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Graphical abstract

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- cDC activates Cytotoxic T cells

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DNASE1L3 enhances antitumor immunity and suppresses tumor progression in colon cancer

Wenling Li¹,²,⁸, Hideki Nakano³,⁸, Wei Fan¹,², Yuanyuan Li¹, Payel Sil¹, Keiko Nakano³, Fei Zhao³, Peer W. Karmaus³, Sara A. Grimm⁴, Min Shi¹, Xin Xu⁵, Ryushin Mizuta⁶, Daisuke Kitamura⁶, Yisong Wan⁷, Michael B. Fessler³, Donald N. Cook³, Igor Shats²*, Xiaoling Li²*, and Leping Li¹*

¹Biostatistics and Computational Biology Branch, ²Signal Transduction Laboratory, ³Immunity, Inflammation, and Disease Laboratory, ⁴Integrative Bioinformatics Support Group, ⁵Epigenetics and Stem Cell Biology Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA
⁶Division of Cancer Cell Biology, Research Institute for Biomedical Sciences, Tokyo University of Science, Yamazaki 2669, Noda, Chiba 278-0022, Japan
⁷Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, NC 27599, USA
⁸Those authors contributed equally

*Correspondence:
Igor Shats (IS), 111 TW Alexander Dr., RTP, NC 27709, USA. Phone: 1-984-287-3485; Email: igor.shats@nih.gov.
Xiaoling Li (XL), 111 TW Alexander Dr., RTP, NC 27709, USA. Phone: 1-984-287-3484; Email: lix3@niehs.nih.gov.
Leping Li (LL), 111 TW Alexander Dr., RTP, NC 27709, USA. Phone: 1-984-287-3836; Email: li3@niehs.nih.gov.
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Conflict of interest

The authors declare no competing interests.
Abstract

DNASE1L3, an enzyme highly expressed in dendritic cells, is functionally important for regulating autoimmune responses to self-DNA and chromatin. Deficiency of DNASE1L3 leads to development of autoimmune diseases in both humans and mice. However, despite the well-established causal relationship between DNASE1L3 and immunity, little is known about the involvement of DNASE1L3 in regulation of anti-tumor immunity, the foundation of modern anti-tumor immunotherapy. In this study, we identify DNASE1L3 as a new regulator of anti-tumor immunity and a tumor suppressor in colon cancer. In humans, DNASE1L3 is downregulated in tumor-infiltrating dendritic cells, and this downregulation is associated with poor patient prognosis and reduced tumor immune cell infiltration in many cancer types. In mice, Dnase1I3 deficiency in the tumor microenvironment enhances tumor formation and growth in several colon cancer models. Notably, the increased tumor formation and growth in Dnase1I3-deficient mice are associated with impaired anti-tumor immunity, including a substantial reduction of cytotoxic T cells and a unique subset of dendritic cells. Consistently, Dnase1I3-deficient dendritic cells directly modulate cytotoxic T cells in vitro. Collectively, our study unveils a previously unknown link between DNASE1L3 and anti-tumor immunity, and further suggests that restoration of DNASE1L3 activity may represent a potential therapeutic approach for anti-cancer therapy.
Introduction

DNASE1L3, a Ca^{2+}/Mg^{2+}-dependent endonuclease (1), is a secreted protein that under normal physiological conditions efficiently digests dietary DNA within the intestinal tract and chromatin released from dead cells (2-4). DNASE1L3 can also digest DNA encapsulated in microparticles released from cells upon activation, malignant transformation, stress, or death (5).

Several lines of evidence suggest that DNASE1L3 is closely associated with systemic immunity. DNASE1L3 is highly expressed in conventional dendritic cells (cDCs) (6-8) and liver sinusoidal endothelial cells (LSECs) (9), gatekeepers of hepatic immunity (10). It is also moderately expressed in plasmacytoid dendritic cells (pDCs), macrophages, and B cells (8). Deficiency in DNASE1L3 has been associated with development of autoimmune diseases in both humans and mice. Genome-wide association studies have uncovered a link between loss of the functional variant of DNASE1L3 and autoimmune diseases such as systemic lupus erythematosus (SLE) (6, 11). Immunochip analysis also identified DNASE1L3 as one of the susceptibility loci for SLE (12). Moreover, autoantibodies against DNASE1L3 are associated with sporadic SLE (13) and DNASE1L3-deficient patients have elevated DNA levels in plasma (6). Consistently, Dnase1l3 deficiency in mice leads to rapid development of autoantibodies to double-stranded DNA (dsDNA) and chromatin, along with hyperinflammatory phenotypes including a reduced marginal zone B cell population and increased fractions of monocytes and activated T cells, hyperactivation of germinal centers, early expansion of T follicular helper cells, elevated plasmablasts in the spleen, and increased incidence of glomerulonephritis (6, 7). Therefore, DNASE1L3 is functionally important for regulating autoimmune responses to self-DNA and chromatin in both humans and mice (6, 7, 14-17).

Despite these evidence of the causal relationship between DNASE1L3, systemic immunity, and autoimmune response, little is known about the involvement of DNASE1L3 in regulation of anti-
tumor immunity. Herein, we systematically investigated the possible role of DNASE1L3 in mediating the interaction between immune cells and cancer cells in both humans and mice using bioinformatics analyses of the Cancer Genome Atlas (TCGA) datasets and various mouse intestinal/colorectal cancer models. Our study demonstrates that DNASE1L3 is downregulated in tumors and this downregulation is associated with poor patient prognosis in many cancer types in humans. Further, Dnase1l3-deficiency in mice delays tissue recovery after damage, increases chronic inflammation and immune cell dysfunction, impairs antitumor immunity, and enhances tumor progression.

Results

**DNASE1L3 is downregulated in human tumors and its downregulation is associated with poor patient survival**

To evaluate the possible involvement of DNASE1L3 in cancer, we first analyzed RNA-seq datasets from TCGA and found that the mRNA levels of *DNASE1L3* were significantly downregulated in a wide range of human cancer types compared to their adjacent normal tissues (Figure 1A). Importantly, downregulation of *DNASE1L3* was associated with poor patient survival for many cancer types (Figure S1A), including colorectal cancer (CRC) (Figure 1B).

*DNASE1L3* is highly expressed in DCs of both humans and mice (6-8) (Figure S1B). To test whether the observed downregulation of *DNASE1L3* in human tumors is due to a reduced number of *DNASE1L3* expressing cells or due to its intrinsic downregulation in those cells, we analyzed a recent public single-cell gene expression profiling study of the CD45-positive (CD45⁺) myeloid population in human colorectal tumors (18). This analysis confirmed that *DNASE1L3* has the highest expression in cDC1 and cDC2 among myeloid cells in normal human colonic tissues (Figure S1C), and further revealed that its expression in both cDC subtypes, particularly cDC2, was significantly downregulated in human CRC tumors compared
to adjacent normal tissues (Figure 1C and S1C). These results suggest that the observed downregulation of \textit{DNASE1L3} in human tumors is likely due to its reduced expression in cDCs in tumors.

Further bioinformatic analyses revealed that several immune cell markers, including \textit{CCR7}, \textit{CD40LG}, and \textit{CD3G}, were among the most positively correlated genes with \textit{DNASE1L3} in human colorectal tumors (Figure 1D), indicative of a possible association between \textit{DNASE1L3} expression and anti-tumor immune activity. Taken together, our \textit{in-silico} analyses of transcriptomic data from human tumors suggest that downregulation of \textit{DNASE1L3} in cDCs may impair anti-tumor immunity, which in turn contributes to tumor progression.

\textbf{Dnase1l3 deficiency in mice results in impaired tissue recovery after DNA damage}

To test the possibility that DNASE1L3 may function as a tumor suppressor, we decided to leverage a previously generated full body constitutive \textit{Dnase1l3}-deficient (knockout, KO) mouse strain (19) in several models of colorectal cancer. We confirmed that expression of \textit{Dnase1l3} in all segments of the intestine was dramatically reduced in this \textit{Dnase1l3} KO mouse strain (Figure S2A). \textit{Dnase1l3}-deficient mice were phenotypically normal with normal body weight, and small intestine and colon length comparable to their wild-type (WT) counterparts (Figure S2B-D). Histological analysis did not reveal any morphological abnormalities in the colon (Figure S2E). Expression of cell type-specific marker genes in the colon, such as those for stem cells (\textit{Lgr5}), endocrine cells (\textit{Sst}), and goblet cells (\textit{Muc2}), was also comparable between the two genotypes (Figure S2F). These data indicate that the GI tracts of young \textit{Dnase1l3} KO mice show no gross abnormalities at baseline.

Since DNASE1L3 is believed to be a secreted protein (3), we further confirmed the inactivation of this enzyme in KO mice by comparing the ability of serum from \textit{Dnase1l3} WT and KO mice to
digest genomic DNA and chromatin. As expected, serum from *Dnase1l3* WT mice efficiently digested genomic DNA, resulting in DNA smears after 30 minutes of incubation (Figure 2A). In contrast, intact genomic DNA remained visible even after a 1-hour incubation with serum from *Dnase1l3* KO mice. Similarly, DNA ladders were clearly visible when isolated nuclei were incubated with serum from *Dnase1l3* WT mice, whereas serum from *Dnase1l3* KO mice failed to generate DNA ladder from nuclei (Figure 2B). Therefore, serum from *Dnase1l3* KO mice is defective in digesting dsDNA and chromatin DNA in vitro.

*Dnase1l3* deficiency has been shown to hamper the clearance of dsDNA/chromatin in peripheral blood (6, 17). To test the possibility that *Dnase1l3* deficiency may also impair recovery after DNA damage in tissues, we injected *Dnase1l3* KO and WT mice intraperitoneally with a single dose of azoxymethane (AOM) and evaluated tissue damage in the colon at two time points, 9 hours and 5 days (D5), after the injection. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining of the colon showed that similar numbers of TUNEL⁺ foci were induced by AOM at the 9 hours early time point in WT and KO mice (Figure 2C and 2D). TUNEL⁺ foci were mostly resolved at the D5 after AOM injection in WT mice. However, significantly more TUNEL⁺ foci were present in the colon of the *Dnase1l3* KO mice at this late time point (Figure 2C and 2D). Consistent with this observation, a different DNA double-strand break agent, doxorubicin, induced similar degrees of TUNEL⁺ foci in the small intestine at an early time point (12h) in WT and KO mice, but significantly more TUNEL⁺ foci were present in the *Dnase1l3* KO mice at both D3 and D5 after the injection (Figure 2E and 2F). Taken together, these data indicate that *Dnase1l3* KO mice are impaired in intestinal tissue recovery after DNA damage, which may affect cell homeostasis and subsequent tumorigenesis.

*Dnase1l3* deficiency impairs tissue recovery and increases susceptibility to intestinal cancer formation
To directly investigate the possible involvement of DNASE1L3 in suppressing tumorigenesis, we employed a well-established chemically induced colorectal cancer model, AOM/dextran sulfate sodium (AOM/DSS) model (20, 21) in Dnase1l3 WT and KO mice (Figure 3A, top). In WT mice, the expression of Dnase1l3 in the colon was markedly induced upon DSS treatment (D10) and remained high during the whole AOM/DSS procedure (Figure S3A). Consistent with our observations that Dnase1l3 KO mice had significantly more damaged cells in the colon than WT mice after AOM dosing (Figure 2C and 2D), Dnase1l3 KO mice recovered more slowly than WT mice after the first cycle of DSS treatment, as indicated by greater body weight loss (Figure 3A) and higher incidence of bleeding and diarrhea (Figure 3B and S3B). At the recovery phase following the first DSS treatment (D19), Dnase1l3 KO mice also had reduced colon length (Figure S3C), impaired recovery of tissue damage (Figure S3D), and showed splenomegaly as previously reported (6) (Figure S3E). Notably, at the final stage of the tumorigenesis process, Dnase1l3 KO mice had more tumors, which were also larger than those in WT mice (Figure 3C-3E). Histopathological evaluations of the colons from AOM/DSS-treated mice further revealed that Dnase1l3 KO mice had more advanced tumors (adenoma and adenocarcinoma) compared to WT mice, whose tumors had characteristics of atypical hyperplasia (Figure 3F and 3G).

Therefore, Dnase1l3 deficiency impairs recovery after short-term treatment and enhanced tumorigenesis after chronic treatment in the AOM/DSS model of inflammation-induced colorectal carcinogenesis. To further confirm these observations, we bred Dnase1l3-deficient mice to APC<sup>min/+</sup> mice, which develop spontaneous intestinal tumors driven by deregulated β-catenin signaling (22, 23). These animals had more adenomas in the small intestine and colon than littermate APC<sup>min/+</sup> mice (Figure 3H and 3I), despite having normal gross tissue morphology (Figure S4). Collectively, our data from two independent colon cancer models demonstrate that Dnase1l3 deficiency in mice promotes tumorigenesis.
**Dnase1l3 deficiency exacerbates early inflammatory responses but reduces late-stage anti-tumor immunity**

To better understand the mechanisms underlying Dnase1l3 deficiency-induced tumorigenesis, we carried out genome-wide transcriptomic analysis of the whole colon tissue from Dnase1l3 WT and KO mice before AOM/DSS treatment (D0), after treatment with one cycle of AOM/DSS plus 5-day recovery (D19), and 24 days after treatment with three cycles of AOM/DSS (D80) as well as the isolated final tumors (For the experimental timeline, please see Figure 3A). As expected, there were few differentially expressed genes (fold change > 2, adjusted p-value < 0.05) between the two genotypes at baseline (D0) (Table S1). However, during AOM/DSS-induced carcinogenesis, a cluster of 207 genes enriched in pathways of immune and inflammatory responses was dramatically induced in the colon of Dnase1l3 KO mice at D19 (Figure 4A, 4B, and Table S2, the purple cluster, KO). In contrast, the expression of these genes was only modestly induced in the colon of WT mice at the same stage, suggesting that Dnase1l3 deficiency exacerbates an early phase inflammatory response in the colon in response to AOM/DSS treatment. Consistent with these observations, expression of the macrophage and DC markers Adgre1 (F4/80), Nos2 (Inos), Arg2 (Arginase-2), and Itgax (Cd11c), and various inflammatory cytokines was significantly higher in the colon of Dnase1l3 KO mice than those of WT mice, mostly around the 1st cycle of AOM/DSS treatment (D19) (Figure 4C). Moreover, phosphorylation of IκBα, an indicator of canonical NF-κB activation (24) involved in regulating the expression of several pro-inflammatory cytokines, was also higher in the colonic tissue of Dnase1l3 KO mice compared to that in WT mice (Figure 4D). Therefore, the colon of Dnase1l3 KO mice displayed an early phase hyper-inflammatory response when challenged with AOM/DSS.

Interestingly, expression levels of the pro-inflammatory genes that were upregulated in the colon of Dnase1l3 KO mice at earlier timepoints did not significantly differ between the final isolated...
tumors from the two genotypes (Figure 4C, tumor). However, RNA-seq analysis revealed that various genes involved in the type I interferon response (25), including Ddx60, Dhx58, Gbp7, Gvin1, Ifit1bl2, Mx2, Oas2, Oas1, Oasl1, Oasl2, and Slfn4, were significantly reduced in the final tumors isolated from Dnase1l3 KO mice compared to those from WT mice (Figure S5), suggestive of a blunted type I interferon response. In addition, Cd8a, a marker of cytotoxic T cells, was also found among the 32 downregulated genes (Figure S5). Consistently, the numbers of CD3+ or CD8+ foci were significantly reduced in the colonic tissue from Dnase1l3 KO mice at D80 compared to WT mice (Figure 4E). Taken together, these observations indicate that the early phase activation of proinflammatory responses in the colon of Dnase1l3 KO mice is followed by a dysfunctional late phase anti-tumor immune response characterized by reduced type I interferon signaling and reduced number of infiltrating cytotoxic T cells (25, 26).

**Dnase1l3 deficiency promotes tumor progression and impairs activity of cDCs in a syngeneic tumor model**

To further elucidate how an early phase activation of proinflammatory responses leads to a late phase reduction of anti-tumor immunity in the colon of Dnase1l3 KO mice, we established a syngeneic colon tumor model by subcutaneously injecting MC38 murine colon cancer cells into Dnase1l3 WT and KO mice. Dnase1l3 KO mice developed significantly larger tumors after 18-21 days (Figure 5A-5C and S6), indicating that Dnase1l3 deficiency in host cells, but not tumor cells, enhances tumor growth. Given that Dnase1l3 is highly expressed in cDCs (6, 7, 18), these observations further suggest that the transition from the early phase of proinflammatory effect to the late phase of immune-suppressive anti-tumor state in Dnase1l3 KO mice may be mediated by immune cells, particularly cDCs, in the tumor microenvironment.

To test this hypothesis, we carried out single cell RNA-seq (scRNA-seq) analyses of DCs (live CD11c+ CD26+ CD45+ CD88- F4/80- Ly-6G- MHC-II+) in MC38 tumors from Dnase1l3 WT and
KO mice (Figure S7A). Clustering analysis of all sequenced DCs identified 14 clusters, which could be further functionally annotated into two major cDC subtypes, cDC1 and cDC2, and minor contamination of plasmacytoid DCs (pDCs) (Figure 5D, 5E, and Table S3). cDC1 represented a small fraction among cDCs in the tumors from both WT and Dnase1l3 KO mice, and tumors from Dnase1l3 KO mice contained increased proportion of cDC1 compared to those from WT mice (Figure 5D, 5E, and Table S3, Cluster 6, 8, and 13). However, differentially expressed gene (DEG) analysis of all intratumoral cDC1 (combined from above three clusters) showed that cDC1 from Dnase1l3 KO mice displayed only significantly reduced genes compared to those from WT mice (Table S4). Further Gene Ontology (GO) and pathway enrichment analyses revealed that these genes were enriched with gene sets associated with antigen processing and presentation as well as protein translation and expression (Figure S7B). These observations suggest that cDC1 in the tumors from Dnase1l3 KO mice might not be fully functional to activate T cells.

The cluster 3 cDC2 was the most dramatically reduced cDC2 cluster in MC38 tumors from Dnase1l3 KO mice (16.75% of total cells from WT compared to 5.8% of total cells from Dnase1l3 KO mice) (Figure 5D, 5F, and Table S3). These cDC2 displayed unique gene expression profile with low levels of Itgax (Cd11c), cDC1 marker Xcr1, and cDC2 markers including Itgam (Cd11b) and Sirpa (Cd172) (Figure S7C, hence CD11b<sup>lo</sup>CD172<sup>lo</sup>), but moderate to high levels of chemokines and cytokines, such as Ccl5, Ccr7, Cxcl10, Ili205, and S100a4 (Figure 5G). These cDC2 resemble a skin-specific cDC subset called double negative (XCR1<sup>lo</sup>CD11b<sup>lo</sup>) cDC2, although skin CD11b<sup>lo</sup> cDC2 express Sirpa at high level (27). cDC2 in cluster 3 also expressed high levels of MHC class I genes such as H2-D1 and H2-K1 and B2m encoding MHC-I-associating molecule β2 microglobulin (Figure S7D) as well as MHC class II genes such as H2-Aa, H2-Ab1, H2-DMb1, H2-Eb1 and Cd74 (Figure S7E), suggesting their potential role in stimulating CD8<sup>+</sup> and CD4<sup>+</sup> T cells. Moreover, Gene Set Variation Analysis (GSVA) indicated
that the cluster 3 cells displayed the most notable alterations between KO and WT in transcriptional profiles among all clusters (Figure S7F). Specifically, cluster 3 cDC2 isolated from MC38 tumors in Dnase1l3 KO mice had decreased GSVA scores in gene sets associated with proteasome, graft-host interaction, autoimmune diseases, and antigen processing and presentation, compared to those from WT mice (Figure S7F, cDC2, 3). The top downregulated genes included several chemokines/cytokines Ccl5, Ccr7 and Cxcl10 (Figure 5G) and activation markers Marcksl1, H2-DMb2, Cd63, and Cd86 (Figure 5H). These observations indicate that tumors in Dnase1l3 KO mice contain a reduced abundance of a special set of active cDC2 than those from WT mice, and these special cDC2 are potentially important to activate anti-tumor T cells.

Consistent with our observations from scRNA-seq analysis, when characterizing the infiltrating innate immune cells in the dLNs and early tumors (10 days after inoculation) from tumor-bearing mice by flow cytometry analyses (Figure S8A), we found that tumors isolated from Dnase1l3 KO mice had reduced abundance of CD86+CD11b+cDC2 compared to tumors in WT mice (Figure 5I). The intratumoral levels of total cDCs and XCR1+cDC1 were comparable between Dnase1l3 KO and WT mice (Figure S8B-S8C). However, relatively few XCR1+cDC1 in dLNs of KO mice expressed an immune activation marker, PD-L1 (28), compared with their counterparts in WT mice (Figure 5J). These data suggest that the enhanced tumor growth in Dnase1l3-deficient mice might be resulting from impaired DC function and consequent reduction in cytotoxic CD8+ and CD4+ T cells in dLNs and tumors.

**Dnase1l3 deficiency alters cytotoxic T cells in mice and in vitro**

In support of the notion that the enhanced tumor growth in Dnase1l3-deficient mice might be a result of impaired DC-mediated activation of cytotoxic T cells, scRNA-seq analysis of the CD45+ cells in MC38 tumors (Figure S9A) revealed that tumors from KO mice were enriched with
dysregulated cytotoxic T cells compared to those from WT mice (Figure S9B and Table S4).
Among 20 CD45\(^+\) immune cells clusters, cluster 13 (GZMB\(^+\)CD8\(^+\) T cells) displayed the biggest reduction in MC38 tumors from Dnase1l3 KO mice compared to those from WT mice, with 2.63 % of total cells from WT compared to 1.57 % of total cells from Dnase1l3 KO mice (Table S4).
In addition to reduction in cellular abundance, GSVA analysis revealed that the top significantly upregulated gene set in this cluster of GZMB\(^+\)CD8\(^+\) T cells from MC38 tumors in Dnase1l3 KO mice was “Primary immunodeficiency” compared to those from WT mice (Figure S9C, CD8, 13), suggesting that they are also dysfunctional. Further DEG analysis of all three clusters of CD8\(^+\) T cells (Cluster 7, 12, and 13) showed that compared to those in WT mice, CD8\(^+\) T cells from MC38 tumors in Dnase1l3 KO mice had significant reduction in numerous genes involved in regulation of stress response, immune response, protein quality control, and apoptosis/cell death (Figure 6A, Table S6). Particularly, the expression of many genes mediating innate immune function, protein degradation, and programmed cell death, such as lysozyme M Lyz2, ubiquitin Ubb, and two subunit of calprotectin, S100a8 and S100a9 (29), were among the most downregulated genes in CD8\(^+\) T cells from MC38 tumors in Dnase1l3 KO mice (Figure 6A).
However, interestingly, CD8\(^+\) T cells from MC38 tumors in Dnase1l3 KO mice also had significantly higher expression of several genes important in T cell activation, differentiation, and immunodeficiency (Figure S9D and Table S6).

To validate observations from scRNA-seq analysis of intratumoral CD45\(^+\) immune cells, we analyzed infiltrating immune cells in the early tumors and dLNs (10-14 days after inoculation) from tumor-bearing mice by flow cytometry (Figure S10A). Consistent with the scRNA-seq results, MC38 tumors in Dnase1l3 KO mice displayed a significant reduction of GZMB\(^+\)CD8\(^+\)CD44\(^hi\) T cells compared to those from WT mice (Figure 6C), along with a trend of reduction in total CD45\(^+\) immune cells and CD8\(^+\) T cells (Figure S10B). In the dLNs, tumor-bearing Dnase1l3 KO mice had fewer CD8\(^+\) T cells and activated GZMB\(^+\) CD4\(^+\) T cells.
compared to WT mice (Figure 6D and S10C). Subsequent qPCR analysis of CD45+ lymphocytes isolated from the dLNs showed that the expression of many upregulated immune genes in intratumoral T cells identified by scRNA-seq (Figure S9D), were significantly reduced or unchanged (but not upregulated) in immune cells from tumor-bearing Dnase1/3 KO mice (Figure 6E and S10D). Together, these observations indicate that Dnase1/3 deficiency alters cytotoxic T cells in LNs and in the tumor microenvironment.

To directly test whether the observed dysregulation of cytotoxic T cells in Dnase1/3 KO is indeed caused by Dnase1/3 deficiency in DCs, we performed an in vitro cell culture experiment in which DCs isolated from WT or Dnase1/3 KO were co-cultured with untreated or oxaliplatin (Oxa)-treated apoptotic MC38 cells overexpressing ovalbumin (MC38-OVA). Apoptotic MC38-OVA cells were tested because DNASE1L3 is known to digest DNA encapsulated in microparticles released from cells upon stress or death (5, 6). Naive CD8+ T cells isolated from OT-I mice, which can specifically recognize ovalbumin expressed by MC38-OVA cells, were then added to these MC38-OVA “elicited” WT or Dnase1/3 KO DCs to analyze their impacts on the proliferation and activation of naive OT-I CD8+ T cells (Figure 6F). Both WT and Dnase1/3 KO DCs co-cultured with apoptotic (Oxa) MC38-OVA had an enhanced ability to promote the proliferation of OT-I CD8+ T cells compared to those co-cultured with untreated (-) MC38-OVA, as revealed by the marked reduction of the CFSE staining intensities of OT-I CD8+ T cells co-cultured with apoptotic MC38-OVA (Figure 6G, left). However, in both conditions, WT and Dnase1/3 KO DCs displayed comparable abilities (Figure 6G, left). The ability of Dnase1/3 KO DCs to induce the expression of CD44 was also comparable with that of WT DCs in both conditions (Figure 6G, right). Further qPCR analysis revealed that Dnase1/3 KO DCs co-cultured with apoptotic MC38-OVA exhibited a reduced ability to alter the expression of several genes involved in activation and/or modulation of T cells (Figure 6H). In particular, the mRNA levels of Lyz2 and S100a9, two top downregulated genes in CD8+ T cells from syngeneic MC38
tumor in KO mice (Figure 6A and Table S6), were dramatically induced in OT-I CD8\(^+\) T cells co-cultured with WT DCs loaded with the apoptotic MC38-OVA (Figure 6H, Lyz2 and S100a9). This induction was significantly blunted in OT-I CD8\(^+\) T cells co-cultured with KO DCs loaded with the apoptotic MC38-OVA (Figure 6H, Lyz2 and S100a9). Given the importance of calprotectin (S100a8 and S100a9 heterodimer) in induction of autophagy and apoptosis (29, 30), this observation suggested that *Dnase1l3* deficiency in DCs could directly affect the tumor killing ability of cytotoxic T cells. Collectively, our analyses indicate that tumors in *Dnase1l3* KO mice have fewer active CD11b\(^+\)CD172\(^+\) cDC2 compared to *Dnase1l3* WT mice, and this deficiency is associated with dysregulation of cytotoxic T cells in *Dnase1l3* KO tumors. These immune defects in *Dnase1l3* KO mice and DCs may directly contribute to impaired anti-tumor immunity, resulting in increased tumor growth and progression.

**Discussion**

Despite a well-established causal relationship between *Dnase1l3*-deficiency and autoimmune diseases (6, 7, 11, 17, 31, 32), little is known about the link between *Dnase1l3* deficiency, anti-tumor immunity, and tumorigenesis/tumor progression. In the present study, we identified the reduced expression of DNASE1L3 as a potential biomarker for poor prognosis and reduced tumor immune infiltration in multiple human cancers through bioinformatics analyses of TCGA datasets (Figure 1 and S1), which was recently confirmed by another bioinformatics study performed in colorectal cancer (33). We further went beyond these observed associations and provided the first genetic evidence for the tumor suppressor role of DNASE1L3 using *Dnase1l3* KO mice. Importantly, in contrast to recent studies that proposed DNASE1L3 suppresses tumor growth via cancer cell-autonomous mechanisms such as increasing apoptosis, modulating glycolysis, or decreasing migration using epithelial cancer cells with artificially overexpressed DNASE1L3 (33, 34), we demonstrate that Dnase1l3 deficiency in the tumor microenvironment rather than in tumor cells is sufficient to induce tumor growth and impair anti-tumor immunity.
Specifically, we showed that downregulation of DNASE1L3 in cancer, observed by others and in the present study, stems from specific downregulation of this gene in dendritic cells (Figure 1C and S1C), the main cell type that physiologically expresses DNASE1L3. Moreover, in the AOM/DSS colorectal cancer model, knockout of Dnase1l3 exacerbated early-phase inflammation that was associated with diminished type I interferon response, which further led to reduced T cell accumulation and enhanced tumor formation at the late stage (Figure 3 and S5). Our syngeneic MC38 tumor experiments, performed in immunocompetent Dnase1l3 KO mice, further demonstrated that deletion of Dnase1l3 in the recipient microenvironment rather than in the subcutaneously grafted epithelial tumor cells, promotes tumor growth, decreases activation of cDC1 and cDC2, and diminishes the abundance and function of cytotoxic T cells in tumors (Figure 5, 6, and S6).

Intriguingly, in MC38 syngeneic tumor model, a unique CD11bloCD172lo subset of cDC2 (cluster 3 in Figure 6A and 6C) was profoundly reduced in the tumors of Dnase1l3-deficient mice. cDC1 are known to activate cytotoxic CD8+ T cells by their superior ability of cross-presentation, but cDC2 are also able to activate CD8+ T cells in addition to CD4+ T cells (35). It has been recently reported that CD11bloCD172lo cDC2 in normal skin stimulate differentiation of type 2 helper T cells (27). Our study suggests that CD11bloCD172lo cDC2 in tumor might play important roles for activation of CD4+ and CD8+ cytotoxic T cells, and reduction of those cDCs might be reflected by tumor progression in Dnase1l3-deficient mice. It will be of great interest to further investigate the role of Dnase1l3 on cDC function in future studies.

Monocytes and macrophages play an important role in control of tumor growth and progression. The expression of markers of monocytes/macrophages were induced in tumors in both WT and Dnase1l3 deficient mice in the AOM/DSS colorectal cancer model (Fig, 4C), but it appeared that proinflammatory cytokines produced by those cells were not sufficient to suppress tumor
progression in *Dnase1l3* KO mice (Figure 3). GSVA of all genes from scRNA-seq analysis of CD45+ immune cells in MC38 tumors (Figure S9C, mono) suggests that this is possibly due to the functional impairment of these cells after inactivation of *Dnase1l3*. Specifically, among all sequenced cell clusters, monocytes from tumors in *Dnase1l3* KO mice displayed the most significant alterations at the transcriptional level and had decreased GSVA scores in gene sets associated with pathways involved in drug metabolism and DNA repair and recombination (Figure S9C, mono). Intriguingly, these cells also had transcriptional upregulation of genes involved in antigen processing and presentation as well as calcium signaling and membrane potential (LTP) (Figure S9C, mono). Further understanding of DNASE1L3-mediated regulation of monocytes/macrophages will help to elucidate the full picture of the role played by this unique DNASE in anti-tumor immunity.

Our study has several important clinical implications. First, the anti-tumor role of DNASE1L3 in colorectal cancer development uncovered in our study has implication in inflammatory bowel disease (IBD) or colon cancer treatment in the clinic, which suggests that DNASE1L3 protein or a *DNASE1L3* carrying vector may be considered as a potential therapeutic tool to help colon recovery or improve colon cancer therapy. Second, our bioinformatic analysis of cancer patients indicate that DNASE1L3 could be evaluated as a predictive biomarker for risk and prognosis of various human cancers, which is also supported by two recent studies (33, 36).

In summary, our study uncovers a direct involvement of DNASE1L3 in promoting tissue recovery after DNA damages and activation of cDCs and cytotoxic CD8+ T cells in the tumor microenvironment and highlights the importance of this enzyme in activation of anti-tumor immunity and suppression of tumor progression. Our findings further suggest that restoration of DNASE1L3 activity may represent a potential therapeutic approach for anti-cancer therapy.
Methods

Animal experiments

All mice were maintained at the National Institute of Environmental Health Sciences (NIEHS) animal facility under strict specific pathogen-free conditions and housed in micro-isolator static cages (Techniplast, Exton, PA) with autoclaved nesting material (Nestlet; Ancare Corp, Bellmore, NY) and on hardwood bedding (Sani-chips; PJ Murphy, Montville, NJ).

For the chronic inflammation-related colon cancer model (AOM/DSS-colon cancer model), 3-month-old *Dnase1I3* WT (*Dnase1I3*+/+) and KO (*Dnase1I3*−/−) mice on the C57BL/6 background were injected with AOM (8 mg/kg, body weight). One week later, mice were challenged with 2.5% DSS water for 7 days followed by a 14-day recovery with regular drinking water for three cycles. Body weight, rectal bleeding and diarrhea were monitored during the entire experiment. Mice with more than 25% weight loss were removed during the experiment.

Additional humane endpoints for the removal of experimental animals on AOM/DSS model include:

Any three of the clinical signs in Category A or any one clinical sign in category B will result in the immediate euthanasia of that animal. **Category A:** ruffled hair coat, hunched posture, lethargy, weight loss of 20%, rectal prolapse greater than 2 mm but, pale extremities, bloating. **Category B:** weight loss of greater than or equal to 25% that does not improve or stabilize in 24h of close observation, inability to move about the cage, inability to right itself, labored breathing, rectal prolapse greater than 5 mm, rectal bleeding score of 3. Diarrhea, mild rectal prolapse (2 mm or less) and mild rectal bleeding (score of 1 or 2) are expected with this model and will not be used as criteria for removal.
We also worked with NIEHS VMS and established appropriate supplemental care and for the close monitoring of the procedures and animals. For example, during DSS-treatment, some mice were given food mash prepared with DSS-water. During the water rest period, mice were given normal food mash every day.

For the AOM model, 2-month-old *Dnase1l3* WT and KO mice were injected intraperitoneally with AOM (8 mg/kg, body weight). Colon tissues were isolated 9 hours, or five days after injection.

For the doxorubicin model, 2-month-old *Dnase1l3* WT and KO old mice were injected intraperitoneally with doxorubicin (10 mg/kg, body weight). Intestine was isolated 12 h, three days, or five days after the injection.

For the syngeneic MC38 cancer model, we performed analysis at two time points – early and late. For the late time point experiment, 8- to 12-week-old *Dnase1l3* WT and KO mice were injected subcutaneously with $1.0 \times 10^5$ MC38 cells into each flank. Mice were monitored twice weekly for 21 days. Tumor length and width were measured by caliper, and tumor volume was calculated using the formula $V = \text{length} \times \text{width}^2 / 2$. Mice were sacrificed at day 21 after the injection of the tumor cells or when their total tumor volume reached the volume of $2,000 \text{ mm}^3$. The experiments were independently performed five times in both male and female mice by two different researchers during this study, and consistent results were obtained. For the early time point, 8- to 12-week-old female WT and KO mice were the injected subcutaneously with $2.5-5.0 \times 10^5$ MC38 cells into the back. Mice were monitored twice weekly for tumor growth and health status. Mice were sacrificed at day 10 or 14 for the analysis of the immune cell populations in tumors and tumor draining lymph nodes.
The group size in each experiment was designed to provide 80 or 90% power (dependent on different experimental outcomes in different mouse strains) to detect the expected magnitude of changes. Animals were randomly allocated into experimental groups to minimize the potential litter/cage effect. During each procedure, WT and KO animals were also treated and harvested in an alternating order to control possible confounding issues. The experimental participants were not blind of animal genotypes and treatments during all stages of experiments and data analysis.

**RNA extraction and quantitative real-time PCR**

RNA was extracted from tissue using RNeasy Mini kit (Qiagen) according to manufacturer’s protocol. 1µg RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Quantitative real-time PCR was performed using iQ SYBR Green Supermix (Biorad) on CFX96 real time PCR instrument (Bio-Rad). Relative mRNA levels were normalized with Lamin A. Fold difference was determined by 2-\(\Delta\DeltaCT\) method. Sequences of qPCR primers can be found in Table S7.

**RNA-seq analysis of colon tissues and colorectal tumors in mice under the AOM/DSS procedure**

RNA was extracted from the whole colon tissue of *Dnase1l3* WT and KO mice at basal condition (D0), after one cycle of AOM/DSS (D19), after three cycles of AOM/DSS (D80) and from final tumors from *Dnase1l3* WT and KO mice during the AOM/DSS procedure. For each group, three to five samples were used. Comparisons were carried out between the two genotypes for each group. RNA was extracted using RNeasy mini kit (QIAGEN) and was of sufficient quality. Single-ended (75 base pairs) sequencing was carried out in house using Illumina NextSeq 500 to at least 30 million reads per sample. The quality of reads was evaluated using *FastQC*.
(v0.11.5). Reads were aligned using STAR (version 020201) (37). The STAR index was built using mouse genome (mm10, GENCODE release vM25). Transcript expression was quantified using the subread package (38) (version 1.6.2) to generate raw counts for 55,293 genes. Lastly, a variance stabilizing transformation was applied to raw counts from all genes using the DESeq2 (39) software (version 1.30.0) in R to identify differential expressed genes, assuming a negative binomial distribution for count values. A gene was considered differentially expressed when its adjusted $p$-value < 0.05 and fold change > 2.0. For tumor samples, differentially expressed genes were declared with nominal $p$-value < 0.01 (without adjusting for multiple comparisons) and fold change > 2.0. Gene set enrichment analysis was carried out using DAVID (40) and gProfiler (https://biit.cs.ut.ee/gprofiler/gost).

**Single cell RNA sequencing (scRNA-seq) analysis of tumor-associated DCs and CD45+ immune cells**

**Isolation of DCs and T cells for scRNA-seq analysis**

MC38 tumor cell line was subcutaneously injected into mouse flanks ($5 \times 10^5$ each side), and tumors were excised from euthanized mice without skin, lymph nodes or connective tissues 10 days after the injection. Minced tumors were digested with Liberase TM (100 µg/mL) (Roche), Collagenase XI (250 µg/mL), Hyaluronidase (1 mg/mL) and DNase I (200 µg/mL) (Sigma Aldrich) for 30 min at 37 °C (41). The reaction was stopped by the addition of EDTA (20 mM final concentration). A single-cell suspension was prepared by sieving the digested tissue through a 70 µm nylon strainer (BD Biosciences). DCs and T cells were enriched by double gradient centrifugation using 14.5% Nycodenz (Accurate Chemical) (low density for DCs) and Histopaque 1083 (Millipore Sigma) (high density for T cells). Cells were collected from each interface then washed with PBS containing 0.5% bovine serum albumin and 2 mM EDTA.

**Flow cytometric analysis and sorting**
Cells were diluted to 0.5-1×10^6/100 µL and incubated with a non-specific binding blocking reagent cocktail of anti-mouse CD16/CD32 Ab (2.4G2) (10% culture supernatant), 5% normal mouse and 5% rat serum (Jackson ImmunoResearch) (41). For DC analysis, cell surface antigens were stained with following fluorochrome-conjugated Abs obtained from BD Biosciences (BD), BioLegend (BL) or eBioscience/ThermoFisher Scientific (eBio) on ice for 30 min. Biotin-anti-mouse CD3e (145-2C11, BD553060), BUV395-anti-mouse CD11b (M1/70, BD 563553), PerCP-Cy5.5-anti-mouse CD11c (N418, eBio 45-0114-82), biotin-anti-mouse CD19 (6D5, BL115504), BV510-anti-mouse CD45.2 (104, BL 109867), FITC-anti-mouse CD86 (GL1, BD561962), PE-anti-mouse CD88 (20/70, BL 135806), eFluor450-anti-mouse MHC class-II I-A^b (AF6-120.1, eBio 48-5320-82), BV711-anti-mouse PD-L1 (10F.9G2, BL 124319), Alexa Fluor 647-anti-mouse XCR1 (ZET, BL 148214). Biotinylated Abs were followed by PE-Dazzle594-conjugated streptavidin (BL 405247). Stained cells were fixed by 1% paraformaldehyde. For T cell analysis, cell surface antigens were stained with following fluorochrome-conjugated Abs. BUV395-anti-mouse CD4 (RM4-5, BD 740208), BV510-anti-mouse CD8α (53-6.7, BD 563030), biotin-anti-mouse CD44 (1M7, BD 553132), APC-anti-mouse CD62L (MEL-14, eBio 17-0621-81), BV711-anti-mouse PD-1 (29F.1A12, BL 135231), PE-anti-mouse TCR Cβ (H57-597, BD 53172), APC-anti-mouse CD62L (MEL-14, eBio 17-0621-81). BV711-rat IgG2a (RTK2758, BL 400551) and FITC-rat IgG2a (R35-95, BD 554688). Dead cells stained with eFluor780-conjugated Live/Dead dye (eBio 65-0865-18). Biotinylated Abs were followed by PerCP-Cy5.5-conjugated streptavidin (BD 551419). For staining intracellular proteins in T cells, cells were fixed and permeabilized using a kit (eBio 00-5521) according to manufacturer instruction then stained with eFluor450-anti-mouse Foxp3 (FJK-16s, eBio 48-5773-80), and FITC-anti-mouse granzyme B (GZMB) (QA16A02, BL 372205). Stained cells were analyzed on LSR-Fortessa flow cytometer (BD), and the data analyzed using FACS Diva (BD) and FlowJo (Treestar) software. Only single cells were analyzed, and dead cells were excluded from analysis. Gating
strategies used for differentiating the immune cell populations are shown in Table S8 and all antibodies used in this study can be found in Table S9.

**Single cell RNA sequencing of tumor-associated DCs and CD45+ immune cells**

Tumor associated DCs and total leukocytes were isolated and enriched as described above. Cell surface antigens were stained with following fluorochrome-conjugated Abs together with eFluor780-conjugated Live/Dead dye (ThermoFisher Scientific). PerCP-Cy5.5-anti-mouse CD11c (N418, eBio 45-0114-82), APC-anti-mouse CD26 (H194-112, BL 137807), BV711-anti-mouse CD45.2 (104, BL 137807), PE-anti-mouse CD88 (20/70, BL 135806), PE-Dazzle594-anti-mouse F4/80 (BM8, BL 123146), eFluor450-anti-mouse MHC class-II I-A\(^{b}\) (AF6-120.1, eBio 48-5320-82), BV510-anti-mouse Ly-6G (1A8, BL 127633). Among stained cells, cDCs (CD11c\(^+\) CD26\(^+\) CD45.2\(^+\) CD88\(^-\) F4/80\(^-\) I-A\(^{b}\) Ly-6G\(^-\) Live/Dead\(^-\)) or leukocytes (CD45.2\(^+\) Live/Dead\(^+\)) were purified using a cell sorter FACS ARIA-II (BD Biosciences). The cells were counted and examined for viability using a TC-20 cell counter (Bio-Rad). About 8000-9900 live cells at 2×10\(^5\) cells/ml concentration with 70% or higher viability were loaded into the Single Cell Chip followed by forming single cell emulsion in Chromium Single Cell Controller (10x Genomics, Chromium Single Cell 3’ Library & Gel Bead Kit v3.1). The mRNA reverse transcription, cDNA generation and amplification, and single cell gene expression library construction were carried out according to the protocols provided by the manufacture. The libraries were then sequenced by the NIEHS Epigenomics and DNA Sequencing Core Laboratory on NovaSeq 6000 (Illumina) with paired-end sequencing (Read 1: 28; Read 2: 90). A total of 8.7×10\(^8\) reads were obtained for the four samples.

**Single-cell RNA-seq data processing**

Raw sequencing data were demultiplexed, aligned, and counted with Cell Ranger pipelines (10x Genomics). First, we used cellranger `mkfastq` command to generate FASTQ files. Then, we extracted expression data at a single-cell resolution using cellranger command `count`. Lastly, we used cellranger `aggr` to combine sequencing data from multiple libraries with mapped
sequencing depth. We obtained four expression files for cDC and CD45\(^+\) populations isolated from Dnase113 KO and WT mice.

**Clustering of scRNA-seq data**

R package Seurat 4.0 (42) was used for gene and cell filtering, expression normalization, principle component analysis (PCA), variable gene finding, clustering analysis, and UMAP analysis. Analyses were performed with default parameters unless specified otherwise. First, the four gene-by-cell expression data matrices from the aggregated library were imported separately to create four Seurat objects. Based on visual inspection, we filtered cells using the following criteria: 1) expressing < 200 or > 5,000 genes; 2) cell counts > 25,000; and 3) if proportions of mitochondrial gene expression in cells were larger than 10%. Cells with more than 10% of mitochondrial genes were also filtered out. Data were then log-transformed \([\log_2(TPM+1)]\) for subsequent analysis. PCA was performed for dimension reduction using the top 2,000 most variable features. We then did a cell cycle analysis to further filter out cell uninteresting variations. We acquired both S phase genes and G2/M phase genes and assigned cell cycle scores by using the CellCycleScoring function. We subtracted (“regress out”) this source of heterogeneity from the data by using ScaleData function with variables to regress using both calculated S and G2/M cell cycle scores. To combine WT and KO datasets together, we first selected features that were repeatedly variable across WT and KO datasets for cDCs and CD45\(^+\) cells separately, using SelectIntegrationFeatures function. We then identified anchors using the FindIntegrationAnchors function, which takes both WT and KO Seurat objects as input, and use these anchors to integrate the two datasets together with IntegrateData function. We run the standard workflow for the visualization and clustering as follows: 1) Used ScaleData function on the integrated data; 2) performed PCA analysis using the RunPCA function with 30 PCs; 3) used RunUMAP with PCA reduction with the first 30 PCs; 4) calculated nearest neighbors by using the FindNeighbors function with the selected 30 PCs; and 5) identified clusters for each dataset by using the FindClusters function with resolution of 0.5. For
cluster analysis, *FindNeighbors* function with the first 10 principal components was used for clustering analysis. We also performed differential gene expression analyses using Student’s T test (*t.test* function in R) between the WT and KO cells in each cluster. We then adjusted for multiple testing by False Discovery Rate (FDR) method (*p.adjust* function with ‘fdr’ in R). Marker genes’ expression levels in different conditions were visualized with the R package *ggplot2* violin plots.

**Gene set variability analysis (GSVA) of scRNA-seq datasets**

GSVA was performed using the GSVA R package (43) in R3.6.1 on gene expression counts from tumor-associated DCs or CD45+ immune cells isolated from WT and *Dnase1L3*–/– mice without background genes. Gene sets were sourced from MSigDB 7.2 (www.gsea-msigdb.org) and TRANSFAC 7.0. We used output from GSVA to fit a linear model using the “lmFit” function and used the “eBayes” function from the limma R package (44) to compute moderated t-statistics, moderated F-statistic, and log-odds of differential expression by empirical Bayes moderation. Resulting p-values were corrected by Benjamini-Hochberg (BH) for multiple comparisons.

**In vitro OT-I CD8+ T cell proliferation and activation induced by MC38-OVA loaded WT or Dnase1L3 KO DCs**

MC38 cells with stable expression of ovalbumin (MC38-OVA) were cultured in regular complete DMEM medium, then treated with or without 400 µM Oxaliplatin for 24 hours to induce apoptosis. Spleens from WT or *Dnase1L3* KO mice were digested and splenocytes were purified by gradient centrifugation with 14.5% Nycodenz. WT or *Dnase1L3* KO DCs were then enriched from purified splenocytes using MACS bead-conjugated anti-CD11c and AutoMACS (posse1-s). Regular MC38-OVA cells and oxaliplatin-treated apoptotic MC38-OVA cells (1x10^6) were washed and mixed with 1x10^6 WT or *Dnase1L3* KO DCs, incubated for 4 hours, then MC38-OVA and DCs culture were irradiated with 30 Gy γ-ray to stop the growth of MC38-OVA cells and to
minimize DC numbers in the subsequent T cell analysis. DCs were washed and resuspended in cRPMI-10 medium at 5x10^5/ml.

Naive CD8^+ T cells were purified from LNs, MLNs, and spleens from naive OT-I mice using Ab cocktail and AutoMACS (depl 025). Naive CD8^+ T cells were then labeled with 3 µM CFSE at 37^oC for 15 min, then resuspend in cRPMI-10 at 1x10^6/ml. Finally, 1x10^5 CFSE-labeled OT-I CD8^+ T cells (100 µl) and 5x10^4 DCs co-cultured with apoptotic MC38-OVA from above steps (100 µl) were added into 96-well U-bottom plate, incubated at 37^oC incubator for 48 hours. Final co-incubated OT-I CD8^+ T cells were stained with CD44 antibody and Live/Dead dye and analyzed by FACS, or directly subjected to qPCR analysis.

Statistics
Values are expressed as mean ± standard error of mean (SEM) from at least three independent experiments or biological replicates. Significant differences between the means with two comparison groups were analyzed by the two-tailed, unpaired, non-parametric Mann-Whitney test (sample size ≥4) or student’s t-test (sample size <4) (45). Significant differences between the means with more than two comparison groups were analyzed by two-way ANOVA with adjustments for multiple comparisons using Tukey’s HSD. For all comparisons, differences were considered significant at p<0.05. P-value less than 0.05, 0.01, 0.001 and 0.0001 were denoted as *, **, ***, ****, respectively. For qPCR analysis in Figure 4C, outliers that fall below Q1 – 3.0*IQR or above Q3 + 3.0*IQR were removed. No samples were excluded from other data analyses.

Data processing and analysis of RNA-seq and scRNA-seq data were described in “RNA-seq analysis of colon tissues and colorectal tumors in mice under the AOM/DSS procedure” and
“Single cell RNA sequencing analysis of tumor-associated DCs and CD45+ immune cells” sections, respectively.

Software and algorithms used in this study for data analysis are included in Table S10.

**Other methods are described in Supplementary Methods.**

**Study approval**

All animal experiments were designed and conducted in accordance with guidelines of NIEHS/NIH Animal Care and Use Committee under the Animal Study Proposal number ASP2018-0018.

**Data availability**

The Gene Expression Omnibus accession number for the bulk RNA-seq dataset generated with colonic tissues or tumors from WT and *Dnase1l3* KO mice under the AOM/DSS procedure at different time points is GSE196077.

The Gene Expression Omnibus accession number for scRNA-seq datasets generated with tumor-associated DCs or CD45+ immune cells from MC38 tumors on WT and *Dnase1l3* KO mice is GSE196054.

Public available TCGA database used in this study is available from [https://gdac.broadinstitute.org/](https://gdac.broadinstitute.org/).

Additional information about DEGs and gene clusters in the bulk RNA-seq dataset included in Table S1 and S2.

The scRNA-seq results on immune cell clusters and DEGs are in Table S3 to S6.
All oligos used in the study are listed in Table S7.

The gating strategies for FACS analysis of the immune cell populations are listed in Table S8.

All antibodies used in the study are listed in Table S9.

Software and algorithms used in this study for data analysis are included in Table S10.

The raw data of all figures are included in the “Supporting data values” file.

Unedited immunoblots for Figure 4D is provided as a supplementary file.
Author contributions

LL conceived the project, designed the study. WL carried out most of the experiments with help from PS, WF, FZ, PK. HN and KN performed the single cell RNA-seq, the flow cytometry analysis, and the in vitro co-culture experiment with help from XX and WF. YL, MS, LL, WL, and SG performed data analysis. XL, IS, DC, MF, YW guided, designed, and coordinated the study. DK and RM provided Dnase1l3 KO mice. WL, XL, and LL wrote the manuscript with input from all co-authors.

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References


Figure legends

Figure 1. DNASE1L3 is downregulated in human tumors and its downregulation is associated with poor patient survival.

(A) The mRNA levels of DNASE1L3 are downregulated in multiple types of human cancer. Violin plots of relative DNASE1L3 expression in paired tumor vs normal tissue for 17 human tumor types based on RNA-seq datasets from TCGA. BLCA: Bladder Urothelial Carcinoma; BRCA: Bladder Urothelial Carcinoma; CHOL: Cholangiocarcinoma; COAD: Cholangiocarcinoma; ESCA: Esophageal carcinoma; HNSC: Head and Neck squamous cell carcinoma; KICH: Kidney Chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; STAD: Stomach adenocarcinoma; THCA: Thyroid carcinoma; UCEC: Uterine Corpus Endometrial Carcinoma. (B) High expression of DNASE1L3 is associated with increased survival in CRC patients. Kaplan-Meier survival probability curves for the TCGA colorectal patients (n=597). Survival of the patients with DNASE1L3 expression level in tumors above the mean are shown in red (favorable) and of those with DNASE1L3 expression level in tumors at or below the mean are shown in blue (unfavorable). P-value was computed based on the log-rank test of the survival distributions of high and low expression groups. (C) DNASE1L3 is downregulated in cDC1 and cDC2 cells from tumors of CRC patients compared to those from normal colorectal tissues. Single-cell RNA-seq datasets of human colorectal tumors (18) were analyzed (n=10 for normal cDC1, 36 for tumor cDC1, 36 for normal cDC2, and 64 for tumor cDC2; Mann-Whitney test, *p<0.05). (D) The expression of DNASE1L3 is positively correlated with that of several immune activation markers, particularly T cell activation markers, in CRC patients. The mRNA levels of DNASE1L3 and those of selected immune activation markers from TCGA colorectal tumor RNA-seq data were analyzed (n=286; Pearson product-moment correlation test).
Figure 2. *Dnase1l3* KO mice display impaired recovery after DNA damage in vitro and in vivo.

(A) Serum from *Dnase1l3* KO mice has a reduced ability to digest genomic DNA in vitro. 500ng genomic DNA was incubated with 5 µl serum isolated from *Dnase1l3* WT and KO mice, respectively. (B) Serum from *Dnase1l3* KO mice has a reduced ability to digest isolated nuclei. 1 x 10⁶ nuclei were incubated with 15 µl serum isolated from *Dnase1l3* WT and KO mice, respectively, for two hours. (C-D) *Dnase1l3* KO mice have a reduced ability to recover in the colon after AOM treatment. 2-month-old female *Dnase1l3* WT and KO mice were injected with AOM (8 mg/kg) and colonic tissues were isolated 9 h, or 5 days after the injection. Colonic tissue sections were stained by TUNEL. (C) Representative images of TUNEL staining of the damaged cell in the colon of *Dnase1l3* WT and KO mice. (D) Quantification of percentages of TUNEL positive signals in C (n=4 WT and 4 KO as basal controls; n=5 WT and 6 KO at 9 h; n=6 WT and 7 KO at D5; Mann-Whitney test, *p<0.05*). (E-F) *Dnase1l3* KO mice have a reduced ability to recover in the small intestine after doxorubicin treatment. 2-month-old female *Dnase1l3* WT and KO mice were injected with doxorubicin (10 mg/kg) and intestine tissues were isolated 12 h, 3 days, and 5 days after the injection. (E) Representative images of TUNEL staining of the damaged cells in the small intestine of *Dnase1l3* WT and KO mice. (F) Quantification of the percentage of TUNEL-positive cells in E (n=4 WT and 4 KO as basal controls; n=4 WT and 5 KO at 12 h; n=6 WT and 6 KO at D3; n=5 WT and 6 KO at D5; Mann-Whitney test, *p<0.05*).

Figure 3. *Dnase1l3* deficiency enhances colon cancer formation in mice.

(A) *Dnase1l3* KO mice have increased body weight loss during AOM/DSS treatment. *Dnase1l3* WT and KO mice were subjected to AOM/DSS colorectal cancer procedure with 3 cycles of DSS treatment (peak) as described in Methods (n=15 WT and 15 KO initial mice.
from one experiment, it was independently repeated and similar result was obtained; Multiple Mann-Whitney tests, *p<0.05. (B) More Dnase1l3 KO mice developed severe bleeding and diarrhea than WT mice during the AOM/DSS colorectal cancer procedure (n=30 WT and 27 KO from two independent experiments). (C) Representative images indicating that Dnase1l3 KO mice have higher tumor burden than Dnase1l3 WT mice when treated with three cycles of AOM/DSS. Bar, 1 cm. (D) Dnase1l3 KO mice have increased tumor number in the colon after the AOM/DSS colorectal cancer procedure (n=36 WT and 29 KO; Mann-Whitney test, ****p<0.0001). (E) Dnase1l3 KO mice have increased colon tumor burden. Tumor burden was quantified by Fiji (n=24 WT and 25 KO; Mann-Whitney test, ***p<0.001). (F) Representative H&E colonic images of Dnase1l3 WT and KO mice in the AOM/DSS colorectal cancer model. (G) Colorectal tumors developed in Dnase1l3 KO mice are at more advanced stages compared to those in WT mice in the AOM/DSS colorectal cancer model. H&E colonic sections from WT and Dnase1l3 KO mice (n=15 WT and 13 KO) were evaluated and scored by a Board-certified veterinary pathologist. (H) Representative images of intestinal adenoma from WT;APC^{min/+} and KO;APC^{min/+}. (I) More Dnase1l3 KO mice developed adenoma in the small intestine and colon than WT mice in the APC^{min/+} model (n=15 WT;APC^{min/+} and 21 KO;APC^{min/+}).

Figure 4. Dnase1l3 deficiency enhances early-stage inflammation yet reducing late-stage colonic tissue accumulation of T cells in mice.

(A-B) Colonic tissues of Dnase1l3 KO mice display increased inflammation during the early stage of AOM/DSS-induced carcinogenesis. Transcriptomes from colonic tissues of WT and Dnase1l3 KO mice at D0, D19, and D80 of the AOM/DSS procedure were analyzed by RNA-seq as described in Methods. (A) The dynamics of four gene clusters are shown as a heatmap. (B) Enriched pathways of the 207 genes shown in the purple cluster. GO, Gene
Ontology; REAC, Reactome Pathway Database; KEGG, Kyoto Encyclopedia of Genes and Genome. (C) Dnase1l3 KO mice have increased expression of various pro-inflammatory markers at different time points of the AOM/DSS-induced carcinogenesis when analyzed by qPCR (n=4, 5, 6, 10, 6, 12, 5 WT and 4, 6, 7, 6, 6, 12, 10, 2 KO at each time point, respectively; outliers that fall below Q1 − 3.0*IQR or above Q3 + 3.0*IQR were removed; Mann-Whitney test, *p<0.05, **p<0.01, ***p<0.001). (D) Colonic tissues of Dnase1l3 KO mice display increased NF-κB signaling. Western blotting of phospho-IκBα in the colonic tissue samples of the Dnase1l3 WT and KO mice at D19 in the AOM/DSS procedure. (E) Dnase1l3 KO mice have reduced colonic tissue accumulation of T cells at the end stage of the AOM/DSS procedure. Left, representative images for CD3 (upper panels) or CD8 (bottom panels) IHC staining in colorectal tissues from Dnase1l3 WT and KO mice after the AOM/DSS colorectal cancer procedure. Right, the percentage of CD3 immunostaining positive areas and CD8 immunostaining positive areas were quantified from the IHC colonic tissue sections by Fiji (n=10 WT and 12 KO; Mann-Whitney test, *p<0.05).

Figure 5. Dnase1l3 deficiency promotes tumor progression and impairs activity of cDCs in a syngeneic tumor model.

(A) Dnase1l3 deficiency increases the growth of MC38 tumors. 1 x 10^5 MC38 colon cancer cells were subcutaneously injected into 8-12 week-old immunocompetent WT or Dnase1l3 KO mice (n=10 WT and 11 KO; Mann-Whitney test, *p<0.05). (B) Representative images of final dissected tumors. (C) Weights of final MC38 tumors (n=30 tumors from WT and 39 tumors from KO from three independent experiments; Mann-Whitney test, *p<0.05). (D) UMAP analysis of cDC single cells sampled from MC38 tumors from Dnase1l3 WT and KO mice (analyzed by scRNA-seq). (E) DCs from MC38 tumors in Dnase1l3 KO mice have reduced cDC2 but increased cDC1 populations. (F) The relative abundance of different subgroups of cDC2 cells. (G) Dnase1l3 deficient cDC2 cells in cluster 3 have reduced expression of chemokines and
cytokines (Mann-Whitney test, *p<0.05, **p<0.001). (H) Dnase1l3 deficient cDC2 cells in cluster 3 have reduced expression of various activation marker genes (Mann-Whitney test, **p<0.01, ***p<0.001, ****p<0.0001). (I) Dnase1l3 KO mice have reduced abundance of CD86⁺CD11b⁺ cDCs in the MC38 tumors at an early stage of tumor development (10 days after inoculation) (n=21 WT and 21 KO from three independent experiments; *p<0.05).

Representative FACS plots from WT and KO mice are shown. Biological replicates were combined in the analysis using a linear regression model to remove the batch effects (see Methods). (J) Dnase1l3 KO mice have reduced abundance of activated cDCs in tumor draining lymph nodes (dLNs) at an early stage of tumor development (10 days after inoculation).

Indicated immune cell population in dLNs was analyzed by flow cytometry (n=16 WT and 16 KO from two independent experiments). Biological replicates were combined in the analysis using a linear regression model to remove the batch effects (see Methods).

**Figure 6. Dnase1l3 deficiency alters cytotoxic T cells and impairs anti-tumor immunity.** (A-B) Top downregulated genes (Log2FC<-0.4) in CD8⁺ T cells from MC38 tumors in Dnase1l3 KO mice were analyzed for pathway enrichment (*padj<0.05, **padj<0.01, ***padj<0.001, ****padj<0.0001). (C) Dnase1l3 KO mice have reduced abundance of activated T cells in MC38 tumors 14 days after inoculation. Indicated immune cell populations in isolated tumors were analyzed by flow cytometry (n=8 WT and 8 KO mice, Student’s t-test, *p<0.05). (D) Dnase1l3 KO mice have reduced abundance of activated T cells in tumor draining lymph nodes (dLNs) 10 days after inoculation (analyzed by flow cytometry, n=16 WT and 16 KO from two independent experiments; Mann-Whitney test, *p<0.05, **p<0.01). (E) Dnase1l3 KO mice have reduced expression of several immune cell genes in CD45⁺ immune cells isolated from tumor draining lymph nodes (dLNs) 14 days after inoculation (analyzed by qPCR, n=5 WT and 5 KO mice; Mann-Whitney test, *p<0.05, **p<0.01). (F) Schematic representation of in vitro co-culture experiment using OT-I CD8⁺ T cells and WT or Dnase1l3 KO DCs in the presence of regular or
apoptotic MC38-OVA cells. Apoptosis of MC38-OVA was induced by treatment with 400 μM oxaliplatin (Oxa) for 24 hours. (G) OT-I CD8⁺ T cells have comparable proliferation and CD44 activation when co-cultured with apoptotic MC38 cells and WT or Dnase1/l3 KO DCs. The mean fluorescence intensity (MFI) of CFSE dye (for cell proliferation) and the MFI of CD44 in OT-I CD8⁺ T cells were analyzed by FACS (n=3 independent experiments, two-way ANOVA test with correction for multiple comparisons, ***p<0.001). (H) OT-I CD8⁺ T cells co-incubated with Dnase1/l3 KO DCs in the presence of apoptotic MC38 cells have reduced expression of some key immune cell markers (analyze by qPCR, n=3 replicates, two-way ANOVA test with correction of multiple comparisons, *p<0.05, ***p<0.001).
Supplementary Tables

Table S1. DEG lists in the colons of WT and Dnase1l3 KO mice at different time points in the AOM/DSS colorectal model.

Table S2. Gene clusters in the colons of WT and Dnase1l3 KO mice at different time points in the AOM/DSS colorectal model.

Table S3. cDC clusters in MC38 tumors from WT and Dnase1l3 KO mice.

Table S4. List of DEGs in cDC1 identified by scRNA-seq analysis of the infiltrating cDCs from MC38 tumors.

Table S5. CD45+ immune cell clusters in MC38 tumors from WT and Dnase1l3 KO mice.

Table S6. List of DEGs in CT8+ T cells identified by scRNA-seq analysis of the CD45+ immune infiltrating cells from MC38 tumors.

Table S7. List of oligonucleotides used in this study.

Table S8. List of antibodies used in this study.
Figure 1. *DNASE1L3* is downregulated in human tumors and its downregulation is associated with poor patient survival.

(A) The mRNA levels of *DNASE1L3* are downregulated in multiple types of human cancer. Violin plots of relative *DNASE1L3* expression in paired tumor vs normal tissue for 17 human tumor types based on RNA-seq datasets from TCGA. BLCA: Bladder Urothelial Carcinoma; BRCA: Bladder Urothelial Carcinoma; CHOL: Cholangiocarcinoma; COAD: Cholangiocarcinoma; ESCA: Esophageal carcinoma; HNSC: Head and Neck squamous cell carcinoma; KICH: Kidney Chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; STAD: Stomach adenocarcinoma; THCA: Thyroid carcinoma; UCEC: Uterine Corpus Endometrial Carcinoma. (B) High expression of *DNASE1L3* is associated with increased survival in CRC patients. Kaplan-Meier survival probability curves for the TCGA colorectal patients (n=597). Survival of the patients with *DNASE1L3* expression level in tumors above the mean are shown in red (favorable) and of those with *DNASE1L3* expression level in tumors at or below the mean are shown in blue (unfavorable). P-value was computed based on the log-rank test of the survival distributions of high and low expression groups. (C) *DNASE1L3* is downregulated in cDC1 and cDC2 cells from tumors of CRC patients compared to those from normal colorectal tissues. Single-cell RNA-seq datasets of human colorectal tumors (18) were analyzed (n=10 for normal cDC1, 36 for tumor cDC1, 36 for normal cDC2, and 64 for tumor cDC2; Mann-Whitney test, *p*<0.05). (D) The expression of *DNASE1L3* is positively correlated with that of several immune activation markers, particularly T cell activation markers, in CRC patients. The mRNA levels of *DNASE1L3* and those of selected immune activation markers from TCGA colorectal tumor RNA-seq data were analyzed (n=286; Pearson product-moment correlation test).
Figure 2. **Dnase113 KO mice display impaired recovery after DNA damage in vitro and in vivo.**

(A) Serum from Dnase113 KO mice has a reduced ability to digest genomic DNA in vitro. 500ng genomic DNA was incubated with 5 µl serum isolated from Dnase113 WT and KO mice, respectively. (B) Serum from Dnase113 KO mice has a reduced ability to digest isolated nuclei. 1 x 10⁶ nuclei were incubated with 15 µl serum isolated from Dnase113 WT and KO mice, respectively, for two hours. (C-D) Dnase113 KO mice have a reduced ability to recover in the colon after AOM treatment. 2-month-old female Dnase113 WT and KO mice were injected with AOM (8 mg/kg) and colonic tissues were isolated 9 h, or 5 days after the injection. Colonic tissue sections were stained by TUNEL. (C) Representative images of TUNEL staining of the damaged cell in the colon of Dnase113 WT and KO mice. (D) Quantification of percentages of TUNEL positive signals in C (n=4 WT and 4 KO as basal controls; n=5 WT and 6 KO at 9 h; n=6 WT and 7 KO at D5; Mann-Whitney test, *p<0.05). (E-F) Dnase113 KO mice have a reduced ability to recover in the small intestine after doxorubicin treatment. 2-month-old female Dnase113 WT and KO mice were injected with doxorubicin (10 mg/kg) and intestine tissues were isolated 12 h, 3 days, and 5 days after the injection. (E) Representative images of TUNEL staining of the damaged cells in the small intestine of Dnase113 WT and KO mice. (F) Quantification of the percentage of TUNEL-positive cells in E (n=4 WT and 4 KO as basal controls; n=4 WT and 5 KO at 12 h; n=6 WT and 6 KO at D3; n=6 WT and 6 KO at D5; Mann-Whitney test, *p<0.05).
Figure 3. \textit{Dnase1/3} deficiency enhances colon cancer formation in mice.

(A) \textit{Dnase1/3} KO mice have increased body weight loss during AOM/DSS treatment. \textit{Dnase1/3} WT and KO mice were subjected to AOM/DSS colorectal cancer procedure with 3 cycles of DSS treatment (peak) as described in Methods \((n=15\) WT and 15 KO initial mice from one experiment, it was independently repeated and similar result was obtained; Multiple Mann-Whitney tests, \(*p<0.05\). The body weight loss was monitored during entire experimental time frame every 3 days. (B) More \textit{Dnase1/3} KO mice developed severe bleeding and diarrhea than WT mice during the AOM/DSS colorectal cancer procedure \((n=30\) WT and 27 KO from two independent experiments). (C) Representative images indicating that \textit{Dnase1/3} KO mice have higher tumor burden than \textit{Dnase1/3} WT mice when treated with three cycles of AOM/DSS. Bar, 1 cm. (D) \textit{Dnase1/3} KO mice have increased tumor number in the colon after the AOM/DSS colorectal cancer procedure \((n=36\) WT and 29 KO; Mann-Whitney test, \(* * * * p<0.0001\). (E) \textit{Dnase1/3} KO mice have increased colon tumor burden. Tumor burden was quantified by Fiji \((n=24\) WT and 25 KO; Mann-Whitney test, \(* * * * p<0.0001\). (F) Representative H&E colonic images of \textit{Dnase1/3} WT and KO mice in the AOM/DSS colorectal cancer model. (G) Colorectal tumors developed in \textit{Dnase1/3} KO mice are at more advanced stages compared to those in WT mice in the AOM/DSS colorectal cancer model. H&E colonic sections from WT and \textit{Dnase1/3} KO mice \((n=15\) WT and 13 KO) were evaluated and scored by a Board-certified veterinary pathologist. (H) Representative images of intestinal adenoma from WT;\textit{APC}^{Min/+} and KO;\textit{APC}^{Min/+}. (I) More \textit{Dnase1/3} KO mice developed adenoma in the small intestine and colon than WT mice in the \textit{APC}^{Min/+} model \((n=15\) WT;\textit{APC}^{Min/+} and 21 KO;\textit{APC}^{Min/+}).
Figure 4. Dnase1I3 deficiency enhances early-stage inflammation yet reducing late-stage colonic tissue accumulation of T cells in mice.

(A-B) Colonic tissues of Dnase1I3 KO mice display increased inflammation during the early stage of AOM/DSS-induced carcinogenesis. Transcriptomes from colonic tissues of WT and Dnase1I3 KO mice at D0, D19, and D80 of the AOM/DSS procedure were analyzed by RNA-seq as described in Methods. (A) The dynamics of four gene clusters are shown as a heatmap. (B) Enriched pathways of the 207 genes shown in the purple cluster. GO, Gene Ontology; REAC, Reactome Pathway Database; KEGG, Kyoto Encyclopedia of Genes and Genome. (C) Dnase1I3 KO mice have increased expression of various pro-inflammatory markers at different time points of the AOM/DSS-induced carcinogenesis when analyzed by qPCR (n=4, 5, 5, 6, 10, 6, 12, 5 WT and 4, 6, 7, 6, 6, 12, 10, 2 KO at each time point, respectively; outliers that fall below Q1 − 3.0*IQR or above Q3 + 3.0*IQR were removed; Mann-Whitney test, *p<0.05, **p<0.01, ***p<0.001). (D) Colonic tissues of Dnase1I3 KO mice display increased NF-κB signaling. Western blotting of phospho-IκBα in the colonic tissue samples of the Dnase1I3 WT and KO mice at D19 in the AOM/DSS procedure. (E) Dnase1I3 KO mice have reduced colonic tissue accumulation of T cells at the end stage of the AOM/DSS procedure. Left, representative images for CD3 (upper panels) or CD8 (bottom panels) IHC staining in coloconic tissues from Dnase1I3 WT and KO mice after the AOM/DSS colorectal cancer procedure. Right, the percentage of CD3 immunostaining positive areas and CD8 immunostaining positive areas were quantified from the IHC colonic tissue sections by Fiji (n=10 WT and 12 KO; Mann-Whitney test, *p<0.05).
Figure 5. Dnase1l3 deficiency promotes tumor progression and impairs activity of cDCs in a syngeneic tumor model.

(A) Dnase1l3 deficiency increases the growth of MC38 tumors. 1 x 10^5 MC38 colon cancer cells were subcutaneously injected into 8-12 week-old immunocompetent WT or Dnase1l3 KO mice (n=10 WT and 11 KO; Mann-Whitney test, p<0.05). (B) Representative images of final dissected tumors. (C) Weights of final MC38 tumors (n=30 tumors from WT and 39 tumors from KO from three independent experiments; Mann-Whitney test, p<0.05). (D) UMAP analysis of cDC single cells sampled from MC38 tumors from Dnase1l3 WT and KO mice (analyzed by scRNA-seq). (E) DCs from MC38 tumors in Dnase1l3 KO mice have reduced cDC2 but increased cDC1 populations. (F) The relative abundance of different subgroups of cDC2 cells. (G) Dnase1l3 deficient cDC2 cells in cluster 3 have reduced expression of chemokines and cytokines (Mann-Whitney test, p<0.05, ***p<0.001). (H) Dnase1l3 deficient cDC2 cells in cluster 3 have reduced expression of various activation marker genes (Mann-Whitney test, **p<0.01, ***p<0.001, ****p<0.0001). (I) Dnase1l3 KO mice have reduced abundance of CD86^+ CD11b^+ cDCs in the MC38 tumors at an early stage of tumor development (10 days after inoculation) (n=21 WT and 21 KO from three independent experiments; p<0.05). Representative FACS plots from WT and KO mice are shown. Biological replicates were combined in the analysis using a linear regression model to remove the batch effects (see Methods). (J) Dnase1l3 KO mice have reduced abundance of activated cDCs in tumor draining lymph nodes (dLNs) at an early stage of tumor development (10 days after inoculation). Indicated immune cell population in dLNs was analyzed by flow cytometry (n=16 WT and 16 KO from two independent experiments). Biological replicates were combined in the analysis using a linear regression model to remove the batch effects (see Methods).
Figure 6. Dnase1f13 deficiency alters cytotoxic T cells and impairs anti-tumor immunity.

(A-B) Top downregulated genes (log2FC<0.4) in CD8+ T cells from MC38 tumors in Dnase1f13 KO vs WT mice were analyzed for pathway enrichment (*padj<0.05, **padj<0.01, ***padj<0.001, ****padj<0.0001). (C) Dnase1f13 KO mice have reduced abundance of activated T cells in MC38 tumors 14 days after inoculation. Indicated immune cell populations in isolated tumors were analyzed by flow cytometry (n=8 WT and 8 KO mice, Student’s t-test, *p<0.05). (D) Dnase1f13 KO mice have reduced abundance of activated T cells in tumor draining lymph nodes (dLNs) 10 days after inoculation (analyzed by flow cytometry, n=16 WT and 16 KO from two independent experiments; Mann-Whitney test, *p<0.05, **p<0.01). (E) Dnase1f13 KO mice have reduced expression of several immune cell genes in CD4+ immune cells isolated from tumor draining lymph nodes (dLNs) 14 days after inoculation (analyzed by qPCR, n=5 WT and 5 KO mice; Mann-Whitney test, *p<0.05, **p<0.01). (F) Schematic representation of in vitro co-culture experiment using OT-I CD8+ T cells and WT or Dnase1f13 KO DCs in the presence of regular or apoptotic MC38-OVA cells. Apoptosis of MC38-OVA was induced by treatment with 400 µM oxaliplatin (Oxa) for 24 hours. (G) OT-I CD8+ T cells have comparable proliferation and CD44 activation when co-cultured with apoptotic MC38 cells and WT or Dnase1f13 KO DCs. The mean fluorescence intensity (MFI) of CFSE dye (for cell proliferation) and the MFI of CD44 in OT-I CD8+ T cells were analyzed by FACS (n=3 independent experiments, two-way ANOVA test with correction for multiple comparisons, **p<0.01). (H) OT-I CD8+ T cells co-incubated with Dnase1f13 KO DCs in the presence of apoptotic MC38 cells have reduced expression of some key immune cell markers (analyze by qPCR, n=3 replicates, two-way ANOVA test with correction of multiple comparisons, *p<0.05, ***p<0.001).