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Notes and Insights

The deficiency of DNASE1L3 does not affect systemic sclerosis pathogenesis in two inducible murine models of the disease

Systemic Sclerosis (SSc), also known as scleroderma, is a rare autoimmune disease that affects connective tissue and is associated with substantial mortality. The physiopathology of SSc involves a complex interplay between aberrant immune activation, vascular dysfunction, and profibrotic mechanisms. Recent studies have highlighted the significant role of innate immune cells in SSc pathogenesis. In particular, Toll Like Receptor (TLR)-dependent activation of plasmacytoid dendritic cells (pDCs) by endogenous DNA has been shown to induce their production of type I interferon and CXCL4 [1], ultimately contributing to SSc pathogenesis [2]. While the recognition of endogenous DNA is an important trigger of SSc, the mechanisms regulating its levels during SSc remain poorly understood.

The abundance, quality, and immunostimulatory potential of extracellular self-DNA are controlled by extracellular deoxyribonucleases (DNASEs), including DNASE1 and DNASE1 like 3 (DNASE1L3) [3]. While DNASE1 function *in vivo* remains controversial, DNASE1L3 has been demonstrated to prevent the development of systemic autoimmunity in both mice and humans [3]. This is likely attributed to DNASE1L3's capacity to digest naked DNA similar to DNASE1, as well as DNA in nucleosomes, microparticles derived from dying cells, and immune complexes [3]. Autoimmunity induced by *Dnase1l3* deficiency has been linked to the accumulation of extracellular DNA in microparticles, which stimulates endosomal TLR7 and TLR9 in B cells and pDCs [4, 5]. Single nucleotide polymorphisms (SNPs) in *DNASE1L3*, such as the one causing the R206C mutation, have been associated with multiple autoimmune syndromes, including SSc [6, 7]. The R206C mutation impairs DNASE1L3 secretion (8) and SSc patients carrying this SNP were found to exhibit the reduced plasmatic activity of DNASE1L3 and increased abundance of large fragments of circulatory cell-free DNA [9]. Despite the clear genetic association between the SNP in *DNASE1L3* and SSc, the specific function of DNASE1L3 in SSc development remains unknown.

To investigate the impact of DNASE1L3 on SSc pathogenesis, we induced SSc-like disease in both *Dnase1l3* proficient (WT) and deficient (KO) mice using bleomycin (BLM for 4 weeks) or hypochlorous acid (HOCL for 6 weeks). Upon analyzing mouse skin by hematoxylin and eosin staining, we observed the same extent of epidermal thickening induced by BLM and HOCL in both WT and *Dnase1l3* KO mice (Fig. 1A and B). mRNA analysis from the skin revealed no significant effect on the expression of key fibrosis-associated genes, *Col1a1*, *Col1a2*, and *Tgfb1*, by BLM, whereas HOCL treatment significantly upregulated their expression (Fig. 1C–E). However, the induction of pro-fibrotic genes by HOCL was similar between WT and *Dnase1l3 KO* mice (Fig. 1C–E). BLM causes very early on (3-5 days) the expression of profibrotic genes, which is likely why we do not see their expression sustained at late time points (4 weeks). Flow cytometry analysis of skin immune cells showed that HOCL significantly increased the frequency of $CD45^+$ skin immune cells, while BLM did not affect it, regardless of mouse genotype (Fig. S1A). However, the absolute number of $CD45^+$ cells remained similar between WT and *Dnase1l3* KO mice treated with PBS, BLM, and HOCL (Fig. S1B). Additionally, BLM induced an accumulation of inflammatory monocytes ($CD11b^+$, $Ly6G^+$), while HOCL preferentially increased the frequency of granulocytes ($CD11b^+$, $Ly6C^+$) in comparison to mice treated with PBS (Fig. 1F–H). Notably, these observations indicate that BLM and HOCL contribute to skin inflammation, but such skin inflammation was unaffected by *Dnase1l3* deficiency (Fig. 1F–H). Analysis of macrophages, conventional (c)DCs, and pDCs in the skin did not show any impact of BLM and HOCL treatments or mouse genotype on their frequency and activation profile, as assessed by their expression of CD86 (Fig. S1C–E). Overall, these findings demonstrate that BLM and HOCL induce skin inflammation contributing to fibrosis, a process unaffected by *Dnase1l3* deficiency.

Lung fibrosis is common and life-threatening complication of SSc. Therefore, we investigated the impact of *Dnase1l3* deficiency on lung pathology in murine SSc models induced by BLM and HOCL. Picrosirius red staining revealed increased lung collagen deposition in BLM-treated mice, but not in HOCL-treated mice (Fig. 2A and B). *Dnase1l3* deficiency did not alter the extent of lung fibrosis induced by BLM (Fig. 2A and B). BLM treatment also significantly increased lung weight in both WT and *Dnase1l3* KO mice (Fig. 2C), likely due to extracellular matrix accumulation. The lack of impact of HOCL on lung fibrosis in our experiments could be due to the reduced susceptibility of C57BL/6 mice to HOCL treatment compared with the commonly used BALB/c mice. We next analyzed the expression of genes associated with fibrosis only in mice that were treated with BLM. We observed that BLM induced a significant upregulation of *Col1a1* and *Col1a2* and fibronectin 1 (*Fn1*) in WT mice (Fig. 2D). While a similar trend was observed in *Dnase1l3* KO mice, the results were not significant for collagen genes (Fig. 2D). The expres-

Correspondence: Dr. Vanja Sisirak
e-mail: vanja.sisirak@u-bordeaux.fr

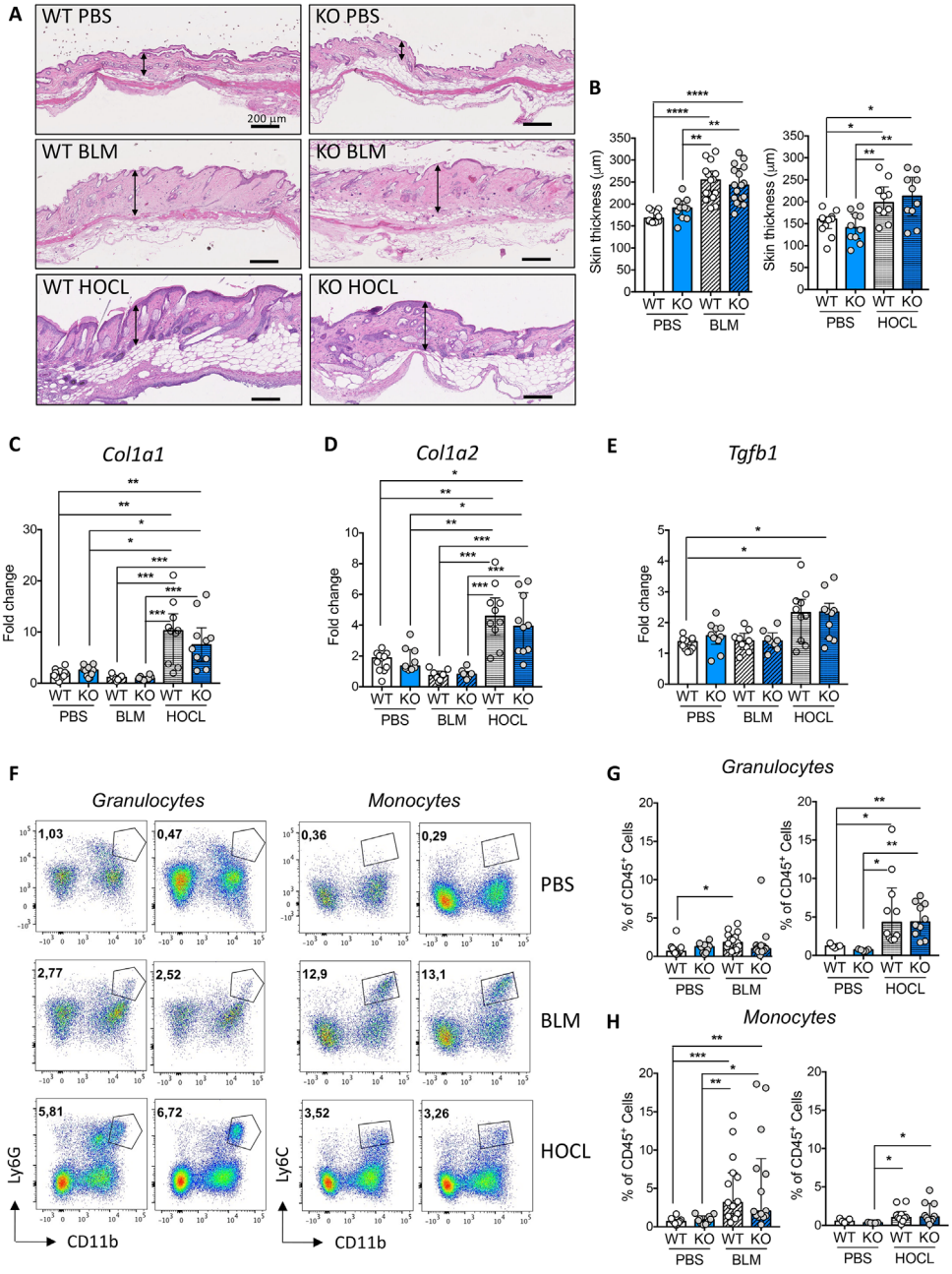

Figure 1. *Dnase1l3* deficiency does not affect skin fibrosis and inflammation in two murine models of SSc. Wild-type (WT) and *Dnase1l3* deficient (KO) mice were subcutaneously injected with PBS, bleomycin (BLM), or hypochlorous acid (HOCL), and their skin was harvested 30 days after BLM treatments or 40 days after HOCL treatment. (A) Representative images of hematoxylin and eosin-stained skin sections obtained from WT and *Dnase1l3* KO mice after the indicated treatments (5× magnification, scale bars are indicated in each image, black arrows indicate the dermis used to quantify skin thickness). (B) Bar graphs representing the median skin thickness in the indicated mice treated with BLM (left panel) and HOCL (right panel). Skin mRNA expression of genes encoding (C) type I collagen α1 (*Col1a1*), (D) type I collagen α2 (*Col1a2*), (E) transforming growth factor β (*Tgfb1*) in the indicated mice. (F) Representative flow cytometry plots of granulocytes identified as $CD11b^+$, $Ly6G^+$ cells and monocytes identified as $CD11b^+$, $Ly6C^+$ cells, among live $CD45^+$ cells in the skin of WT and *Dnase1l3* KO mice after the indicated treatments. Bar graph representing the median percentage of (G) granulocytes and (H) monocytes among live $CD45^+$ cells from the skin of the indicated mice. Individual mice data are presented, with bar graphs representing medians with the interquartile range. Data are representative of two independent experiments (including $n = 15$ mice/group in BLM experiments and $n = 10$ mice/group in HOCL experiments). Statistical significance was evaluated using the nonparametric Kruskal Wallis test followed by a post hoc Dunn multiple comparison test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

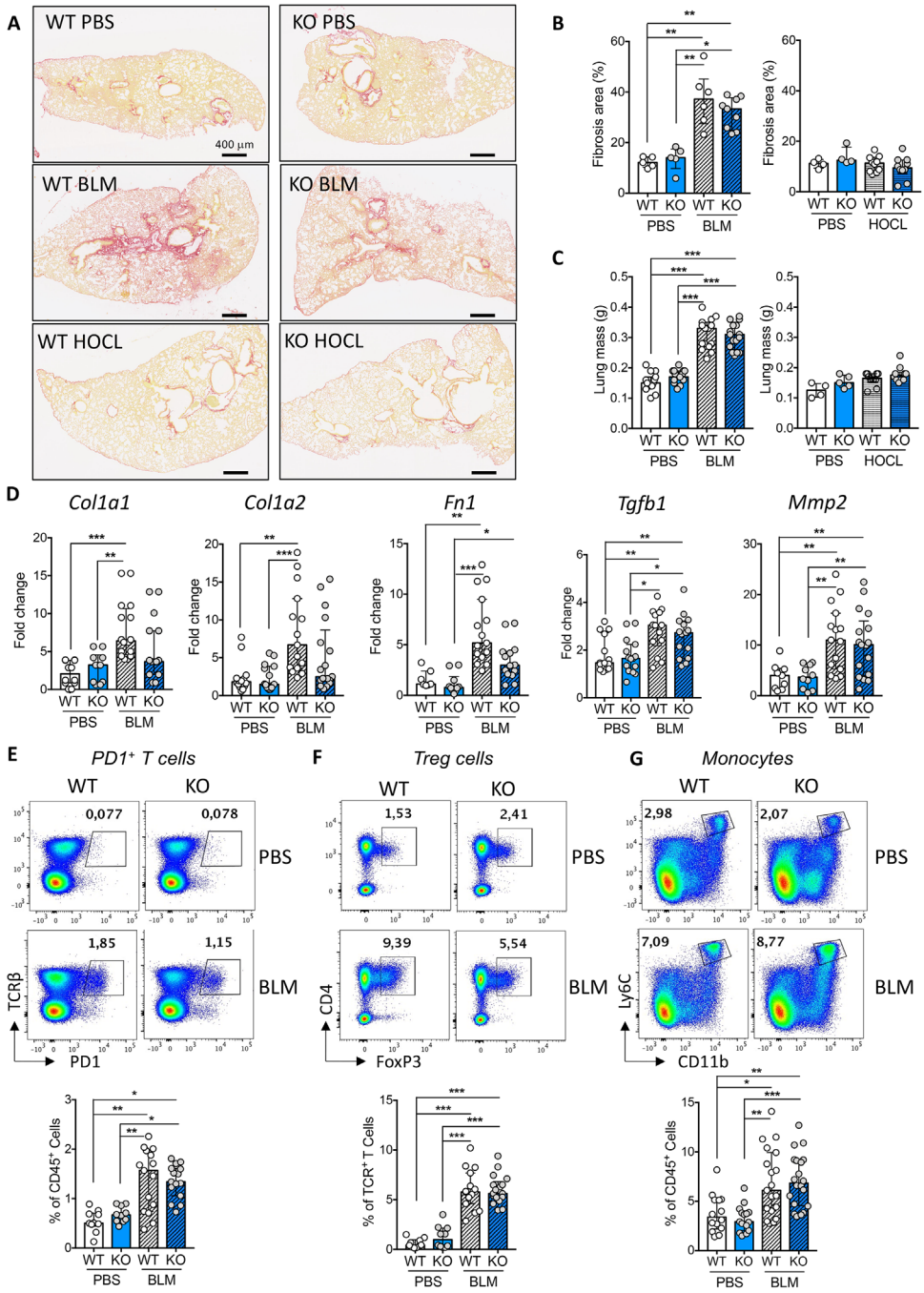

Figure 2. *Dnase1l3* deficiency does not affect lung fibrosis and inflammation induced by BLM. Wild-type (WT) and *Dnase1l3* deficient (KO) mice were injected subcutaneously with PBS, bleomycin (BLM), or hypochlorous acid (HOCL), and their lungs were harvested 30 days after BLM treatments or 40 days after HOCL treatment. (A) Representative images of Picro Sirius Red stained lung sections from WT and *Dnase1l3* KO mice after the indicated treatments (2,5× magnification, scale bars are indicated in each image). (B) Bar graphs showing the median lung fibrosis as quantified by the QuPath integrated tools of the indicated mice. (C) Bar graph depicting the median lung mass in the indicated animals. (D) Lung mRNA expression of genes encoding type I collagen α1 (*Col1a1*), type I collagen α2 (*Col1a2*), fibronectin 1 (*Fn1*), transforming growth factor β (*Tgfb1*), and matrix metalloproteinase-2 (*Mmp2*) in the indicated mice. Representative flow cytometry plots and bar graph representing the median percentage (E) $PD1^+$, $TCR\beta^+$ T cells among live $CD45^+$ cells, (F) $CD4^+$, $FoxP3^+$ regulatory T cells among $TCR\beta^+$ T cells, and (G) $CD11b^+$, $Ly6C^+$ monocytes among live $CD45^+$ cells, in WT and *Dnase1l3* KO mice after the indicated treatments. Individual mice data are presented, with bar graphs representing medians with the interquartile range. Data are representative of two independent experiments (including $n = 15$ mice/group in BLM experiments and $n = 10$ mice/group in HOCL experiments). Statistical significance was evaluated using the nonparametric Kruskal Wallis test followed by a post hoc Dunn multiple comparison test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

sion of *Tgb1* and of the matrix metallopeptidase 2 (*Mmp2*) was however significantly increased in response to BLM treatment in both WT and *Dnase1l3* KO mice (Fig. 2D). To assess tissue inflammation and immune activation we analyzed both innate and adaptive lung immune cells. BLM increased the frequency of PD1 expression in lung infiltrating T cells in both WT and *Dnase1l3 KO* mice (Fig. 2E). This upregulation of PD1 by BLM was detected in CD4, CD8 T cells and most strikingly in regulatory T cells (Treg) (Fig. S2A–D). HOCL treatment did not affect PD1 expression (Fig. S2A–C). Given that $PD1^+$ T cells were previously shown to contribute to lung tissue fibrosis, these results are consistent with our observations showing that only BLM-induced lung fibrosis. Furthermore, BLM treatment led to the expansion of lung Tregs ($CD4^+$, $FoxP3^+$) in both WT and *Dnase1l3* KO mice (Fig. 2F), consistent with their described profibrotic function in SSc-like murine models. However, lung cDCs, pDCs, and their activation profile were unaffected by BLM treatment or mouse genotype (Fig. S2E and F). BLM-induced lung inflammation was evidenced by increased infiltration of inflammatory monocytes, independent of the presence or absence of DNASE1L3 (Fig. 2G). In summary, BLM-induced lung fibrosis, T-cell modifications, and inflammation were not influenced by *Dnase1l3* deficiency.

Collectively, our study indicates that the deficiency of DNASE1L3 does not influence the development of SSc-like disease caused by BLM and HOCL. Conversely, systemic defects in B cells (Fig. S3A–D) and the production of anti-DNA antibodies (Fig. S3E and F) resulting from the deficiency of *Dnase1l3* [4, 5] were not aggravated by the treatment of mice with either BLM or HOCL. Although SNPs affecting DNASE1L3 function have been associated with SSc pathogenesis in humans [6, 7, 9], our findings fail to demonstrate any significant role of DNASE1L3 in SSc-like pathogenesis in mice. These results could be explained by several non-mutually exclusive hypotheses. First, the deficiency of DNASE1L3 might be compensated by other DNASEs, including DNASE1 and DNASE1L2. However, given that these DNASEs display unique expression profile, functions, and substrates, this hypothesis seems unlikely [3]. Additionally, *Dnase1l3* KO mice exhibited an inability to digest nuclear DNA (Fig. S3G) and showed a diminished capacity to digest naked DNA (Fig. S3H), suggesting that the absence of DNASE1L3 is not counteracted by enhanced DNASE1 activity, particularly in digesting naked DNA. Furthermore, the deficiency of *Dnase1l3* did not affect the expression of *Dnase1* and *Dnase1l2* in various tissues (Fig. S3I). Second, impaired DNASE1L3 activity alone may not be sufficient to contribute to SSc pathogenesis. This is supported by the observation that SNPs affecting DNASE1L3 were also detected in healthy individuals [10]. Thirdly, the mouse models of SSc that we utilized may not be the most appropriate for investigating the role of DNASE1L3 in SSc. SNPs in DNASE1L3 were associated with limited cutaneous forms of SSc that are positive for anti-centromere autoantibodies [6, 7]. Typically, anti-centromere autoantibodies-positive SSc patients have a better prognosis, exhibit limited fibrosis, and vascular dysfunction. Currently, there are no animal models, either inducible or genetic, that replicate all these features, particularly the autoreactivity to centromeres, which could be suitable for studying the function of DNASE1L3 in SSc pathogenesis. Lastly, the differential expression profiles between murine and human DNASE1L3 may also explain the lack of impact of *Dnase1l3* KO on SSc development *in vivo*. In humans, *DNASE1L3* is expressed in pDCs, while in mice, *Dnase1l3* is predominantly expressed by cDCs and to a lesser extent by macrophages [5]. Considering that pDCs have been shown to contribute to SSc pathogenesis in both mice and humans through aberrant recognition of endogenous nucleic acids [1, 2], it is tempting to speculate that human DNASE1L3 may exert pDC-intrinsic functions which could play a crucial role in SSc development in humans. Therefore, future studies, particularly in SSc patients with an SNP in *DNASE1L3* will be essential for elucidating DNASE1L3's specific role in SSc pathogenesis.

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Anne Garreau[#1], Pauline Santa[#1], Maxime Dubois[1], Damien Brisou[1], Émeline Levionnois[1], Paôline Laurent[1], Amandine Ferriere[1], Anaïs Roubertie[1], Séverine Loizon[1], Dorothée Duluc[1], Patrick Blanco[1,2], Cécile Contin-Bordes[1,2], Marie-Elise Truchetet[1,2,3] and Vanja Sisirak[1]

[1] CNRS-UMR 5164, ImmunoConcEpT, Bordeaux University, Bordeaux, France
[2] Department of Immunology and Immunogenetics, Bordeaux University Hospital, Bordeaux, France
[3] Department of Rheumatology, Bordeaux University Hospital, Bordeaux, France

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Abbreviations: **BLM**: bleomycin · **cDC**: conventional dendritic cell · **DNASE**: Deoxyribonuclease · **HOCL**: hypochlorous acid · **KO**: knock out · **pDC**: plasmacytoid dendritic cell · **SNP**: single nucleotide poly-

morphism · **SSc**: systemic sclerosis · **TLR**: Toll like receptor

[#]Anne Garreau, Pauline Santa contributed equally to this study.

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