Dnase2a Deficiency Uncovers Lysosomal Clearance of Damaged Nuclear DNA via Autophagy

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Dnase2a Deficiency Uncovers Lysosomal Clearance of Damaged Nuclear DNA via Autophagy

Graphical Abstract

Highlights

- Cells deficient for lysosomal Dnase2a or autophagy accumulate extranuclear DNA
- Cells treated with DNA damaging agents also accumulate extranuclear DNA
- Extranuclear DNA is found in buds or small speckles
- Sting-mediated inflammation is induced by accumulated DNA

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In Brief
Deficiencies in DNA nucleases can lead to the accumulation of self DNA, activation of innate immunity, and development of autoimmune disease. The source of immunostimulatory DNA is not known in most cases. Lan et al. now find that damaged nuclear DNA accumulates outside the nucleus and stimulates Sting-dependent DNA-sensing when mice lack a lysosomal nuclease, Dnase2a. The results support a model in which damaged chromosomal DNA is normally exported from the nucleus and cleared by autophagy.

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Dnase2a Deficiency Uncovers Lysosomal Clearance of Damaged Nuclear DNA via Autophagy

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SUMMARY

Deficiencies in DNA-degrading nucleases lead to accumulation of self DNA and induction of autoimmunity in mice and in monogenic and polygenic human diseases. However, the sources of DNA and the mechanisms that trigger immunity remain unclear. We analyzed mice deficient for the lysosomal nuclease Dnase2a and observed elevated levels of undegraded DNA in both phagocytic and nonphagocytic cells. In nonphagocytic cells, the excess DNA originated from damaged DNA in the nucleus based on colocalization studies, live-cell imaging, and exacerbation by DNA-damaging agents. Removal of damaged DNA by Dnase2a required nuclear export and autophagy-mediated delivery of the DNA to lysosomes. Finally, DNA was found to accumulate in Dnase2a −/− or autophagy-deficient cells and induce inflammation via the Sting cytosolic DNA-sensing pathway. Our results reveal a cell-autonomous process for removal of damaged nuclear DNA with implications for conditions with elevated DNA damage, such as inflammation, cancer, and chemotherapy.

INTRODUCTION

Mammalian cells express numerous nucleic sensors that detect and induce responses to invading viruses and other microbes (Barber, 2011; Paludan and Bowie, 2013). Although this suite of sensors can also recognize self DNA and RNA, several mechanisms are in place to avoid such innate immune responses to self. First, a subset of the RNA and DNA sensors—TLR3, 7, 8, and 9—are localized to endosomal compartments that appear to be spatially segregated from self nucleic acids (Ishii and Akira, 2006; Stetson and Medzhitov, 2006). For example, when TLR9 is engineered to localize on the surface of cells, it recognizes circulating DNA and induces inflammation (Barton et al., 2006). Second, self nucleic acids may have modifications that render them undetectable to some sensors (Iwasaki, 2012). Finally, nucleases clear cells of excess nucleic acids that might otherwise activate sensors. Evidence for this last mechanism is abundant, because mutations in DNases and RNases lead to autoimmunity in mouse models and in human monogenic and polygenic diseases.

Specifically, mutations in Dnase1 (Napirei et al., 2000; Yasutomo et al., 2001), Dnase2 (Kawane et al., 2006, 2010), and Dnase3/Trex1 (Crow et al., 2006; Lee-Kirsch et al., 2007; Morita et al., 2004) enable self DNA originating from apoptotic cells (Yoshida et al., 2005) or retroelements (Stetson et al., 2008) to accumulate and result in autoimmune diseases, including systemic lupus erythematosus and Aicardi-Goutières syndrome in humans (Crow et al., 2006; Lee-Kirsch et al., 2007; Yasutomo et al., 2001), and autoimmune arthritis (Kawane et al., 2006, 2010), nephritis (Gall et al., 2012), and myocarditis (Morita et al., 2004) in mice. Studies of the Trex1/Dnase3 gene that degrades DNA (Chowdhury et al., 2006; Mazur and Perrino, 2001) clearly demonstrate the consequences of cells overreacting to self DNA. Trex1 −/− mice were shown to develop a lethal autoimmune cardiomyopathy (Morita et al., 2004) with associated accumulation of single-stranded DNA produced in S phase (Yang et al., 2007); subsequent studies implicated undegraded retroelements as a source of immunostimulatory DNA (Stetson et al., 2008) and demonstrated the importance of the Sting DNA-sensing pathway in triggering inflammation and autoimmunity (Gall et al., 2012).

Another example is Dnase2a, a ubiquitously expressed lysosomal DNA endonuclease that degrades DNA to oligonucleotides and nucleotides (Evans and Aguilera, 2003). Dnase2a −/− mice spontaneously produce high levels of type I interferons (IFNs) and show embryonic lethality that is rescued by removing the IFN receptor (Yoshida et al., 2005). Conditional ablation of Dnase2a in adult mice leads to production of immune cytokines (e.g., tumor necrosis factor [TNF−α], interleukin 6 [IL-6], CXCL10, IL-1β), and type I IFNs), autoantibodies (rheumatoid factor and anti-double-stranded DNA [dsDNA]) and development of chronic polyarthritis that resembles human rheumatoid arthritis (Kawane et al., 2001). Dnase2a deficiency also exacerbates autoimmunity in mice and in monogenic and polygenic human diseases. However, the sources of DNA and the mechanisms that trigger immunity remain unclear. We analyzed mice deficient for the lysosomal nuclease Dnase2a and observed elevated levels of undegraded DNA in both phagocytic and nonphagocytic cells. In nonphagocytic cells, the excess DNA originated from damaged DNA in the nucleus based on colocalization studies, live-cell imaging, and exacerbation by DNA-damaging agents. Removal of damaged DNA by Dnase2a required nuclear export and autophagy-mediated delivery of the DNA to lysosomes. Finally, DNA was found to accumulate in Dnase2a −/− or autophagy-deficient cells and induce inflammation via the Sting cytosolic DNA-sensing pathway. Our results reveal a cell-autonomous process for removal of damaged nuclear DNA with implications for conditions with elevated DNA damage, such as inflammation, cancer, and chemotherapy.
et al., 2009). Consistent with the role of lysosomes in recycling materials ingested via endocytosis, undigested DNA (partly derived from discarded erythrocyte nuclei) was found in fetal liver macrophages in Dnase2a knockout mice (Kawane et al., 2001; Kriese et al., 2002).

These two models of DNase deficiency demonstrate that defective degradation of nucleic acids can activate autoimmune responses, and yet the source and mechanism of clearance of DNA remain unclear. We focused on the Dnase2a-knockout as a genetic model to dissect the source and subcellular localization of DNA and the mechanisms that trigger immunity and observed unexpected accumulation of nuclear DNA outside the nucleus. Additional experiments led us to define a mechanism by which lysosomal Dnase2a clears damaged nuclear DNA via autophagy.

RESULTS

DNA Accumulates in Phagocytic and Nonphagocytic Dnase2a-Deficient Cells

We hypothesized that phagocytic cells likely accumulate the highest levels of DNA in Dnase2a-deficient animals based on their ability to ingest erythrocyte nuclei and apoptotic cells (Kriese et al., 2002; Yoshida et al., 2005). Consistent with the hypothesis, we detected excessive DNA content in professional phagocytes (using propidium iodine [PI] and other DNA dyes such as Ruby and Hoechst), including bone-marrow-derived dendritic cells (BMDCs) and splenic DCs from Dnase2a−/− mice (Figure 1A), as well as primary BMDCs silenced for Dnase2 by stably expressed small hairpin RNAs (shRNAs). To determine the distribution of DNA in these mice, we stained tissue sections with DAPI and observed higher levels of DNA in cells of joints, kidney and liver of knock out (Dnase2a−/−) relative to wild-type (Dnase2a+/+) mice (Figure 1B). Concomitant with the heightened inflammation previously observed in Dnase2a−/− mice, we detected elevated levels of inflammatory genes (the IFN-inducible chemokine, Cxcl10 and the cytokines, Il6 and Tnfa) in all cell types (B and T cells, DCs, macrophages) of the spleen from knockout mice relative to control littermates. This global activation could occur as a result of independent, autonomous events in each cell type or a cascade of inflammation initiated by a small number of cells.

We were surprised to observe increased DNA levels in non-phagocytic lymphocytes (primary T and B cells, Figure 1C; with the small fraction of genomic DNA representing a significant absolute level of DNA) and nonimmune cells, including primary lung and skin fibroblasts, long-term cultured mouse lung fibroblasts (MLFs), in knockout versus wild-type mice, and p53−/− mouse embryonic fibroblasts (MEFs) silenced with Dnase2a-targeting shRNAs (Figure S1A). To exclude the possibility that Dnase2a−/− cells are more often in S phase (>2n DNA) relative to wild-type cells, we synchronized nonphagocytic MLFs of both genotypes and observed a similar increase in DNA by PI staining (Figure 1D; Ruby staining showed same result). We also confirmed the higher DNA content in Dnase2a−/− cells with independent measurements, including UV absorbance of purified genomic DNA (Figure 1E), which correlated well with mean intensity of dye-stained cells by flow cytometry (Figure S1B). These results imply that Dnase2a is essential for preventing the accumulation of DNA, not only as expected in phagocytic cells, but also in non-phagocytic cells.

Damaged DNA Is Found outside the Nucleus of Dnase2a-Deficient Cells

We suspected that the excess DNA could be derived from an intracellular source. To pinpoint the location of accumulated DNA within Dnase2a-deficient cells, we stained MLFs with anti-dsDNA. We observed small and large DNA aggregates at perinuclear regions (Figure 2A, untreated), and interestingly, adjacent to condensed chromosomes of dividing cells (Figure S2A, untreated). We thus hypothesized that damaged chromosomal DNA would need to be discarded during replication or DNA damage and repair and exported from the nucleus for degradation by Dnase2a.

To visualize DNA fragments generated by damage or repair, we stained tissues and cultured cells for phosphorylated histone 2AX (γ-H2AX), an established marker of double-stranded DNA breaks (DSBs). In vivo staining of γ-H2AX was dramatically elevated in the kidneys of Dnase2a-deficient relative to wild-type mice (Figure 2B), and similarly in vitro in Dnase2a-deficient MLFs (Figure 2C, untreated), suggesting that damaged DNA accumulates to high levels in the absence of Dnase2a in the steady state. We also noted that there was increased nuclear DNA and γ-H2AX signal in the nucleus of Dnase2a−/− MLFs (Figures 2A, 2C, and 2D). Whether excess extranuclear DNA may hinder the export of damaged nuclear DNA and thus promote DNA damage in the nucleus is an open question.

To increase the burden of endogenous damaged DNA, we treated cells or mice with cytarabine (Ara-C), a nucleoside analog that causes replication stalls, and observed even higher levels of extranuclear DNA in cells (Figure 2A, treated), whereas cell viability remained high (Figure S2B). In addition, p53−/− MEFs, which have defects in the regulation of DNA repair, showed clear dose-responsive DNA accumulation to Ara-C by flow cytometry and immunostaining (Figures S2C and S2D). Two different anti-dsDNA antibodies (sc-58749, DNA11-M) and serum from NZB/NZW lupus mice gave similar staining results. DAPI staining also showed similar patterns but required acetone fixation and did not reveal the small DNA speckles in the cytosol (Figure S2E).

Preincubation with DNA, including salmon DNA, calf thymus DNA and IFN-stimulatory DNA (ISD), and digested with Dnase1 confirmed the specificity of the anti-dsDNA antibody (Figures S2F and S2G). We note that MLFs with excessive levels of damaged DNA did not detectably transfer their DNA to cells with normal levels of DNA after 24 hr of coculture, indicating that the observed extranuclear DNA aggregates were not derived from extracellular sources (Figure S2H).

Importantly, the γ-H2AX signal, which was higher in Dnase2a−/− cells after Ara-C treatment (Figure 2C, treated), co-localized with dsDNA both at perinuclear buds and cytosolic speckles (Figure 2D). Furthermore, in vivo treatment of Ara-C aggravated joint swelling in Dnase2a−/− mice suggesting that damaged DNA could exacerbate arthritis in this model (Figure 2E). Thus, the extranuclear DNA accumulating in Dnase2a-deficient cells—spontaneously or induced by
DNA-damaging agents—appears to consist of damaged dsDNA fragments.

Accumulated DNA Originates in the Nucleus
Supporting the nuclear origin of accumulated DNA, three major spatial patterns of extranuclear DNA were observed by confocal microscopy (Figure 3A, relative frequencies on the right): (1) nuclear buds that appear continuous with the nucleus (found in Dnase2a−/− but rarely in wild-type cells); (2) cytosolic speckles that fan out from the nucleus (most prominent upon Ara-C treatment); and (3) large extranuclear aggregates that are likely to be detached nuclear buds based on their shape, size, and proximity to the nucleus (mostly in Ara-C-treated cells). High-resolution fluorescence and electron microscopy staining for dsDNA further confirmed the presence of DNA buds and speckles (emanating at specific points) in close apposition with the

Figure 1. Accumulation of DNA in Dnase2a-Deficient Cells
(A) DNA content of BMDCs and splenic DCs from wild-type and Dnase2a−/− mice examined by PI staining (MFI shown), representative of three independent experiments with MFI ratio of Dnase2a−/− to Dnase2a+/+ of 1.15 ± 0.13 SD (BMDCs) and 1.19 ± 0.24 SD (splenic DCs).
(B) DAPI staining of tissues from wild-type and Dnase2a-deficient mice. Mean density of DAPI-positive areas is based on five to ten total fields from two independent comparisons of matched wild-type and knockout mice; values are mean ± SEM; scale bar represents 10 μm.
(C) Overall DNA content of live immune cells from spleens of wild-type and Dnase2a-deficient mice assessed by flow cytometry using Ruby dye. Each symbol represents the ratio of the MFI from cells of Dnase2a−/− versus matched Dnase2a+/+ mice. Six independent experiments were analyzed using a regression model to account for technical batch effect and biological variation; mean MFI ratios for all experiments per cell type are indicated next to the horizontal bars that mark the mean.
(D) DNA content of nocodazole-synchronized (100 ng/ml for 16 hr) Dnase2a−/− and Dnase2a+/+ MLFs by PI staining, representative of three independent experiments with MFI shown, p < 0.05.
(E) Relative amount of purified genomic DNA content from Dnase2a−/− or Dnase2a−/− MLFs by PI staining, representative of three independent experiments with MFI shown, p < 0.05.

See also Figure S1.
nucleus (Figure 3B). Buds and speckles were often encircled by or colocalized with the nucleoporin protein, NUP98, a docking site for transport at the nuclear pore complex and marks the nuclear envelope (Figure 3C). When the fate of newly synthesized nuclear DNA was tracked with bromodeoxyuridine (BrdU), we observed comparable patterns of perinuclear buds and cytosolic speckles emanating from the nucleus (Figure 3D), indicating that replicated DNA contributed to extranuclear DNA.

We tested the potential of mitochondria to contribute excess DNA, and found that MitoTracker signal (Figure S3A) and
Figure 3. Export of DNA from Nucleus to Cytosol

(A) Left, immunostaining of anti-dsDNA shows three distinct extranuclear DNA patterns quantified in MLFs: nuclear buds, cytosolic speckles, large extranuclear aggregates; scale bar represents 10 µm. Right, frequencies in Dnase2a+/+ and Dnase2a−/− MLFs, without or with Ara-C treatment (10 µM, 24 hr), based on ten fields at 20×. Values are mean ± SEM. Frequency of normal nuclei is not shown.

(B) Representative images of immunofluorescence staining (anti-NUP98, green; anti-dsDNA, red; scale bar represents 500 nm; N, nucleus) in MLFs. Untreated cell with clear cytoplasm is a Dnase2a+/+ MLF.

(C) Anti-dsDNA (red) and anti-NUP98 (green) staining in MLFs; scale bar represents 10 µm.

(D) MLFs pulsed with BrdU (15 µM for 6 hr) show newly replicated DNA in buds and speckles; scale bar represents 10 µm.

(E) Sequences from time-lapse imaging of GFP-H2B-infected MLFs illustrating nuclear DNA budding (top series) and thread formation/detachment (bottom series); scale bar represents 5 µm; asterisks highlight changes.

See also Figure S3 and Movies S1 and S2.
PCR-based quantitation of mitochondrial DNA (Figure S3B) was not significantly different between Dnase2a+/+ and Dnase2a−/− cells. Only a minor fraction of extranuclear DNA colocalized with mitochondria by immunofluorescence (Figure S3C). To further exclude mitochondria as a source of DNA, we used aphidicolin to inhibit nuclear but not mitochondrial replication (Ikegami et al., 1978) and observed abrogation of the cytosolic DNA accumulation in the presence of Ara-C (Figure S3D). The observations of dsDNA accumulation along with experiments excluding mitochondria indicate that the nucleus is the major source of accumulated DNA in Dnase2a knockout and Ara-C-treated cells.

DNA Is Exported from Nucleus
To study the spatial and temporal dynamics of excess DNA, we infected wild-type and Dnase2a−/− MLFs with a retrovirus carrying GFP-H2B. Although DNA dyes (e.g., Hoechst and Ruby) induce phototoxicity and limit their use for live capture, histone proteins are known to associate tightly with DNA and have been used as surrogate markers for DNA (Kanda et al., 1998). We confirmed the colocalization of GFP-H2B with dsDNA in the cytosol by immunofluorescence (Figure S3E). Time-lapse live imaging of Ara-C-treated cells with a spinning disc confocal microscope revealed several steps of the nuclear DNA export process, including nuclear DNA bud formation and detachment of nuclear DNA from the nucleus into the cytosol (through unusual thread-like structures that are first connected to the nucleus and then fragment in the cytosol) after 2–8 hr of Ara-C treatment (Figure 3E; Movies S1 and S2). These live observations demonstrate that endogenous DNA moves out of the nucleus when cells are under genotoxic stress.

Accumulated DNA Induces Inflammation
The abnormally high level of damaged DNA outside the nucleus has the risk of activating innate immune DNA-sensing pathways that induce cytokine and chemokines. Indeed, Ara-C as well as topoisomerase inhibitors (doxorubicin and etoposide that act through independent mechanisms) not only caused a rise in DNA levels but also upregulated chemokine and cytokine gene expression (including Cxcl10, Ifnb, I6, Tnfα) in a dose-dependent manner in p53−/− MEFs (data not shown). In MLFs, we found that the upregulation of Cxcl10 expression as a result of Ara-C treatment was most pronounced when Dnase2a was absent (Figure 4A). Confirming that the excessive DNA and Cxcl10 levels observed in Dnase2a−/− MLFs were indeed due to lack of degradation by Dnase2a, we successfully rescued the inflammatory and extranuclear DNA phenotypes with inducible Dnase2a expression (Cxcl10, Figure 4B; dsDNA, Figure 4C). Furthermore, the known adaptor Sting/Tmem173 and mediator Tbk1 of the cytosolic DNA-sensing pathway, were expressed at higher levels at knockout compared to wild-type MLFs (Figure 4A), and small interfering RNA (siRNA)-mediated silencing of either mediator ablished the heightened activation observed in Dnase2a-deficient and Ara-C-treated cells (Figure 4D), consistent with loss of arthritis in Dnase2a−/−Sting−/− mice (Ahn et al., 2012). Finally, corroborating the nuclear origin of the accumulated DNA, inhibition of nuclear export by leptomycin B (LMB) almost completely abolished cytokine production (Figure 4E) in MLFs, whereas ISD-induced Cxcl10 expression was only partially reduced (Figure S4B). Thus, exported nuclear DNA that accumulates as a result of a Dnase2a deficiency or induction of DNA damage engages a Sting-dependent cytosolic nucleic acid sensing pathway to induce inflammation.

DNA Localizes with Autophagosomes and Lysosomes
Given that Dnase2a colocalizes with DNA in a LAMP1+ compartment, a key question is how extranuclear DNA is transported to the lysosome for degradation by Dnase2a. We considered the possibility that autophagy, a major mechanism for recycling of cytosolic cargo, would deliver the excess DNA to lysosomes. Indeed, canonical autophagy genes, including Atg5, Atg7, and Beclin1, were significantly upregulated in Dnase2a-deficient MLFs compared with wild-type cells (Figure 5A). The autophagosomal marker LC3-II and the lysosomal protein LAMP1 were also elevated in Dnase2a−/− and Ara-C-treated MLFs (Figures 5B and S5C) and in Dnase2a−/− kidneys (Figure 5D) compared with those in wild-type. LC3 formed the typical punctate cytosolic pattern found during active autophagy.

To further visualize the overlap of autophagosomes and lysosomes with cytosolic DNA in the DNA degradation process, we used antibodies against LC3 and LAMP1 to study their colocalization with dsDNA. In Ara-C-treated wild-type cells, excess DNA colocalized with LC3 and LAMP1, likely reflecting autophagosome-lysosome fusion and the depositing of nuclear DNA into the lysosomal degradation pathway (Figure 5E, top, seen also in untreated wild-type cells [left], but this is a rare event). Confirming our findings, we note that the nucleocytoplasmic distribution of LC3 that we observe has been reported (Drake et al., 2010) and that GFP-LC3 showed similar intensity, punctate pattern, and colocalization with DNA (Figures S5A and S5B). In contrast, in Dnase2a-deficient MLFs, DNA aggregates did not localize with LAMP1 yet remained localized with LC3 (Figure 5E, bottom; also Figure S5C showing enlarged nuclear buds, and reduced DNA/LAMP1 and LC3/LAMP1 colocalization in Figure 5F). We suspect that aborted fusion may be a quality-control mechanism for avoiding lysosomes that lack degradation enzymes and results in accumulation of autophagosomes similar to what is observed in lysosomal storage disorders lacking hydrolases (Settembre et al., 2008).

Requirement for Autophagy and Lysosomes in Autonomous DNA Removal
Based on these data, we predicted that a defect in autophagy, like a deficiency in Dnase2a, would lead to elevated levels of DNA in the cell. We found that Atg5−/− MEFs amassed higher levels of DNA (Figure 6A, Ruby staining; Figure 6B, anti-dsDNA immunostaining), with elevated cytosolic speckles and aggregates (Figure 6C), but lacked colocalization of DNA with lysosomes (Figure 6D). Consistent with a role for autophagy in removing immunostimulatory DNA that could engage cytosolic DNA sensing pathways, basal increase of DNA levels in Atg5−/− MEFS was sufficient to mount an immune response, whereas Ara-C treatment heightened the level of cytokine expression (Figure 6E).

To test the role of the lysosome in DNA clearance, we blocked vesicular fusion to lysosomes using bafilomycin A1 (which
inhibits V-ATPases and lysosomal acidification) and found enhanced accumulation of extranuclear DNA in wild-type MLFs treated with Ara-C (Figure S6). In contrast, the autophagy inducer rapamycin reduced the levels of cytosolic DNA in knockout cells (Figure S6). These data reveal a nuclear-to-autophagosome-to-lysosome transport pathway that targets nuclear DNA for degradation and is upregulated in the context of DNA damage. We note that silencing Atg5 resulted in elevated DNA levels and Cxcl10 expression that were beyond those observed in the Dnase2a<sup>−/−</sup> cells (Figure 6F). The synergistic phenotype of Atg5/Dnase2a deficient cells suggests the existence of additional autophagy-dependent mechanisms of DNA clearance.

Figure 4. Accumulated DNA Activates Sting-Dependent Inflammation
(A) Expression of Cxcl10 mRNA in untreated or Ara-C-treated wild-type and Dnase2a<sup>−/−</sup> MLFs.
(B) Cxcl10 expression of Dnase2a<sup>−/−</sup> MLFs rescued with Dnase2a ORF using a Tet-on vector with doxycycline induction (3 μg/ml, 24 hr); eGFP ORF was used as negative control.
(C) Immunostaining of anti-dsDNA for KO and rescued cells (as in B); scale bar represents 20 μm; right, quantitation of fluorescent signals based on five fields of 20x.
(D) Cxcl10 mRNA levels in Dnase2a<sup>+/+</sup> and Dnase2a<sup>−/−</sup> MLFs transfected with siRNAs targeting Sting or Tbk1, untreated or treated with 10 μM Ara-C for 24 hr.
(E) Cxcl10 expression of KO or rescued MLFs after doxycycline induction, untreated or treated with Ara-C and leptomycin (20 nM, 24 hr) as indicated.
Values are mean ± SEM. Data are representative of two (B–D) or three (A and E) independent experiments. See also Figure S4.
beyond Dnase2a. Importantly, double knockdown of Atg5 with Sting or Tbk1 eliminated the immune response (Figure 6F), consistent with a role for STING in sensing damaged DNA (and its observed association with autophagosomes in the presence of DNA [Saitoh et al., 2009; Watson et al., 2012]).

**DISCUSSION**

An important question in immunology is whether self DNA can trigger the normal viral sensing pathways, and, if it does, what is the source of self DNA. We addressed these questions using Dnase2a−/− mice that develop arthritis and accumulate self DNA. Previous studies show that Dnase2a degrades DNA from ingested apoptotic cells (Kawane et al., 2001) and can even degrade entire genomes autonomously when cells die during development in fly (Bass et al., 2009). In contrast, our study focuses on an unexpected process by which lysosomal Dnase2a autonomously degrades damaged DNA that is exported from the nucleus in living cells (schematic in Figure S7).

In our study, we provide multiple lines of evidence that excess DNA in Dnase2a-deficient cells consists of exported nuclear DNA fragments. An important question is why the presence of extranuclear DNA has not been reported more regularly. We hypothesize that Dnase2a removes the exported DNA rapidly—before it can be visualized—analogous to the historical difficulties in observing apoptotic cells in animals due to their immediate removal by phagocytic cells. The observation of increased buds at the nuclear envelope in Dnase2a-deficient cells may thus indicate a higher rate of damaged DNA generation than removal. Nevertheless, other studies have detected DNA fragments arising from replication in budding yeast (Sogo et al., 2007) and fly (Blumenthal and Clark, 1977), overrepresented dsDNA fragments from chromosomes as free dsDNA molecules in human cells during S phase (Gómez and Antequera, 2008), and release of short DNA fragments into the cytosol prompted by physical (Kawashima et al., 2011) or radiation-induced injury (Pang et al., 2011). Interestingly, unrepaired or irreparable DNA has been found to relocalize to the nuclear periphery (Nagai et al., 2011). Importantly, BCN1-depleted cells and reduced ATG5 or p53 has been strongly implicated in autophagy and the DNA sensing pathway interact requires further studies. A recent report that finds increased radiosensitivity of ATG5 or BCN1-depleted cells and reduced ATG5−/− tumor growth after irradiation (Ko et al., 2014) may be related to our finding that autophagy affects trafficking of damaged DNA. Furthermore, the ATG5 locus has been shown to affect susceptibility to lupus (Delgado-Vega et al., 2010; Graham et al., 2009; Kaiser and Criswell, 2010), suggesting that perhaps the risk allele causes patient cells to amass DNA and induce excessive inflammation.

Testing the in vivo impact of autonomous damaged DNA on inflammation is not feasible at this time because we cannot eliminate the effects of extracellular DNA in animals. However, prior studies of DNA repair gene KO models, in which generation of damaged DNA is elevated, are consistent with our cellular model. For example, ATM−/− cells accumulate cytoplasmic double-strand telomeric DNA in mouse and human cells (Hande et al., 2001), and Atm−/− and p53−/− MEFs show increased basal interferon-stimulated genes (ISGS) (Sugihara et al., 2011). Furthermore, p53 has been strongly implicated in autoimmune suppression, and its overexpression limits arthritis development through STAT-mediated regulation (Park et al., 2013). A more detailed analysis of the excess DNA in Dnase2a−/− cells will be informative for tracing the chromosomal origin of the observed damaged DNA fragments (e.g., to mutation-prone common fragile sites [Ozeri-Galai et al., 2012], or early replication fragile sites [Barlow et al., 2013], regions of increased synthesis at rereplication sites [Green et al., 2010], or nucleosome-free gaps [Gómez and Antequera, 2008]).

We also observed that extranuclear DNA localizes in small speckles and large buds/aggregates and is often associated with the nuclear envelope. Furthermore, these structures colocalized with the autophagy machinery that is required for their clearance. These steps resemble the recently described phenomenon of nucleophagy (by which autophagy removes pieces of the nucleus [Mijaljica et al., 2010]), which is best described in yeast where piecemeal microautophagy of the nucleus pinches and degrades nuclear components in rapidly dividing cells, a process often induced by nutrient deprivation or rapamycin treatment (Krick et al., 2008; Roberts et al., 2003). Other examples include whole-nuclei removal in fungi (Shoji et al., 2010) as well as micronuclei in human cancer cells (Rello-Varona et al., 2012). In humans, nuclear laminopathies (caused by mutations in Lmna, encoding lamin A and C that form the structural support of the nucleus) and envelopathies (caused by mutations in emerin, a transmembrane protein on the inner nuclear membrane binding to lamin) (Dauer and Worman, 2009) show resemblance to our cellular observations—in cells with mutations in lamin A, giant perinuclear autophagosomes, or autolysosomes containing DNA form apparently because of loss of integrity in the nuclear structure and extrusion of damaged nuclei into the cytoplasm (Park et al., 2009). Nucleophagy has not been dissected in detail in mammals, and its exact relationship to our findings is not known (Mijaljica et al., 2010). Finally, a recent study reports that γ-H2AX-positive cytosolic chromatin fragments are processed by autophagy in senescent cells (Ivanov et al., 2013).

Our observation that undigested DNA is not present in lysosomes of Dnase2a KO cells indicates an additional regulatory step controlling the trafficking (Cai et al., 2007) and fusion of autophagosomes to lysosomes (e.g., analogous to lack of accumulated products in lysosomes in the absence of lysosomal hydrolases (Settembre et al., 2008). More importantly, how autophagy and the DNA sensing pathway interact requires further studies. A recent report that finds increased radiosensitivity of ATG5 or BCN1-depleted cells and reduced ATG5−/− tumor growth after irradiation (Ko et al., 2014) may be related to our finding that autophagy affects trafficking of damaged DNA. Furthermore, the ATG5 locus has been shown to affect susceptibility to lupus (Delgado-Vega et al., 2010; Graham et al., 2009; Kaiser and Criswell, 2010), suggesting that perhaps the risk allele causes patient cells to amass DNA and induce excessive inflammation.

Prior studies have shown that ATM and NF-kB are critical in the induction of inflammation by DNA damage ([Hinz et al., 2010; Yang et al., 2011]) and that damaged DNA appears to stimulate the type I IFN response (Brzostek-Racine et al., 2011). Here, we add to these mechanistic studies by showing a requirement for Sting and Tbk1 in the induction of inflammation in response to excess damaged DNA (consistent with Dnase2a−/− Sting−/− mice not developing arthritis [Ahn et al., 2012]). Surprisingly, recent studies have found that DNA damage response (DDR) components Mre11 (Kondo et al., 2013), DNA-PK (Ferguson et al., 2012), and Ku70 (Zhang et al., 2011) (as well extrachromosomal histone H2B [Kobiyama et al., 2010]) are also required for induction of the cytosolic DNA-sensing pathway. Interestingly, some of the DNA repair proteins have also been found to be
Figure 5. Autophagy in Dnase2a Deficiency
(A) Levels of autophagy gene mRNAs in wild-type and Dnase2a−/− MLFs.
(B) Immunostaining with anti-LC3 (red) in Dnase2a+/+ and Dnase2a−/− MLFs showing punctate patterns, DAPI (blue) as counterstain; scale bar represents 10 μm; right, quantitation of LC3 signal.

(legend continued on next page)
cleared by autophagy after DSB repair (Robert et al., 2011), suggesting that DDR proteins may bind the exported DNA fragments we observe and perhaps play a role in their stabilization, transport, and immune sensing. Together, these results point to an interesting yet poorly understood pathway that links damaged DNA, DNA repair proteins, and the innate immune response.

Why are there many nucleases involved in clearance of self DNA? It is likely that they specialize by targeting particular substrates within different subcellular compartments. For example, Dnase2a and Trex1 are both critical nucleases that prevent cells from accumulating self DNA and triggering autoimmunity. Although deficiencies in either nuclease lead to Sting-dependent autoimmunity (Ahn et al., 2012; Gall et al., 2012), Trex1 associates with cytosolic face of the ER and targets single-stranded DNA (ssDNA), whereas Dnase2a sits in the lysosome and degrades dsDNA. Interestingly, a recent study observed upregulation of lysosomes in Trex1−/− cells (Hasan et al., 2013), suggesting that extra DNA may be targeting autophagy/Dnase2a-mediated clearance to compensate for lack of Trex1. Trex1 is able to degrade retroelement ssDNA (Stetson et al., 2008); however, consistent with the role of Dnase2a in degrading dsDNA, our preliminary deep sequencing of cytosolic DNA from Dnase2a−/− cells did not show enrichment of retroelements (e.g., SINE, LINE, LTR sequences). Thus, whereas both nucleases are equally important for homeostasis, their DNA substrates, localization, and impact on disease appear to be distinct.

Finally, some of our findings may help explain the therapeutic effect of DNA-damaging agents in cancer. Such agents have been found to induce antitumor immunity (Galluzzi et al., 2012), and in other studies to activate the IFN-β–Stat1–ISG axis (Brzostek-Racine et al., 2011; Novakova et al., 2010). Our results suggest that the damaged DNA induced by these treatments would be exported from the nucleus and engage innate immune sensing pathways that could modulate tumor immunity.

**EXPERIMENTAL PROCEDURES**

**Mice**

Dnase2a−/− and Mx1-Cre mice, a gift from Dr. Shigekazu Nagata (Kyoto University, Japan), were bred and genotyped to obtain conditional Dnase2aflox/−; Mx1-Cre mice. Wild-type littermates with matched age and sex were selected as controls. Both genotypes were injected intraperitoneally (i.p.) with 1.5 μg/weight (g) of poly I:C three times every other day at 12–16 weeks of age to induce deletion of Dnase2a as described (Kawane et al., 2006). Mice were housed in a specific pathogen-free facility at MGH, and protocols were approved by MGH SRAC in accordance with the institutional animal ethics guidelines.

**Cell Isolation and Culture**

Lungs of mice were finely cut and digested with 1 mg/ml collagenase D (Roche) and 20 U/ml DNase I (Roche). Mouse lung fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM), 15% FBS, 1% penicillin-streptomycin (P/S), NEAA, sodium pyruvate, HEPES, and L-glutamate.

**Immunofluorescence Cell Staining**

Cells were stained with Vybrant DyeCycle Ruby stain (Invitrogen) and Vybrant DyeCycle Cyan stain (Invitrogen) and fixed with 3% paraformaldehyde (PFA). Cells were permeabilized with 0.1% Triton X-100 and stained with antibodies against Dnase2, LAMP1, Atg5, Atg7, and LC3 (Novus Biologicals). Lungs of mice were digested and stained with antibodies against gH2AX, NUP98 (Cell Signaling Technology), LC3 (Novus Biologicals), and biotin-LAMP1 and biotin-LC3 (BDU Biologend). Images were acquired with a Nikon Eclipse Ti-E confocal microscope equipped with a Nikon C1 (photons, 40X AR, 100X) or a Zeiss LSM710 confocal microscope, processed with NIS elements AR 2.30 or Carl Zeiss microimaging software, and analyzed with ImageJ across five to 20 random fields from at least two different experiments. Live-cell imaging was performed on MLFs grown on glass-bottom tissue culture dishes (FluroDish, World Precision Instruments) to reach 60%–80% confluence. Cell images were visualized using a spinning-disk confocal system (UltraView confocal scanner, PerkinElmer) equipped with a Nikon Eclipse TE2000-U microscope and an environmental chamber (37°C, 5% CO2) (Solent Scientific). Images were acquired with a plan APO TIRF Nikon oil immersion objective (60 X 1.45) every 3–5 min for 2–8 hr using Z code stack. The images were processed with Volocity software (Volocity 6, PerkinElmer).

Three-color confocal staining with anti-dsDNA (blue), anti-LC3 (red), and anti-LAMP1 (green) in wild-type and Dnase2a−/− MLFs (arrowheads indicate extranuclear DNA aggregates); scale bar represents 10 μm.

**Quantitative Real-Time PCR**

Cells were infected with puromycin-resistant lentivector vector encoding shRNAs to Dnase2a, Atg5, or control or transfected with 150 nM of siGENOME pool siRNA (Dharmacon) targeting Atg5, Tbk1, Sting, or negative control, using Lipofectamine RNAiMAX (Life Technologies). Knockdown efficiency was confirmed by quantitative real-time PCR.

**Live-Cell Imaging**

Live-cell imaging was performed on MLFs grown on glass-bottom tissue culture dishes (FluroDish, World Precision Instruments) to reach 60%–80% confluence. Cell images were visualized using a spinning-disk confocal system (UltraView confocal scanner, PerkinElmer) equipped with a Nikon Eclipse TE2000-U microscope and an environmental chamber (37°C, 5% CO2) (Solent Scientific). Images were acquired with a plan APO TIRF Nikon oil immersion objective (60 X 1.45) every 3–5 min for 2–8 hr using Z code stack. The images were processed with Volocity software (Volocity 6, PerkinElmer).
Figure 6. Role of Autophagy and Lysosomes in Autonomous DNA Removal

(A) DNA content of Atg5+/+ and Atg5−/− MEFs by Ruby staining.
(B) Immunostaining of anti-dsDNA in Atg5+/+ versus Atg5−/− MEFs with or without Ara-C treatment; scale bar represents 20 µm.
(C) Relative frequencies of bud, speckle, and aggregate patterns in Atg5+/+ and Atg5−/− MEFs.
(D) Anti-LAMP1 (green) and anti-dsDNA (red) dual staining in Atg5−/− MEFs versus Atg5+/+ cells; scale bar represents 2 µm; N, nucleus.
(E) Ratio of Cxcl10 mRNA levels in Atg5+/+ versus Atg5−/− MEFs with or without Ara-C treatment.
(F) Relative Cxcl10 expression in wild-type or Dnase2a−/− MLFs silenced with siRNAs.

All data are results of independent duplicate experiments. Values are mean ± SEM. See also Figure S6.
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Y.Y.L. performed all other experiments, and N.H. supervised the work. M.S.R. performed the computational analysis of DNA deep sequencing and fluorescence imaging. R.B. performed the time-lapse imaging experiment. W.T. and N.H. conceived the project, designed experimental strategies, and wrote the manuscript. D.L. performed, analyzed, and quantified histological six figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cellrep.2014.08.074.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cellrep.2014.08.074.

AUTHOR CONTRIBUTIONS

Y.Y.L. and N.H. conceived the project, designed experimental strategies, and wrote the manuscript. D.L. performed, analyzed, and quantified histological and fluorescence imaging. R.B. performed the time-lapse imaging experiments. M.S.R. performed the computational analysis of DNA deep sequencing and assisted in statistics. All authors contributed to interpretation and discussions. Y.Y.L. performed all other experiments, and N.H. supervised the work.

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Statistical Analyses

All statistical analyses were performed using GraphPad PRISM 4 (GraphPad Software). All values were expressed as mean ± SEM. Samples were analyzed using Student’s t test or as indicated, with *p < 0.05, **p < 0.01, and ***p < 0.001.

REFERENCES


