, incubated at 37°C for 20 h in the presence of 0.1 μg/ml mouse MFG-E8 with or without chloroquine, stained with DAPI, and observed by microscopy. (left and right) Original magnification, 100; bars, 50 μm. A single MEF carrying apoptotic cells is shown in the middle. Original magnification, 200; bar, 50 μm. (B) Activation of the CXCL10 gene in *DNase II*$^{-/-}$ MEF. *DNase II*$^{+/+}$ (open bar) or *DNase II*$^{-/-}$ MEF were allowed to engulf apoptotic *CAD*$^{-/-}$ thymocytes for 20 h in the presence of 0.1 μg/ml mouse MFG-E8 with or without 10 μM chloroquine. The expression level for CXCL10 mRNA was determined by real-time PCR and was normalized to the expression level of β-actin mRNA. The mean values from three independent experiments are plotted with standard deviations (error bars). (C) Activation of the *IFNβ* gene in *DNase II*$^{-/-}$ MEF. Immortalized MEF from wild-type (+/+) or *DNase II*$^{-/-}$ (−/−) embryos were incubated with (+) or without (−) apoptotic thymocytes, and the IFNβ mRNA in MEF was analyzed by RT-PCR. RT-PCR for the RNA samples from *DNase II*$^{-/-}$ MEF was performed in the presence (+) or absence (−) of reverse transcriptase (RT). As a control, β-actin mRNA was analyzed by RT-PCR. (D) Effect of the reexpression of DNase II in the activation of *CXCL10* gene in *DNase II*$^{-/-}$ MEF. Integrin αβ-expressing immortalized *DNase II*$^{-/-}$ MEF was transfected with the retrovirus expression vector carrying the DNA fragment coding for the Flag-tagged DNase II gene or the empty vector. The stable transformants were then incubated with or without apoptotic *CAD*$^{-/-}$ thymocytes as described above, and stained with DAPI. Original magnification, 100; bar, 50 μm. In the right panel, the CXCL10 mRNA level was quantified by real-time PCR. The relative expression level against β-actin mRNA is shown. The experiments were performed three times, and the mean values are shown with standard deviations (error bars).

the liver of these double-deficient embryos indicated that the IFNβ, CXCL10, and TNFα mRNAs were expressed as abundantly as in the *DNase II*$^{-/-}$ embryos (Fig. 4). To further confirm no involvement of TLR in activation of the innate immune system in the *DNase II*$^{-/-}$ embryos, the *DNase II*$^{+/-}$ *MyD88*$^{-/-}$*TRIF*$^{-/-}$ mice were established. As shown in Table I, the intercross of these *DNase II*$^{+/-}$ *MyD88*$^{-/-}$*TRIF*$^{-/-}$ mice did not produce triple mutant mice that carry the *DNase II*$^{-/-}$ *MyD88*$^{-/-}$*TRIF*$^{-/-}$ genotype. These results indicated that TLR3, TLR9, or other TLRs that use MyD88 or TRIF adaptor were not involved in activating the *IFNβ* gene by the endogenous DNA accumulated in lysosomes.

DISCUSSION

Systemic lupus erythematosus is characterized by the production of autoantibodies against single-stranded or double-

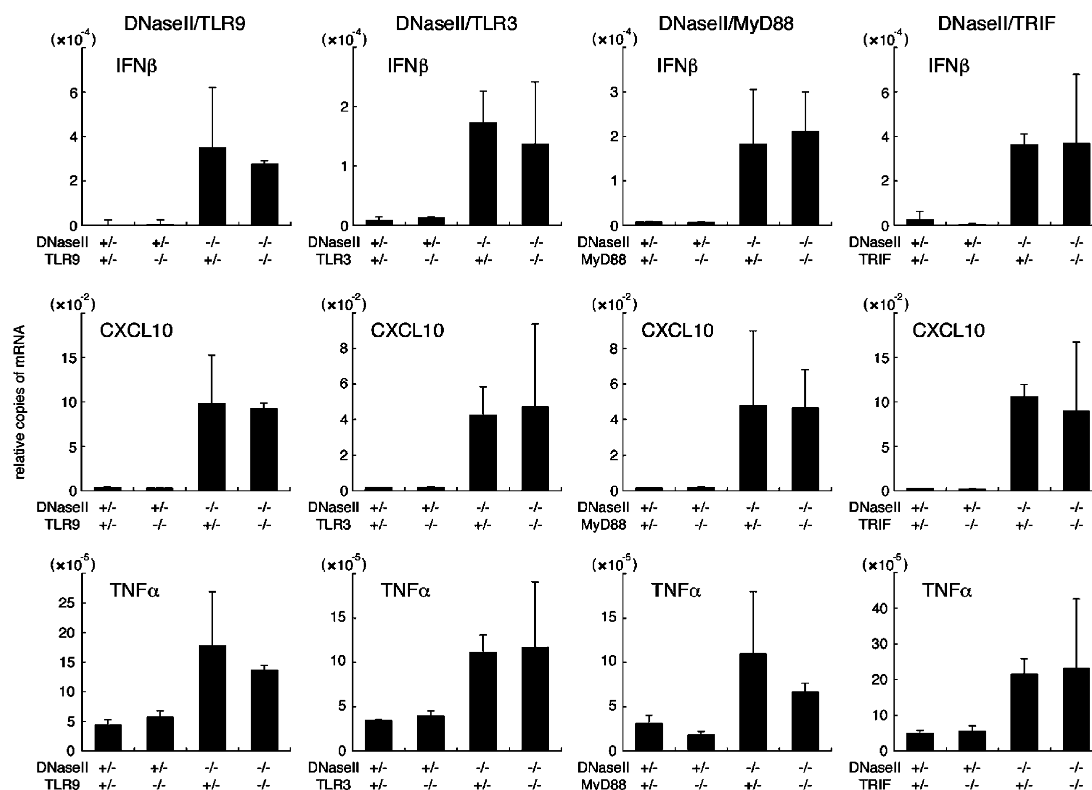

Figure 4. No involvement of TLR9, TLR3, MyD88, or TRIF in the *IFNβ, CXCL10, and TNFα* gene activation in *DNase II$^{-/-}$* embryos. RNA was prepared from the liver of wild-type, *TLR9$^{-/-}$*, *TLR3$^{-/-}$*, *MyD88$^{-/-}$*, *TRIF$^{-/-}$*, *DNase II$^{-/-}$*, *DNase II$^{-/-}$TLR9$^{-/-}$*, *DNase II$^{-/-}$TLR3$^{-/-}$*, *DNase II$^{-/-}$MyD88$^{-/-}$*, and *DNase II$^{-/-}$TRIF$^{-/-}$* embryos at E12.5. The mRNA level for IFNβ, CXCL10, and TNFα was determined by real-time PCR, and is plotted as a relative value to β-actin mRNA with standard deviations.

stranded DNA. Patients with systemic lupus erythematosus often carry circulating DNA in their serum, suggesting that DNA activates the immune system (18). In fact, bacterial DNA activates the innate immune system, leading to inflammation and septic shock, and infection by DNA viruses activates the IFNα and IFNβ genes (10). We previously showed that DNA derived from apoptotic cells or nuclei expelled from erythroid precursor cells accumulates in the macrophages of *DNase II$^{-/-}$* embryos, activates a group of genes, and kills the embryos by inducing severe anemia (5, 7). Among the genes activated in the *DNase II$^{-/-}$* fetal liver, several, such as the *IFNβ* and *CXCL10* genes, seem to be directly activated in the macrophages by the undigested DNA, whereas others are activated secondarily through the IFN system (this report and reference 7).

Many groups have studied the signal transduction for activation of the innate immune system by DNA using in vitro and in vivo systems. The exposure of macrophages to naked bacterial, but not mammalian, DNA activates the innate immunity in a TLR9- and MyD88-dependent manner (12, 13, 19). This finding led to the proposal that the unmethylated CpG motif that is abundant in the bacterial genome is responsible for the induction of innate immunity by DNA (10); and, this idea was supported by the observation that TLR9 recognizes this DNA motif (20). In contrast to this hypothesis, when a DNA–liposome complex is used to introduce DNA into macrophages, inflammatory responses, including induction of the *IFNβ* gene, are induced not only by bacterial DNA but also by mammalian DNA (21). This response depends on endosomal acidification (21), and partly requires TLR9 (22). In addition, mammalian DNA–immunoglobulin complex activates B cells via TLR9 (23); but, it activates dendritic cells by both TLR9-dependent and -independent pathways (24). Apoptotic cells are engulfed by macrophages, and their DNA is degraded by DNase II in lysosomes (5), implying that the undegraded DNA left in the *DNase II$^{-/-}$* macrophages is localized to lysosomes. Thus, we expected that the *IFNβ* gene induction in *DNase II$^{-/-}$* macrophages occurred via TLR9, which can be translocated to the late endosomes and lysosomes (25). However, a deficiency of *TLR9* or its adaptor *MyD88* had no effect on the activation of the *IFNβ* and *CXCL10* genes in *DNase II$^{-/-}$* embryos. The treatment of *DNase II$^{-/-}$* phagocytes with chloroquine, which inhibits the TLR9-mediated signaling (26), had no effect on the expression of the *CXCL10* gene expression, confirming that TLR9 is not involved in this system. In addition, *TLR3* deficiency also did not rescue the lethality of the *DNase II$^{-/-}$* embryos, indicating that TLR3, which is responsible for poly(I)(C)-induced *IFNβ* gene ex-

pression (27), does not play a role in the *IFNβ* gene activation in *DNase II*$^{-/-}$ macrophages. Signaling from the other TLRs (TLR1–2 and TLR5–8) is mediated by MyD88 or TRIF adaptor (12, 13). Single- and double-null mutations of the *MyD88* or *TRIF* gene failed to rescue the lethality of *DNase II*$^{-/-}$ embryos. These results indicate that TLRs, at least the known TLRs, are not likely to be involved in activation of the *IFNβ, CXCL10*, and *TNFα* genes by mammalian DNA accumulated in macrophages.

The *IFNβ* and *CXCL10* genes carry a similar element called IFN stimulus response element (ISRE) or the IRF element on their promoter (28, 29). LPS, poly(I)(C), and viruses activate the *IFNβ* and *CXCL10* genes by activating IRF3 or IRF7, which binds to the ISRE on their promoter (30, 31). Recently, a TLR-independent pathway that activates IRF3 via an RNA helicase was established for viral RNA (32, 33). It will be interesting to examine the possible involvement of IRF3 and/or IRF7 in the activation of *IFNβ* and *CXCL10* genes by undigested mammalian DNA in lysosomes. The *TNFα* gene promoter carries NF-κB and AP-1 binding sites. LPS, bacterial DNA, and viral RNA activate the *TNFα* gene through a TLR-dependent JNK–NF-κB pathway. Our results indicate that the *TNFα* gene induction in *DNase II*$^{-/-}$ macrophages is also TLR independent. How this gene is activated by the mammalian DNA that escaped from lysosomal degradation remains to be studied.

Type I IFN and TNFα have a major effect on various autoimmune diseases (34, 35). A large amount of chromosomal DNA, which is potentially immunogenic, is degraded in our bodies during erythropoiesis and programmed cell death. Elucidation of the molecular mechanism by which undigested mammalian DNA leads to the IFN and TNFα production will help us identify candidate targets for the therapeutic intervention of these autoimmune diseases.

MATERIALS AND METHODS

Mice. The *CAD*$^{-/-}$, *DNase II*$^{+/-}$, *MyD88*$^{-/-}$, *TRIF*$^{-/-}$, *TLR3*$^{-/-}$, and *TLR9*$^{-/-}$ mice were described previously (4, 5, 20, 36–38). The mice were housed in a specific pathogen-free facility at Osaka University Medical School or Oriental Bioservice Inc. All animal experiments were performed in accordance with protocols approved by the Osaka University Medical School Animal Care and Use Committee. The genotype of the *DNase II, MyD88*, *TRIF*, *TLR3*, and *TLR9* genes was determined by PCR using the primers listed in the supplemental materials (available at http://www.jem.org/cgi/content/full/jem.20051654/DC1). All mice except *TRIF*$^{+/-}$ mice, that were backcrossed three times, were backcrossed to C57BL/6 strain at least six generations.

Cells and reagents. A derivative of mouse T cell lymphoma (WR19L, ATCC TIB 52) expressing mouse Fas and a caspase-resistant form of ICAD-L (W3/Ildm) was described previously (15). Fetal liver macrophages were prepared essentially as described previously (39). In brief, the livers from E14.5 embryos were dissected and passed through a 22-gauge needle five times. The cells were washed with PBS, and cultured in a 10-cm suspension culture dish (Corning) with α-MEM containing 10% FBS supplemented with mouse macrophage colony-stimulating factor (40). After 2 d, the cells were vigorously washed, and adherent cells were recovered by incubating at 37°C in PBS containing 0.02% EDTA. The cells were further cultured for a week and were used as fetal liver macrophages.

MEF were established from E13.5 embryos as described previously (41). In brief, after removing the head and liver, the torso was minced and dispersed in PBS containing 0.05% trypsin and 0.5 mM EDTA. After incubating at 37°C for 40 min, DMEM containing 10% FBS was added to the mixture, and the tissues were dissociated by vigorous pipetting. After allowing the large pieces of tissue debris to settle, the suspended cells were cultured in DMEM containing 10% FBS. In some cases, the cells were cultured for 1 mo by splitting the cultures at 1:4 until they stopped proliferating. The cells were further maintained by changing the medium twice a week, and the immortalized cells that started to grow after 1 mo were expanded. MEF cell lines expressing $\alpha_v\beta_3$-integrin or DNase II were established by transforming MEF with a retrovirus carrying α_v- or β_3-integrin or Flag-tagged DNase II as described previously (16).

Leucine zipper–tagged human Fas ligand (42) and mouse MFG-E8 (16) were produced in monkey COS7 and human 293T cells, respectively, and purified to homogeneity as described previously.

Cell sorting and FACS analysis. Cell populations enriched in primary macrophages from the E14.5 fetal liver were obtained using a MACS column (MS$^+$ Separations; Miltenyi Biotec) according to the instructions provided by the manufacturer. In brief, the fetal liver cells (2.0×10^7 cells) were incubated at 4°C for 20 min with 2.5 μg/ml of biotinylated F4/80 antibody (a gift from Dr. M. Tanaka; clone 6-16A) in 4 ml of MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA) containing 10 μg/ml anti-FcγIII/II receptor (BD Biosciences). The cells were further incubated at 4°C for 20 min with anti-biotin microbeads (80 μl, Miltenyi Biotec), and loaded onto a column placed in a magnetic field. The cells that passed through and were retained on the column were collected, and used as F4/80-negative and positive cells, respectively. To monitor the sorting procedure, aliquots of the fractionated cells were stained for 30 min at 4°C with Alexa 488-conjugated streptavidin (Molecular Probes) and propidium iodide (PI) in staining solution (PBS containing 2% FBS and 0.05% NaN_3), and analyzed by a FACScan flow cytometer (Becton Dickinson) using Cell Quest software (Quest Software).

Phagocytosis. Phagocytosis was assayed essentially as described previously (16). In brief, fetal liver macrophages or MEF were cultured in 6-well plates. Thymocytes from *CAD*$^{-/-}$ mice or W3/Ildm lymphoma cells were treated for 2 h at 37°C with Fas ligand to induce apoptosis in ~50% of the cells, and then incubated with phagocytes. To stain the DNA of the engulfed apoptotic cells, the phagocytes were washed to remove the surface-bound cells, fixed with methanol containing 4% formaldehyde and 5% acetic acid, and subjected to Feulgen or DAPI staining.

Real-time PCR. For real-time PCR, total RNA was prepared using the RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN), and was reverse transcribed using Superscript III reverse-transcriptase (Invitrogen) with oligo$(dT)_{12\text{-}18}$ as primer. Aliquots of the reverse transcriptase products were amplified in a reaction mixture containing LightCycler-FastStart DNA Master SYBER green I (Roche Molecular Biochemicals) according to the instructions provided by the manufacturer.

Online supplemental material. Online supplemental material contains primers that were used to determine the genotype of the *DNase II*, *MyD88*, *TRIF*, *TLR3*, and *TLR9* genes and primers for real-time PCR. It is available at http://www.jem.org/cgi/content/full/jem.20051654/DC1.

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