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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Regulatory TCR$\alpha\beta^+$ Double Negative T Cells Suppress $\gamma\delta$ T Cells and Alleviate Colitis

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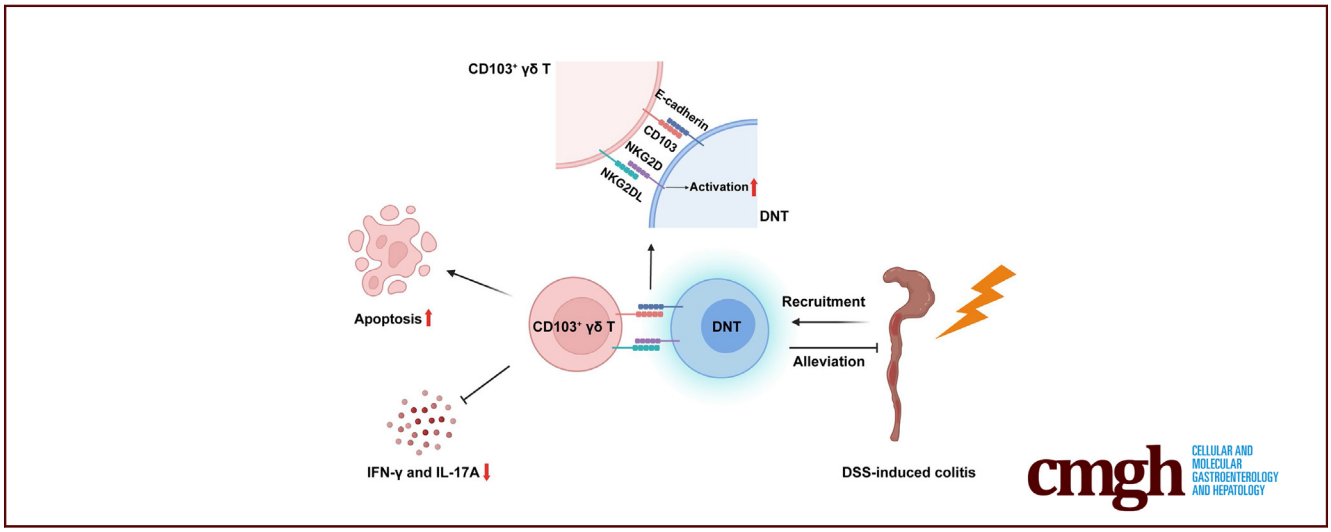

SUMMARY

E-cadherin, in collaboration with NKG2D, mediates the selective suppression of CD103$^+$ $\gamma\delta$ T-cell populations by double-negative T cells during the process of colitis. The adoptive transfer of double-negative T cells may represent a novel therapeutic strategy for inflammatory bowel disease.

BACKGROUND AND AIMS: Inflammatory bowel disease arises from dysregulated immune activations triggered by a myriad of factors. The maintenance of immune tolerance within the intestinal milieu and the suppression of inflammatory responses remain the most efficacious strategies for alleviating enteritis. Regulatory TCR$\alpha\beta^+$ double-negative T (DNT) cells play pivotal roles in orchestrating the homeostasis of various organs; however, their specific influence on colitis has yet to be thoroughly elucidated.

METHODS: Single-cell RNA sequencing and adoptive transfer were used to delineate the distinct signatures of DNT cells and to investigate their role in the context of colitis, respectively.

RESULTS: Our observations revealed that the proportions of DNT cells within the CD3$^+$TCRβ^+NK1.1$^-$ populations in the naive murine colonic lamina propria and intraepithelial layer were significantly elevated compared with those in the mesenteric lymph node, with further augmentation noted in the colon of colitis mice. The adoptive transfer of DNT cells conferred relief from colitis symptoms. E-cadherin facilitated the interaction between DNT cells and CD103$^+$ $\gamma\delta$ T cells, thereby collaboratively enhancing the cytotoxicity of DNT cells against $\gamma\delta$ T-cell populations in concert with NKG2D, ultimately promoting the remission of colitis. Furthermore, mice that received allogeneic DNT cells exhibited ameliorated colitis without inducing graft-versus-host disease.

CONCLUSIONS: Among the diverse populations of DNT subsets, regulatory DNT cells are capable of accumulating in the inflamed colon, thereby preventing the progression of colitis through the suppression of CD103$^+$ $\gamma\delta$ T-cell responses. The adoptive transfer of DNT cells could be a novel therapeutic strategy for inflammatory bowel disease. *(Cell Mol Gastroenterol Hepatol 2025;19:101553; https://doi.org/10.1016/j.jcmgh.2025.101553)*

Keywords: Regulatory DNT Cells; Colitis; γδ T Cells; E-cadherin; CD103.

Inflammatory bowel disease (IBD), encompassing Crohn's disease and ulcerative colitis, constitutes a chronic inflammatory condition with an alarming rise in incidence globally.[1–3] Beyond the confines of intestinal damage, the systemic inflammation may also extend its deleterious effects to such organs as joints and skin, and even lead to the occurrence of colorectal cancer, which is associated with a decreased quality of life.[4–6] IBD arises from a convergence of genetic risks, environmental triggers, and perturbation of microbiota, which collectively foster aberrant immune activation (encompassing innate and adaptive responses) and compromise the integrity of the mucosal barrier.[7–9] Therapies that target proinflammatory factors have been proved to curtail the progression of IBD; however, many patients are resistant to the biopharmaceutical interventions.[10–12] Therefore, the demand for a novel way to mitigate inflammation is still urgent.

TCRαβ$^+$ double-negative T (DNT) cells represent a distinct subset of unconventional T cells characterized by the absence of CD4 and CD8 expression.[13,14] In contrast to the thymic DNT populations, which are precursors of most T cells, the peripheral DNT cells exhibit remarkable versatility. Both suppressive and proinflammatory functions of DNT cells have been reported in different scenarios because of their inherent heterogeneity.[15,16] For instance, interleukin (IL)17$^+$ DNT cells have been implicated in immunepathologies, such as lupus, whereas their Granzyme B$^+$ counterparts demonstrate regulatory functions and hold significant promise for alleviating inflammation through adoptive transfer.[17–19] We previously reported that the Granzyme B$^+$ DNT cells confer protective effects in models of allergic asthma, nonalcoholic steatohepatitis, and psoriasis.[20–22] These findings emerging from mucosal tissues prompt us to explore the role of regulatory DNT cells in maintaining intestinal homeostasis.

Regulatory DNT cells typically mitigate excessive immune activation by Granzyme B and Perforin dependent cell killing.[13,23] Some studies also proved that DNT cells could eliminate effector T cells through Fas-mediated apoptosis.[15,24] Given the absence of CD4 and CD8, the mechanisms for DNT cells recognizing other targets and performing the suppressive functions are well interested. Our prior researches have elucidated that Lag3 contributes to the MHC-II antigen recognition of DNT cells, and that NK receptors (NKG2D and NKG2A) may collaboratively regulate DNT cells activation state; nevertheless, the selective suppressive functions of DNT cells remain poorly understood.[20,22,25]

Thus, we made our initial goal to investigate the effect of DNT cells in murine model of dextran sulfate sodium (DSS)-induced colitis. To our surprise, the adoptive transfer of DNT cells markedly ameliorated colitis by inhibiting the response of γδ T cells. Furthermore, we discovered that DNT cells expressed the epithelial cell marker, E-cadherin, which facilitated their interaction with inflammatory CD103$^+$ γδ T cells, thereby promoting the selective suppression. The impact of regulatory DNT cells on DSS-induced colitis in allogeneic mice was also investigated.

Results

Accumulation of Regulatory DNT Cells in the Colon of DSS-Treated Mice

As illustrated in Figure 1*A*, naive mice possessed significantly higher percentages of TCRβ$^+$NK1.1$^-$ DNT cells within the colonic lamina propria (LP) and intraepithelial (IE) layer than those in the mesenteric lymph node (MLN). To elucidate the role of DNT cells in murine colitis, we compared the proportions of DNT cells in mice subjected to DSS treatment against those not treated. Following the successful establishment of the colitis model, a significant increase in the percentages of DNT cells was observed in the MLN, colonic LP, and IE layer (Figure 1*A*). To further delineate the alterations in DNT cells and their interactions with other populations in the colonic microenvironment, live colonic cells from healthy control animals and mice administered DSS for 1, 3, and 7 days were subjected to single-cell RNA sequencing (scRNA-seq). After quality control, 4 major groups including T cells, B cells, myeloid cells, and epithelial cells were identified (Figure 1*B*).

According to the criterion for identifying TCRαβ-expressing cells while excluding populations positive for *Cd4*, *Cd8α*, *Klrb1c*, and TCRγδ-associated genes, we extracted 1091 cells possessing DNT cell signatures from the T-cell populations for further analysis. Consistent with the flow cytometry analysis, scRNA-seq revealed an elevation in the percentage of DNT cells within T-cell clusters throughout the progression of colitis (Figure 1*C*). Using uniform manifold approximation and projection analysis, we discerned 5 distinct clusters within the transcriptomic DNT populations (Figure 1*D*). As the largest constitute of DNT cells, cluster 0 expressing higher level of *Fcer1g*, *Cd69*, and killer cell lectin-like receptors (*Klrk1*, *Klre1*, *Klrd1*, *Klra4*), was thought to be activated DNT cells (aDNT cells). In contrast, cluster 1 sharing the features of resting T cells (expressing *Ccr7*, *Sell*, *Lef1*) was named as *Ccr7*$^+$ DNT cells (Figure 1*E* and *F*). Distinct from other populations, cluster 2 exhibited up-regulation of exhausted features, such as *Pdcd1* and *Tox2*, whereas proinflammatory effector molecules linked to IL17 signaling (*Il23r*, *Il17a*, *Il22*) were enriched in cluster 4. Among the DNT clusters, both cluster 0 (aDNT) and cluster 3 (*Ccr5*$^+$ DNT), which express *Gzmb* (a signature molecule of regulatory DNT cells), may represent regulatory DNT populations (Figure 1*E* and *F*). Transcriptomic analysis indicated a

Abbreviations used in this paper: **aDNT, activated DNT; DNT cells, double-negative T cells; DSS, dextran sulfate sodium; GFP, green fluorescent protein; IBD, inflammatory bowel disease; IE, intraepithelial; IFN, interferon; IL, interleukin; LP, lamina propria; MLN, mesenteric lymph node; PBS, phosphate-buffered saline; scRNA-seq, single-cell RNA sequencing.**

Most current article

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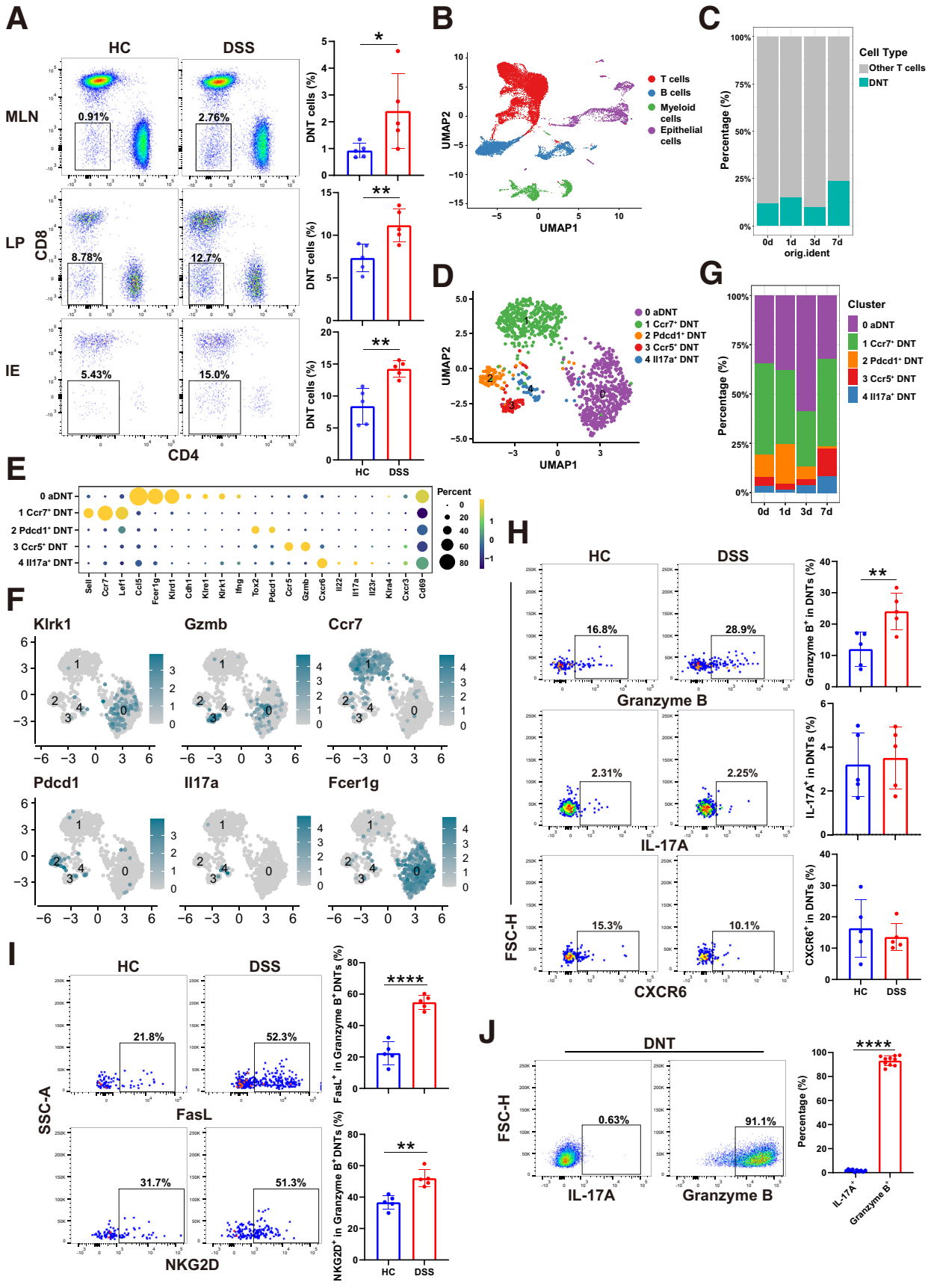

A
HC
DSS
MLN
LP
IE
0.91%
2.76%
8.78%
12.7%
5.43%
15.0%
CD8
CD4
DNT cells (%)
HC
DSS
B
T cells
B cells
Myeloid cells
Epithelial cells
UMAP1
UMAP2
C
Cell Type
Other T cells
DNT
Percentage (%)
orig.ident
0d
1d
3d
7d
D
0 aDNT
1 Ccr7+ DNT
2 Pdcd1+ DNT
3 Ccr5+ DNT
4 Il17a+ DNT
G
Cluster
E
Percent
Sell
Ccr7
Lef1
Ccl5
Fcer1g
Klrd1
Cd1d
Klre1
Klrk1
Ifng
Tox2
Pdcd1
Ccr5
Gzmb
Cxcr6
Il22
Il17a
Il23r
Klra4
Cxcr3
Cd69
F
Klrk1
Gzmb
Ccr7
Pdcd1
Il17a
Fcer1g
H
16.8%
28.9%
Granzyme B
Granzyme B+ in DNTs (%)
2.31%
2.25%
IL-17A
IL-17A+ in DNTs (%)
15.3%
10.1%
FSC-H
CXCR6
CXCR6+ in DNTs (%)
I
21.8%
52.3%
SSC-A
FasL
FasL+ in Granzyme B+DNTs (%)
31.7%
51.3%
NKG2D
NKG2D+ in Granzyme B+DNTs (%)
J
DNT
0.63%
91.1%
IL-17A
Granzyme B
Percentage (%)
IL-17A+
Granzyme B+

tendency for an increase in the percentages of regulatory DNT cells (clusters 0 and 3) and proinflammatory DNT cells (cluster 4) during the progression of colitis (Figure 1*G*). However, flow cytometry analysis confirmed that colonic LP DNT cells exhibited low level of IL17A expression, with comparable proportion of CXCR6$^+$ cells (previously identified as a marker for IL17A$^+$ DNT cells[16]) observed in the colonic LP DNT cells from colitis and healthy mice. Notably, an increased percentage of Granzyme B$^+$ cells was observed in DNT cells derived from the inflamed colonic LP (Figure 1*H*). Moreover, these Granzyme B$^+$ DNT cells from the inflamed colonic LP exhibited enhanced expression of FasL and NKG2D, compared with their counterparts in healthy mice (Figure 1*I*). Collectively, these findings demonstrate that regulatory DNT cells accumulate in the inflamed colon. To explore the significance of these regulatory DNT cells in colitis and to address the limitations in cell numbers for therapeutic studies, we used expanded peripheral-derived DNT cells, characterized by high Granzyme B expression and minimal IL17A expression (sharing a similar phenotype with colonic regulatory DNT), as substitutes for colonic regulatory DNT cells in subsequent investigations (Figure 1*J*).

Adoptive Transfer of DNT Cells Alleviates DSS-Induced Colitis

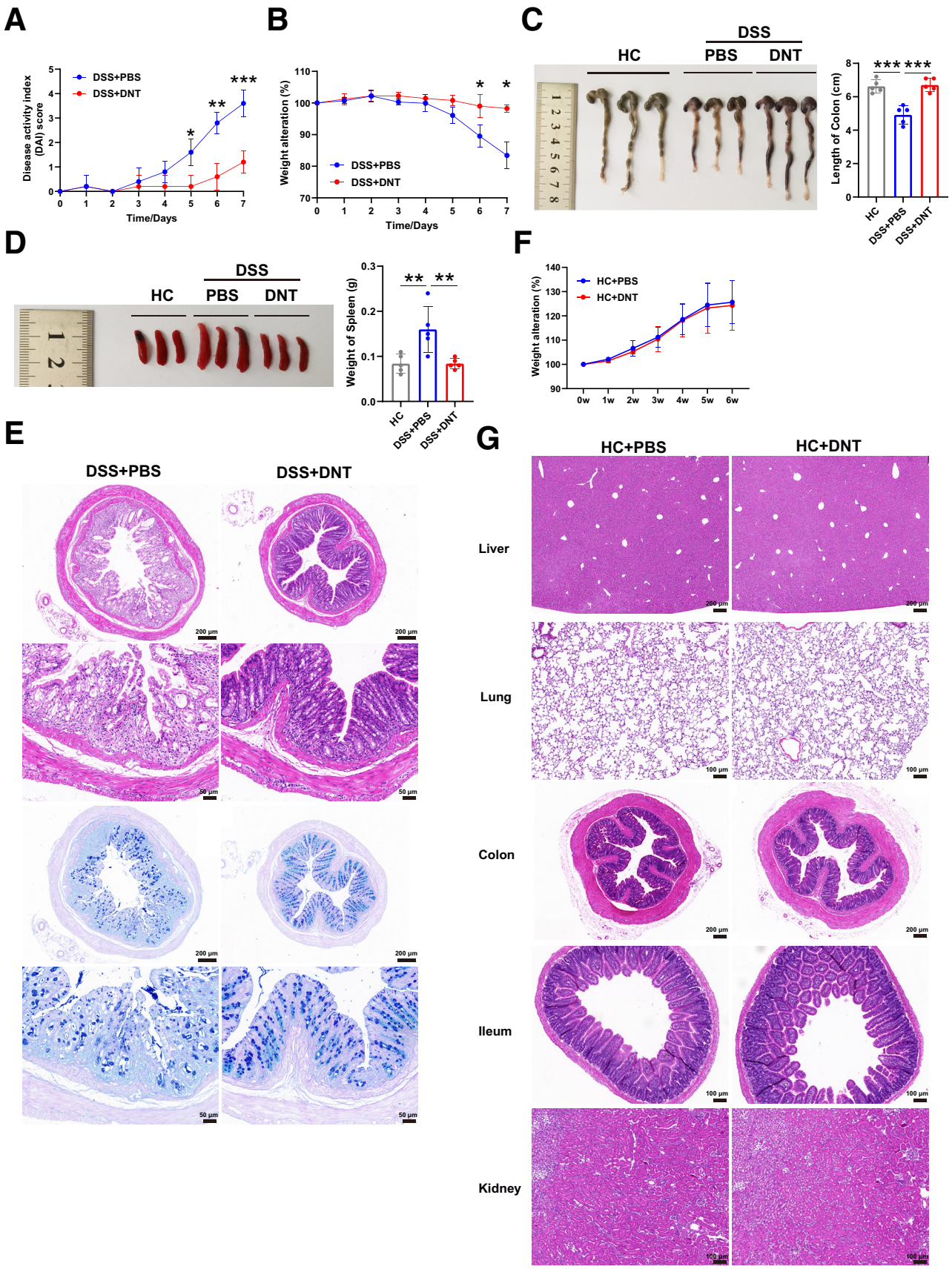
To assess the impact of DNT cells on colitis, we adoptively transferred expanded peripheral-derived DNT cells into recipient mice before the induction of colitis. Remarkably, DNT cells significantly ameliorated colitis, as evidenced by a reduction in the disease activity index score and a mitigation of weight loss (Figure 2*A* and *B*). Mice receiving DNT cells also exhibited an elongated colon and a smaller spleen following DSS treatment (Figure 2*C* and *D*). Compared with phosphate-buffered saline (PBS) controls, DNT cells alleviated the infiltration of inflammatory cells and restored the structural integrity of the mucosal layer in the colon of DSS-treated mice (Figure 2*E*). Furthermore, we injected healthy mice with an equivalent quantity of DNT cells and observed no significant changes in body weight or inflammatory responses in peripheral organs, thereby affirming the safety of DNT cell therapy during a 6-week period (Figure 2*F* and *G*). These results collectively suggest that regulatory DNT cells possess the capacity to prophylactically prevent DSS-induced colitis.

Next, we examined the distribution of adoptively transferred cells using DNT derived from green fluorescent protein (GFP) transgenic mice (mice in which most cells express GFP). As depicted in Figure 3*A-C*, the absolute numbers and proportions of GFP$^+$ DNT cells were significantly elevated at Day 7 post–adoptive transfer in the MLN and colonic LP of colitis mice compared with healthy control animals. Particularly in the colonic LP, the frequency of transferred DNT cells in healthy mice remained relatively stable at a lower level. In contrast, GFP$^+$ DNT cells dramatically infiltrated the inflamed colonic LP throughout the course of DSS treatment (Figure 3*A-C*). To elucidate the factors that recruit DNT cells to the colonic inflammatory sites, we conducted CellPhoneDB analysis on our scRNA-seq data. The results indicated that myeloid and T-cell populations may recruit regulatory DNT clusters (clusters 0 and 3) to the inflamed colon via CXCR3 and CCR5, both of which have been reported to play crucial roles in DNT cell chemotaxis (Figure 3*D*).[19,21,24,26] Consistent with these findings, bulk RNA sequencing revealed the up-regulation of CXCR3 and CCR5 ligands (*Ccl3*, *Ccl4*, *Ccl5*, *Cxcl9*, and *Cxcl10*) in inflamed colon (Figure 3*E*). These data suggest that CXCR3 and CCR5 may facilitate the accumulation of regulatory DNT cells in the colon of colitis mice.

Regulatory DNT Cells Suppress $\gamma\delta$ T-Cell Responses in Murine Colitis

To investigate the targets of regulatory DNT cells in colitis, we first examined the alterations in immunocyte proportions within the inflamed colon. The percentages of NK, NKT, CD4$^+$ T, CD8$\alpha\alpha^+$ T, and CD8$\alpha\beta^+$ T cells were comparable in the colonic LP between PBS- and DNT-treated mice (Figure 4*A* and *B*). However, the percentages of $\gamma\delta$ T cells were significantly diminished in the LP and IE layer of colon from colitis mice receiving DNT cells (Figure 5*A*). The adoptive transfer of regulatory DNT cells also suppressed the expression of interferon (IFN)-γ and IL17A in colonic $\gamma\delta$ T cells (Figure 5*B* and *C*). The cytotoxic effect of DNT cells may underlie the suppression of $\gamma\delta$ T-cell responses, because DNT cells obviously elevated the level of apoptosis in $\gamma\delta$ T cells from the inflamed colon (Figure 5*D*). By coculturing expanded DNT cells with $\gamma\delta$ T cells derived from the colitis mice, we further demonstrated that DNT cells could directly induce apoptosis in $\gamma\delta$ T cells in vitro (Figure 5*E*). Collectively, these findings indicate that DNT cells could inhibit the survival and function of $\gamma\delta$ T cells during colitis.

Figure 1. (***See previous page***). **Regulatory DNT cells accumulate in the inflamed colon.** Mice were treated with DSS and harvested at days 0, 1, 3, and 7 for further analysis. (*A*) Flow cytometry analysis of DNT percentages in CD45$^+$CD3$^+$TCRβ^+NK1.1$^-$ populations obtained from the MLN, colonic LP, and colonic IE layer of mice treated with or without DSS. (*B-G*) Colonic cells isolated at the indicated time points post-DSS treatment were sent for single cell RNA sequencing (CRA012515). Uniform manifold approximation and projection (UMAP) visualization of live cell clusters is shown (*B*). The proportion of DNT clusters in T-cell populations was compared (*C*). UMAP visualization of DNT cell clusters is displayed (*D*). The selected genes are illustrated in DNT clusters by bubble plot (*E*) and feature plots (*F*), respectively. (*G*) Comparison of the percentage of DNT clusters was performed. (*H*) Flow cytometry analysis of Granzyme B, IL17A, and CXCR6 expression in DNT cells from the colonic LP of mice treated with or without DSS. (*I*) Flow cytometry analysis of FasL and NKG2D expression in Granzyme B$^+$ DNT cells from the colonic LP of mice. (*J*) Comparison of IL17A and Granzyme B expression in expanded peripheral-derived DNT cells. At least 2 independent experiments were performed with 5 mice in each group. The *t* test was performed to compare the differences described previously.


A
Disease activity index (DAI) score
DSS+PBS
DSS+DNT
Time/Days
B
Weight alteration (%)
DSS+PBS
DSS+DNT
Time/Days
C
DSS
HC
PBS
DNT
Length of Colon (cm)
HC
DSS+PBS
DSS+DNT
D
DSS
HC
PBS
DNT
Weight of Spleen (g)
HC
DSS+PBS
DSS+DNT
F
Weight alteration (%)
HC+PBS
HC+DNT
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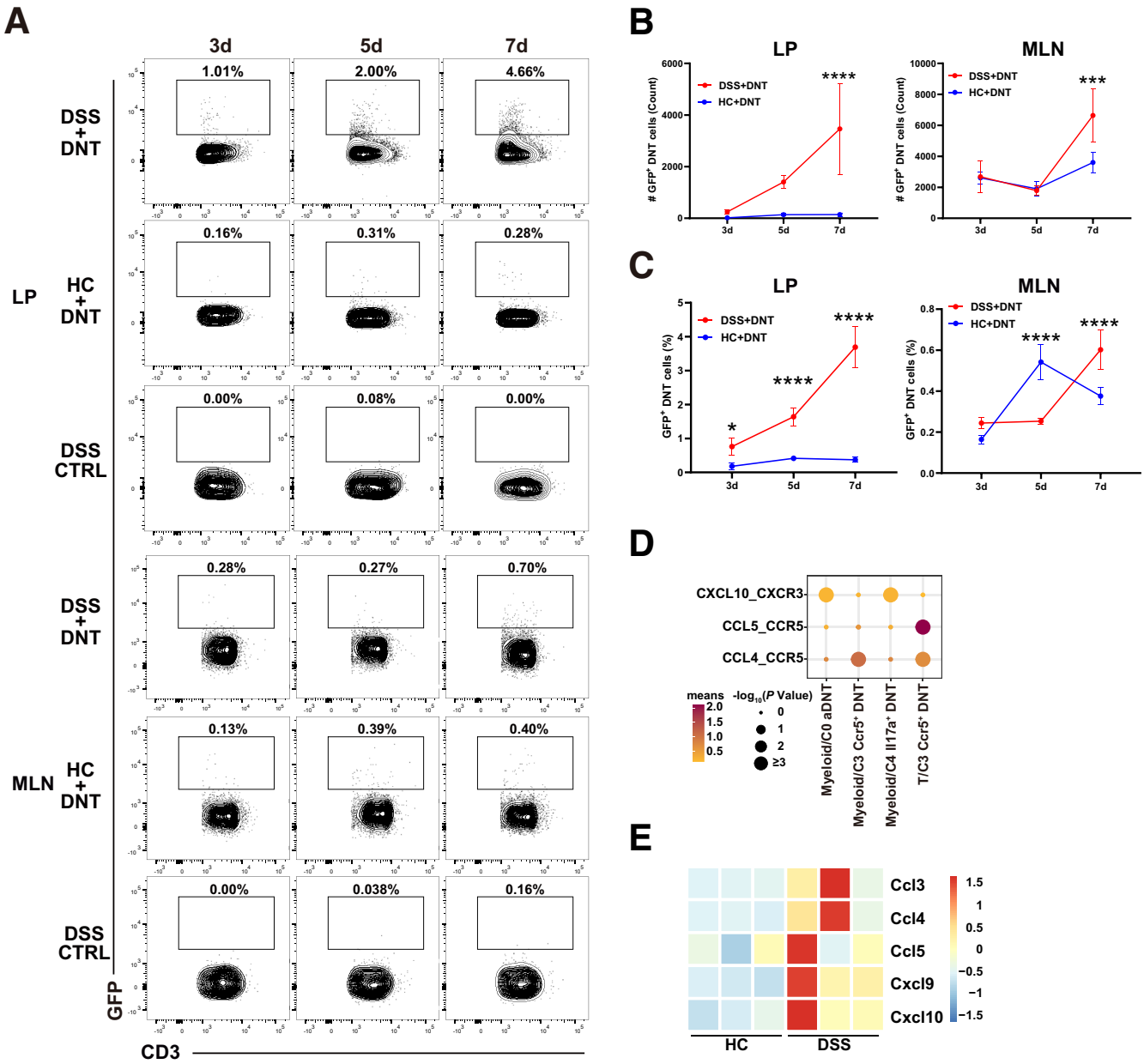


Figure 3. Adoptively transferred DNT cells localize in the inflamed colon. (*A-C*) GFP$^+$ DNT cells were respectively transferred into healthy mice and mice that were about to receive DSS. Mice treated exclusively with DSS were used as control animals. Comparisons of the absolute numbers of DNT cells (*B*) and the percentages of GFP$^+$ DNT cells in CD3$^+$ T cells (*C*) were performed at the indicated time after adoptive transfer. (*D*) Cell-cell interactions between DNT clusters and other cell types in the scRNA-seq data were analyzed using CellPhoneDB (CRA012515). *P* value is represented by the size of the *circle*, whereas the average expression level of the interacting pairs is indicated by *color*. (*E*) Heatmaps depicting the selected chemokines in the colon of healthy and DSS-treated mice (CRA019332). At least 2 independent experiments were performed with 5 mice in each group. The 2-way analysis of variance with multiple comparisons was applied to compare the differences described previously.

Figure 2. (***See previous page***). **Adoptive transfer of regulatory DNT cells alleviates DSS-induced colitis.** DNT cells were transferred into mice scheduled to undergo DSS treatment (*A-E*) or into healthy mice (*F, G*), respectively. Mice receiving an equivalent volume of PBS served as control animals. (*A, B*) Comparisons of the disease activity index scores (*A*) and weight alterations (*B*) between colitis mice treated with DNT cells and those treated with PBS. (*C, D*) Gross morphologic assessments of colon (*C*) and spleen (*D*) from the colitis mice treated with DNT cells or PBS, alongside healthy control animals. The comparisons of colon length and splenic size were also conducted. (*E*) Representative hematoxylin and eosin (H&E) (*top*) and alcian blue/periodic acid–Schiff (*bottom*) stained sections of the colon. (*F*) Comparison of weight alterations between healthy mice treated with DNT cells or PBS. (*G*) Representative H&E staining of the liver, lung, colon, ileum, and kidney sections. At least 2 independent experiments were performed with 5 mice in each group. Analysis of variance with multiple comparisons was applied to compare the differences described previously.

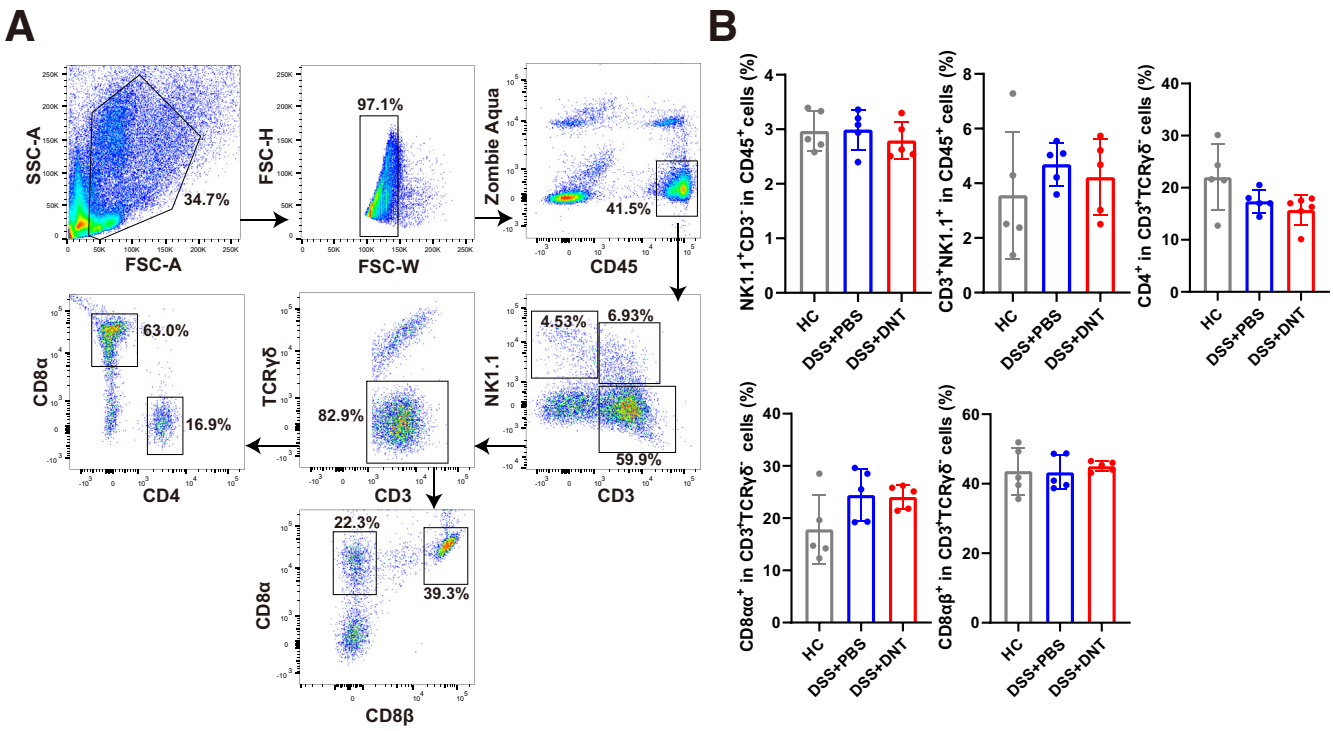


Figure 4. Alterations of immune cells in the colon of mice adoptively transferred with or without DNT cells. (*A*) Gating strategy for inflammatory cell populations in the colonic LP. (*B*) Mice were given DNT cells or PBS, and subsequently prepared for DSS-induced colitis. Healthy mice served as control animals. The comparisons of NK ($NK1.1^+CD3^-$), NKT ($NK1.1^+CD3^+$), $CD4^+$ T, $CD8\alpha\alpha^+$ T, and $CD8\alpha\beta^+$ T-cell proportions in the colonic LP were performed using flow cytometry. Each experiment was repeated at least 2 times with 5 mice in each group. The 1-way analysis of variance with multiple comparisons was used to compare the differences mentioned previously.

Selective Suppression of CD103$^+$ γδ T-Cell Populations by DNT Cells in DSS-Treated Mice

To elucidate the recognition process of DNT cells toward γδ T cells, we characterized the colonic γδ T cells in DSS-treated mice using our scRNA-seq data. Among the colonic T-cell clusters, clusters 1 and 2, exhibiting elevated levels of *Trdc* expression, were identified as γδ T cells (Figure 6*A* and *B*). During the progression of murine colitis, these γδ T-cell clusters progressively increased their expression of *Itgae* (encoding CD103), an intestine homing integrin, which has also been linked to the colitogenic functions in T-cell populations (Figure 6*B* and *C*).[27,28] We confirmed that the colonic LP and splenic γδ T cells from DSS-treated mice contained a higher proportion of $CD103^+$ cells, compared with healthy control animals (Figure 6*D* and *E*). Furthermore, the adoptive transfer of DNT cells resulted in decreased proportions of $CD103^+$ cells in γδ T populations from the colonic LP and spleen, whereas no significant changes were observed in the colonic IE layer (Figure 6*D* and *E*). Thus, the level of apoptosis in γδ T cells from the spleen and colonic LP of colitis mice was furtherly investigated. We found that DNT cells increased the percentages of Annexin V^+ cells among $CD103^+$ γδ T cells, without affecting the apoptosis levels of $CD103^-$ γδ T populations in both the colonic LP and spleen from colitis mice (Figure 6*F* and *G*). To confirm the direct cytotoxicity of DNT cells toward $CD103^+$ γδ T cells, we cocultured DNT cells with the inflammatory γδ T cells obtained from DSS-treated mice and observed a notable reduction in the proportion of $CD103^+$ γδ T cells (Figure 6*H*). Consistent with the in vivo findings, the level of apoptosis in $CD103^+$ γδ T cells was significantly elevated by DNT cells, whereas the $CD103^-$ γδ T cells remained unaltered (Figure 6*I*). These findings suggest that regulatory DNT cells may directly suppress γδ T-cell responses by targeting $CD103^+$ γδ T-cell populations, thereby contributing to the amelioration of colitis.

Given that CD103 is not exclusively expressed in γδ T cells, we also assessed the impact of DNT cells on conventional $CD8^+$ T cells, which are well-documented as $CD103^+$ populations in the intestine.[29,30] As shown in Figure 7*A* and *B*, the adoptive transfer of DNT cells neither reduced the percentages of $CD103^+$ cells in $CD8\alpha^+$ T cells nor enhanced the apoptosis levels of $CD103^+CD8\alpha^+$ populations from the colonic LP and spleen of DSS-treated mice. The obviously increased expression of NKG2D ligands (MULT-1 and Rae-1) and Fas in inflammatory $CD103^+$ γδ T cells compared with $CD103^+CD8\alpha^+$ T cells and their $CD103^-$ counterparts may account for the selective suppression of γδ T cells by DNT cells (Figure 7*C-E*). Specifically, the activation of NKG2D enhances the regulatory capacity of DNT cells, whereas the heightened Fas expression in $CD103^+$ γδ T cells facilitates FasL-mediated cytotoxicity.[15,25,31] Together, these results

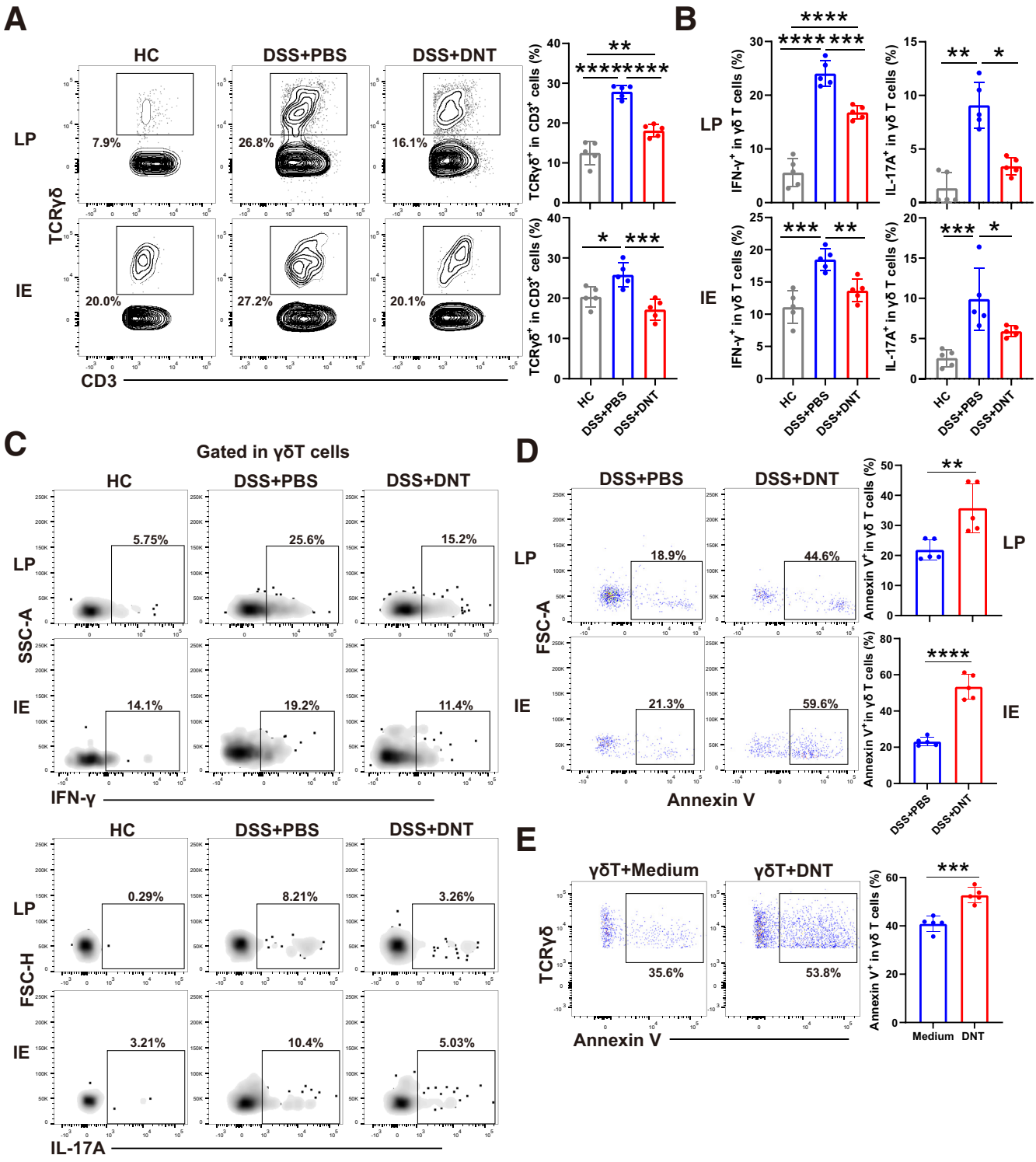
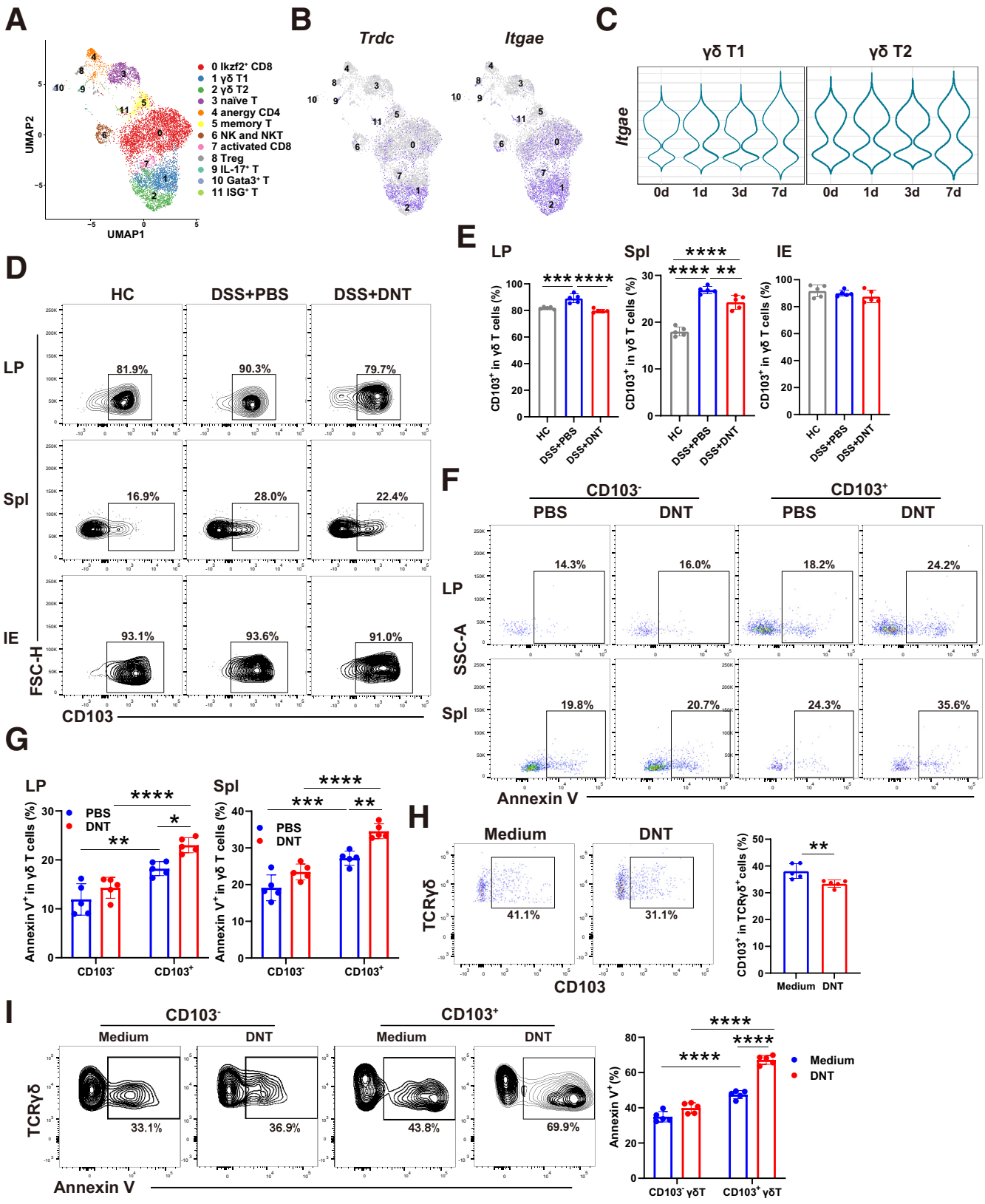


Figure 5. Regulatory DNT cells suppress γδ T cells in colitis mice. (*A*) Comparisons of γδ T-cell percentages in $CD3^+$ T cells obtained from the colon. (*B, C*) Flow cytometry analysis of IFN-γ and IL17A expression in γδ T cells obtained from the colonic LP and IE layer of mice. (*D*) Comparisons of Annexin V^+ cells in γδ T cells from the inflamed colon. (*E*) Evaluation of the capacity of DNT cells to induce γδ T-cell apoptosis in vitro. At least 2 independent experiments were performed with 5 mice in each group. The 1-way analysis of variance with multiple comparisons and the *t* test were used to compare the differences mentioned previously.


A
0 Ikzf2+ CD8
1 γδ T1
2 γδ T2
3 naïve T
4 anergy CD4
5 memory T
6 NK and NKT
7 activated CD8
8 Treg
9 IL-17+ T
10 Gata3+ T
11 ISG+ T
UMAP1
UMAP2
B
Trdc
Itgae
C
γδ T1
γδ T2
Itgae
0d 1d 3d 7d
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HC
DSS+PBS
DSS+DNT
LP
Spl
IE
81.9%
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79.7%
16.9%
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FSC-H
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E
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IE
CD103+ in γδ T cells (%)
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PBS
DNT
14.3%
16.0%
18.2%
24.2%
19.8%
20.7%
24.3%
35.6%
SSC-A
Annexin V
G
LP
Spl
PBS
DNT
Annexin V+ in γδ T cells (%)
CD103-
CD103+
H
Medium
DNT
41.1%
31.1%
TCRγδ
CD103
CD103+ in TCRγδ+ cells (%)
I
CD103-
CD103+
Medium
DNT
33.1%
36.9%
43.8%
69.9%
TCRγδ
Annexin V
Annexin V+ (%)
CD103- γδT
CD103+ γδT

demonstrate that the selective suppression of CD103$^+$ γδ T cells by regulatory DNTs occurs in murine colitis.

E-cadherin Connects DNT Cells and CD103$^+$ γδ T Cells to Facilitate the Selective Suppression

Given that DNT cells selectively suppress the colitogenic CD103$^+$ γδ T cells, CD103, as a binding integrin, was hypothesized to play a pivotal role in this suppressive response. By adding an anti-CD103 neutralizing antibody to the in vitro suppressive assay, the increase of Annexin V$^+$ cells in γδ T populations induced by DNT treatment was effectively blocked (Figure 8*A*). This finding substantiates the involvement of CD103 in the inhibitory effects of DNT on CD103$^+$ γδ T cells. To investigate the CD103-mediated recognition mechanism, we performed CellPhoneDB analysis using our scRNA-seq data. Notably, the E-cadherin and CD103 interactions were identified between the aDNT cluster and γδ T-cell clusters (γδ T1 and γδ T2) within the T-cell populations (Figure 8*B*). Unexpectedly, the aDNT cluster derived from the colon of DSS-treated mice exhibited elevated expression of *Cdh1* (encoding E-cadherin, the ligand for CD103) (Figure 1*E*), a crucial adhesion molecule involved in epithelial behaviors.[32–34] This observation was further substantiated by flow cytometry, which confirmed the up-regulation of E-cadherin expression in Granzyme B$^+$ DNT cells from the inflamed colonic LP (Figure 8*C*). Compared with CD4$^+$ and CD8$^+$ conventional T cells, mixed DNT cells from the spleen and lymph node of healthy mice contained a higher proportion of E-cadherin$^+$ cells, albeit at relatively low levels (Figure 8*D*). Following 3 days of in vitro stimulation, DNT cells further up-regulated their expression of E-cadherin, which was clearly higher than that observed in CD4$^+$ and CD8$^+$ T cells (Figure 8*E*). Notably, the expanded DNT cells used for adoptive transfer were predominantly positive for E-cadherin, in contrast to naive DNT populations (Figure 8*F*). These findings reveal a distinct expression of E-cadherin in DNT cells.

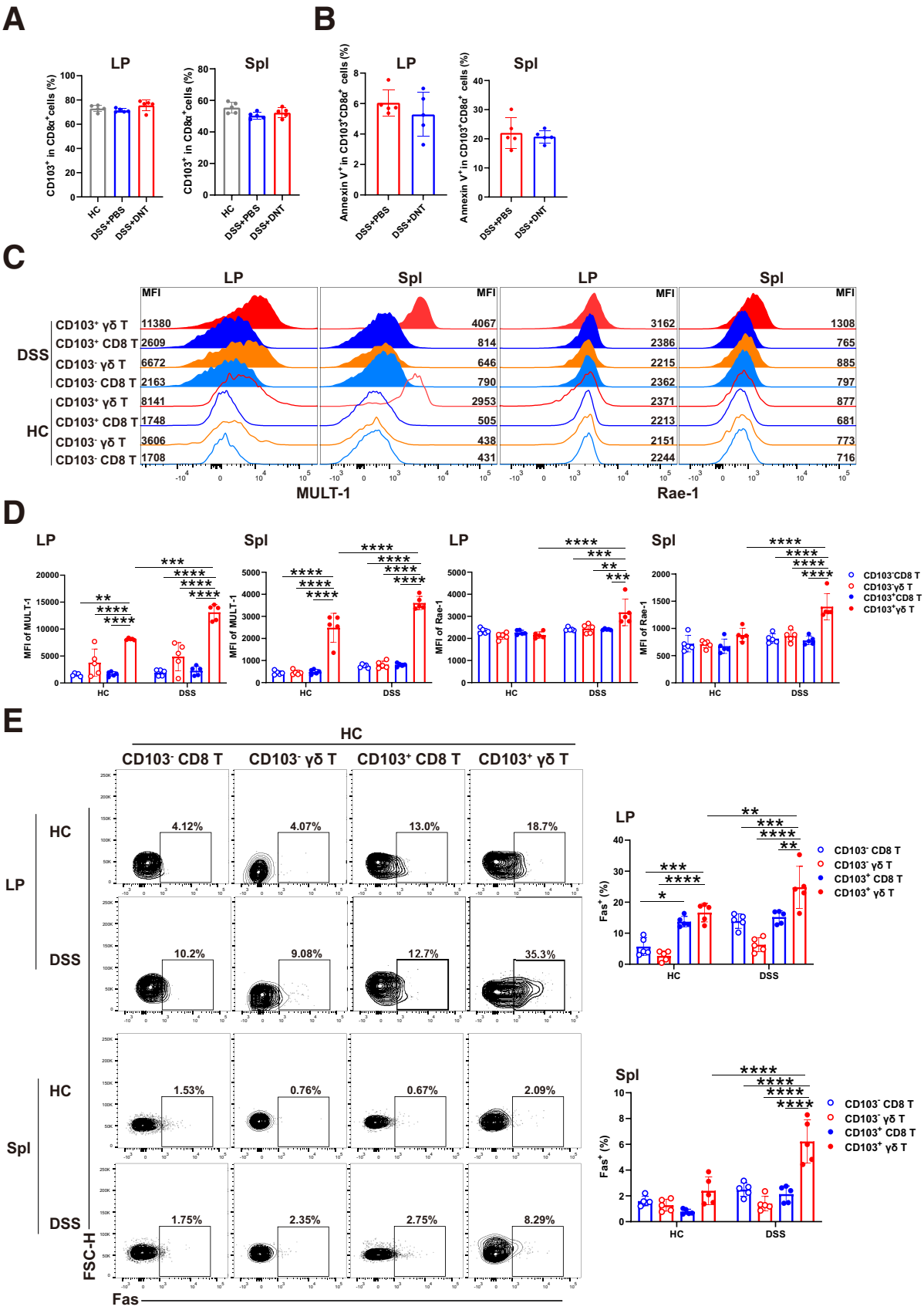
To explore the effect of E-cadherin in the suppression of CD103$^+$ γδ T cells by DNT cells, we introduced an anti-E-cadherin antibody to obstruct the E-cadherin-CD103 interaction within the coculture system. As anticipated, the blocking antibody diminished the apoptosis level of γδ T cells compared with the IgG control (Figure 8*G*). More CD103$^+$ cells in γδ T populations were observed in the coculture system following treatment with the E-cadherin blocking antibody (Figure 8*H* and *I*). On the addition of anti-E-cadherin, the DNT-mediated increase in Annexin V$^+$ cells among CD103$^+$ γδ T cells was also reversed (Figure 8*J*). By administering the anti-E-cadherin antibody to colitis mice treated with DNT cells, we obtained results consistent with those observed in our in vitro experiments. Compared with mice given DNT cells and isotype IgG, colitis mice receiving DNT cells combined with the anti-E-cadherin antibody exhibited impaired weight gain, diminished colon length, and heightened inflammatory cell infiltration, indicating that the blockade of E-cadherin interferes with the protective effects of DNT cells against colitis (Figure 9*A-C*). Both the colonic LP and spleen of colitis mice receiving E-cadherin blocking treatment had significantly elevated percentages of CD103$^+$ γδ T cells (Figure 9*D*). Moreover, CD103$^+$ γδ T cells from the colonic LP and spleen of mice subjected to the E-cadherin blockade showed markedly decreased apoptosis levels and contained higher proportions of cells expressing IFN-γ and IL17A, compared with those from colitis mice treated with DNT cells and isotype IgG (Figure 9*E-G*). Collectively, these findings illustrate that E-cadherin serves as a bridge between DNT cells and CD103$^+$ γδ T cells, thereby mediating the suppressive effects of DNT cells on γδ T-cell populations.

Additionally, we observed that NKG2D blockade significantly attenuated DNT cell-induced apoptosis in total γδ T cells and CD103$^+$ γδ T cells in vitro (Figure 10*A* and *B*). Consistently, the colonic LP and spleen of colitis mice treated with DNT cells and the anti-NKG2D antibody contained elevated percentages of CD103$^+$ γδ T cells with reduced apoptosis levels, as compared with those from mice administered DNT cells and isotype IgG (Figure 10*C* and *D*). These results together suggest that NKG2D-NKG2DL interaction may synergize with E-cadherin-CD103 cell contact to promote the suppression of CD103$^+$ γδ T cells by DNT cells.

Regulatory DNT Cells Mitigate Allogeneic Murine Colitis

To enhance the clinical applicability of DNT cells in the treatment of colitis, we further examined whether allogeneic DNT cells could impede the progression of colitis. The possibility of graft-versus-host disease was initially excluded, because the adoptive transfer of BALB/c mice-derived DNT cells neither affected the weight gain of healthy C57BL/6 mice nor induced inflammatory infiltration in the peripheral organs (Figure 11*A* and *B*). After confirming the safety of allogeneic DNT cell therapy, we transferred DNT cells from BALB/c mice into C57BL/6 mice, subsequently constructing DSS-induced colitis. Anticipatedly, the adoptive transfer of allogeneic DNT cells also successfully reduced the disease activity index score and

Figure 6. (***See previous page***). **Regulatory DNT cells selectively suppress CD103$^+$ γδ T cells in colitis mice.** (*A*) UMAP analysis identified T-cell clusters in scRNA-seq data from the inflamed colon (CRA012515). (*B*) Feature plots of *Trdc* and *Itgae* expression in T-cell clusters. (*C*) Expression of *Itgae* in γδ T clusters as illustrated by the violin plots. (*D-G*) Mice were given DNT cells or PBS, and subsequently prepared for DSS-induced colitis. (*D*, *E*) Comparisons of CD103 expression in the colonic and splenic γδ T cells were conducted using flow cytometry. (*F*, *G*) The proportions of Annexin V$^+$ cells in CD103$^+$ and CD103$^-$ γδ T cells were also quantified. (*H*, *I*) γδ T cells isolated from the colitis mice were cultured with or without DNT cells for 24 hours and subsequently harvested for further analysis. Flow cytometry analysis of CD103 expression in γδ T cells was performed (*H*). The percentage of Annexin V$^+$ cells in γδ T cells was also examined (*I*). At least 2 independent experiments were performed with 5 mice in each group. The analysis of variance with multiple comparisons and the *t* test were used to compare the differences mentioned previously.


A
LP
Spl
CD103+ in CD8α+cells (%)
HC
DSS+PBS
DSS+DNT
B
LP
Spl
Annexin V+ in CD103+CD8α+ cells (%)
Annexin V+ in CD103+CD8α+ cells (%)
C
LP
Spl
LP
Spl
MFI
DSS
HC
CD103+ γδ T
CD103+ CD8 T
CD103- γδ T
CD103- CD8 T
11380
2609
6672
2163
8141
1748
3606
1708
4067
814
646
790
2953
505
438
431
3162
2386
2215
2362
2371
2213
2151
2244
1308
765
885
797
877
681
773
716
MULT-1
Rae-1
D
LP
Spl
LP
Spl
MFI of MULT-1
MFI of MULT-1
MFI of Rae-1
MFI of Rae-1
HC
DSS
CD103-CD8 T
CD103-γδ T
CD103+CD8 T
CD103+γδ T
E
HC
CD103- CD8 T
CD103- γδ T
CD103+ CD8 T
CD103+ γδ T
LP
Spl
HC
DSS
4.12%
4.07%
13.0%
18.7%
10.2%
9.08%
12.7%
35.3%
1.53%
0.76%
0.67%
2.09%
1.75%
2.35%
2.75%
8.29%
FSC-H
Fas
LP
Spl
Fas+ (%)
Fas+ (%)
CD103- CD8 T
CD103- γδ T
CD103+ CD8 T
CD103+ γδ T

restored the impaired weight gain of colitis mice (Figure 11C). As shown in Figure 11*D*, mice receiving allogeneic DNT cells exhibited longer colon compared with mice treated with PBS, which again substantiates the alleviation of colitis by allogeneic DNT cells. In addition, the $CD3^+$ T cells from the colonic LP and IE layer of allogeneic DNT-treated mice contained fewer $\gamma\delta$ T cells than those from mice treated with PBS (Figure 11*E*). An increased level of apoptosis in colonic $\gamma\delta$ T cells was also found in allogeneic DNT-treated mice (Figure 11*F*). In contrast to the effects of syngeneic DNT cell transfer, the suppression of IFN-γ and IL17A production in $\gamma\delta$ T cells by allogeneic DNT cells was only significant in the colonic LP but not in the IE layer of colitis mice (Figure 11*G*). Collectively, these findings indicate that regulatory DNT cells can alleviate colitis in allogeneic mice, at least in part, by suppressing $\gamma\delta$ T-cell responses.

Discussion

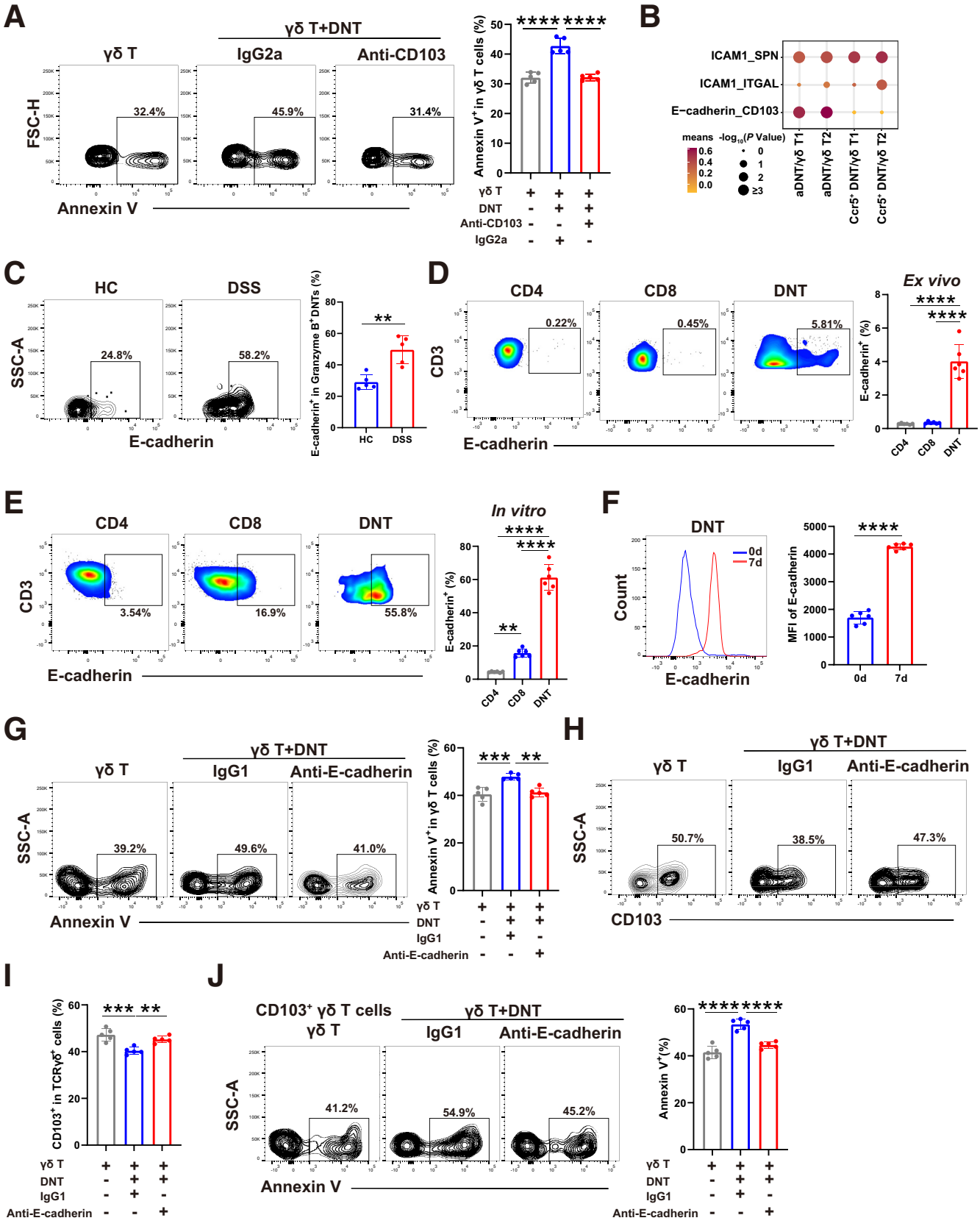
The interplay of disrupted microbiota, compromised mucosal barrier, and dysregulated immune response collectively contributes to the intestinal destruction.[35] Among the strategies to address colitis, it is widely acknowledged that maintaining a state of tolerance within the microenvironment is crucial for alleviating the sufferings.[36,37] Here, we demonstrate that regulatory DNT cells accumulate in the colon of DSS-treated mice, and the adoptive transfer of peripheral-derived DNT cells, which mimic the colonic regulatory populations, impedes the progression of colitis. Most importantly, E-cadherin is found to link DNT to $CD103^+$ $\gamma\delta$ T cells, thereby promoting the target recognition of DNT cells and ultimately ameliorating colitis.

DNT cells that possess a distinct feature of $CD3^+TCR\beta^+NK1.1^-CD4^-CD8^-$, are less studied compared with the conventional $CD4^+$ and $CD8^+$ T cells.[15,17] Both pathogenic and regulatory functions have been reported in peripheral DNT cells because of their inherent heterogeneity.[15,17] Pathogenic DNT cells are characterized by the production of IL17 and the promotion of autoimmune disorders, whereas regulatory DNT cells suppress inflammatory cells through mechanisms involving Perforin and Granzyme B–mediated or Fas-mediated apoptosis.[13,15,17] To elucidate the phenotypic landscape of DNT cells in the colon, we performed scRNA-seq on colonic cells from DSS-treated mice and identified 2 regulatory DNT clusters (aDNT and *Ccr5*$^+$ DNT clusters) alongside a proinflammatory DNT cluster (*Il17a*$^+$ DNT cluster). The scRNA-seq data indicate that regulatory and pathogenic DNT clusters increased during the progression of colitis; however, flow cytometry analysis confirmed only the elevation of Granzyme B^+ DNT cells in DSS-treated mice. Indeed, the effector molecules (NKG2D and FasL) of regulatory DNT cells also exhibited up-regulated expression in Granzyme B^+ DNT cells from the colonic LP of DSS-treated mice. The accumulation of these regulatory DNT cells within the inflamed colon implies that these cells are actively recruited to suppress the pathogenic immune responses. Consequently, we focused on the impact of Granzyme B^+ regulatory DNT cells on colitis, using peripheral-derived DNT cells, which exhibited high Granzyme B expression and minimal IL17A expression, as a mimic for the colonic regulatory DNT populations in prophylactic treatment assay. Remarkably, the adoptive transfer of DNT cells alleviated DSS-induced colitis.

To investigate the target cells of DNT cells in colitis, we examined the alterations in immune cell populations from the colitis mice treated with or without DNT cells. A pronounced reduction in the proportion of $\gamma\delta$ T cells was observed in the inflamed colon of mice receiving DNT cells, whereas other immune cell types remained relatively unaffected. The role of $\gamma\delta$ T cells in colitis is contentious, with some studies highlighting their protective functions against pathogen invasion, whereas others underscore their pathogenic potential by inducing excessive immune responses.[38,39] The divergent subsets of $\gamma\delta$ T cells, deriving from distinct origins and exhibiting specific TCR $V\gamma/V\delta$ chain usage patterns, have contributed to the inconsistent and conflicting outcomes observed in colitis models.[38,40] Given that the functions of $\gamma\delta$ T cells are highly context-dependent, cytokine profiles may facilitate a more precise delineation of their respective roles. Two major effector subsets of $\gamma\delta$ T cells can be categorized based on their cytokine production: $\gamma\delta$ T1 cells, which produce IFN-γ to target intracellular pathogens; and $\gamma\delta$ T17 cells, which secrete IL17, protecting against bacterial and fungal infections and contributing to autoimmune diseases.[38,40,41] Recent findings by Suhail et al[39] revealed that mice lacking $\gamma\delta$ T cells were resistant to DSS-induced colitis, and the depletion of $\gamma\delta$ T cells reduced the sources of IFN-γ and IL17A in colitis mice. Similarly, our findings showed that the adoptive transfer of DNT cells diminished the production of IFN-γ and IL17A in $\gamma\delta$ T cells from the inflamed colon during the amelioration of colitis.

$CD103^+$ $\gamma\delta$ T cells represent a pathogenic subset in intestinal inflammation.[27] The interaction between CD103 and E-cadherin promotes the retention of $\gamma\delta$ T cells in intestine.[42] Furthermore, the expression of CD103 on T cells has been reported to enhance T-cell receptor antigen sensitivity and boost their energetic potential, thereby enabling more rapid antigen recognition and augmenting IFN-γ production.[43] Thus, CD103 may endow $\gamma\delta$ T cells with the capacity to generate more robust and rapid immune responses

Figure 7. (***See previous page***). **Elevated expression of NKG2D ligands and Fas in $CD103^+$ $\gamma\delta$ T cells during colitis progression.** (*A, B*) Mice were given DNT cells or PBS, and subsequently prepared for DSS-induced colitis. (*A*) The percentages of $CD103^+$ cells in $CD8^+$ T cells obtained from the colonic LP and spleen of mice were compared. (*B*) The proportions of Annexin V^+ cells in colonic LP and splenic $CD103^+CD8^+$ cells were also evaluated. Flow cytometry analysis of MULT-1, Rae-1 (*C, D*), and Fas (*E*) expression in $\gamma\delta$ T and $CD8^+$ T cells isolated from the colonic LP and spleen of DSS-treated and healthy mice. Each experiment was repeated at least 2 times with 5 mice in each group. The analysis of variance with multiple comparisons and the *t* test were used to compare the differences mentioned previously.


A
γδ T+DNT
γδ T
IgG2a
Anti-CD103
FSC-H
Annexin V
32.4%
45.9%
31.4%
Annexin V⁺ in γδ T cells (%)
γδ T
DNT
Anti-CD103
IgG2a
B
ICAM1_SPN
ICAM1_ITGAL
E−cadherin_CD103
means
-log10(P Value)
aDNT/γδ T1
aDNT/γδ T2
Ccr5⁺ DNT/γδ T1
Ccr5⁺ DNT/γδ T2
C
HC
DSS
SSC-A
E-cadherin
24.8%
58.2%
E-cadherin⁺ in Granzyme B⁺ DNTs (%)
D
CD4
CD8
DNT
CD3
E-cadherin
0.22%
0.45%
5.81%
Ex vivo
E-cadherin⁺ (%)
E
In vitro
3.54%
16.9%
55.8%
F
Count
0d
7d
MFI of E-cadherin
G
IgG1
Anti-E-cadherin
39.2%
49.6%
41.0%
H
50.7%
38.5%
47.3%
CD103
I
CD103⁺ in TCRγδ⁺ cells (%)
J
CD103⁺ γδ T cells
41.2%
54.9%
45.2%
Annexin V⁺(%)

during colitis. In fact, the adoptive transfer of CD103$^+$ γδ T cells could enhance Th1/Th17 cell generation in intestine and aggravate the severity of disease.[27] Moreover, IE CD103$^+$ γδ T cells could exacerbate the pathogenic epithelial cell shedding via Granzyme release, which accelerates the intestinal injury.[42] Our data indicate that regulatory DNT cells mediate the selective suppression of CD103$^+$ γδ T populations in colitis, with E-cadherin playing a pivotal role in this process. As an epithelial cell effector molecule, E-cadherin has been shown to regulate spindle alignment and promote early stage T-cell development, while also facilitating CD8$^+$ T cells accumulation in salivary glands.[44,45] Recently, Davies et al[46] also reported that E-cadherin promotes CD8$^+$ T-cell invasion into biliary epithelial cells, a process thought to be correlated with biliary injuries in primary biliary cholangitis. Nevertheless, the additional effects of E-cadherin on T cells remain largely unexplored. In our study, we demonstrated that E-cadherin bridges regulatory DNT cells to CD103$^+$ γδ T cells and mediates the selective suppression. Beyond the E-cadherin-CD103 contact, the interaction pairs, such as NKG2D-NKG2DL, also constitute a pivotal element in the recognizing approach through which DNTs suppress γδ T cells. Indeed, blockade of either E-cadherin or NKG2D markedly attenuates the suppression of CD103$^+$ γδ T cells by DNT cells. Additionally, the elevated expression of NKG2D ligands and Fas in inflammatory CD103$^+$ γδ T cells, which could respectively promote DNT cell regulatory function and facilitate FasL-Fas mediated apoptosis, supports the notion that DNTs selectively suppress CD103$^+$ γδ T cells without affecting other CD103 positive populations, such as CD8$^+$ T cells. Thus, these data preliminarily demonstrate that the recognition complex comprising E-cadherin-CD103 and NKG2D-NKG2DL pairs collaboratively enhances the suppression of CD103$^+$ γδ T cells by DNT cells.

To augment the clinical potential of regulatory DNT cells in alleviating colitis, we also introduced allogeneic DNT cells into the model of DSS-induced colitis. As expected, mice receiving allogeneic DNT cells demonstrated ameliorated colitis without inducing graft-versus-host disease, which may ensure a sufficient source of DNT cells in clinical settings and propel the development of "off-the-shelf" regulatory DNT cells for therapeutic applications.

Overall, among the heterogeneous populations of DNT subsets, regulatory DNT cells could accumulate in the inflamed colon and prevent colitis by suppressing γδ T-cell responses. E-cadherin establishes a connection between DNT cells and CD103$^+$ γδ T cells, thereby collaboratively enhancing the cytotoxicity of DNT cells toward γδ T-cell populations in conjunction with NKG2D. The adoptive transfer of syngeneic or allogeneic DNT cells may represent a novel therapeutic strategy for the treatment of IBD.

Materials and Methods

Mouse

GFP transgenic mice (C57BL/6-Tg[UBC-GFP]30Scha/J) and *Rag1*$^{-/-}$ mice were maintained on a C57BL/6 background and purchased from The Jackson Laboratory. Wild-type C57BL/6 and BALB/c mice were obtained from HFK Laboratory (Beijing, China). Mice were allowed to adapt for 5 days and then prepared for the colitis models. All mice were housed in a specific pathogen-free, comfortable temperature environment with a 12-hour light/dark cycle. The animal studies were performed in compliance with the guidelines of the Institutional Animal Care and Ethics Committee of Beijing Friendship Hospital (Approval NO.23-2020).

Expansion of DNT Cells In Vitro

To acquire sufficient DNT cells for in vivo assays, murine DNT cells (CD3$^+$TCRβ$^+$NK1.1$^-$CD4$^-$CD8$^-$) from the mixture of spleen and lymph node were sorted and subsequently cultured with dendritic cells in RPMI 1640 medium, which contained 10% fetal bovine serum and 50 ng/mL IL2 (PeproTech). Following 7 days stimulation, cells were collected and further purified according to DNT cells features (CD3$^+$TCRβ$^+$NK1.1$^-$CD4$^-$CD8$^-$) using a FACS Aria II cell sorter (BD Bioscience). The resulting DNT cells were used for subsequent analyses.

Induction and Clinical Evaluation of DSS-Induced Colitis

Seven-week-old mice were administered 2.5% DSS (MP Biomedicals) in drinking water ad libitum for 7 consecutive days, after which they were harvested for further analysis. To assess the impact of DNT cells on DSS-induced colitis, 5×10^6 DNT cells or an equivalent volume of PBS was adoptively transferred via tail vein injection. Following a 12-hour resting period, the mice were treated with DSS. To evaluate the effects of DNT cells on allogeneic murine colitis, C57BL/6 mice that received DNT cells derived from BALB/c

Figure 8. (***See previous page***). **E-cadherin links DNT cells to CD103$^+$ γδ T cells and facilitates the suppression.** (*A*) γδ T cells and DNT cells were cultured with anti-CD103 or isotype IgG2a in vitro. The percentage of Annexin V$^+$ cells in γδ T cells was measured. (*B*) Interactions between regulatory DNT clusters and γδ T clusters were identified using CellPhoneDB analysis (CRA012515). *P* value is represented by the size of the *circle*, whereas the average expression level of the interacting pairs is indicated by *color*. (*C*) Flow cytometry analysis of E-cadherin expression in Granzyme B$^+$ DNT cells from the colonic LP of mice. (*D*) Flow cytometry analysis of E-cadherin expression in CD4$^+$, CD8$^+$, and DNT cells isolated from a mixed population derived from the spleen and lymph node. (*E*) Following stimulation with dendritic cells for 3 days, the expression of E-cadherin in CD4$^+$, CD8$^+$, and DNT cells was quantified. (*F*) Comparison of E-cadherin expression between DNT cells expanded for 0 days and 7 days. (*G-J*) γδ T cells and DNT cells were cultured with anti-E-cadherin or isotype IgG1 in vitro. After 24 hours, γδ T cells were collected for further analysis. Comparisons of Annexin V$^+$ cell percentages (*G*) and CD103$^+$ cell proportions (*H, I*) in γδ T cells were conducted using flow cytometry. The apoptosis level of CD103$^+$ γδ T cells was also evaluated (*J*). At least 2 independent experiments were performed with 5 mice in each group. The 1-way analysis of variance with multiple comparisons and the *t* test were used to compare the differences described previously.

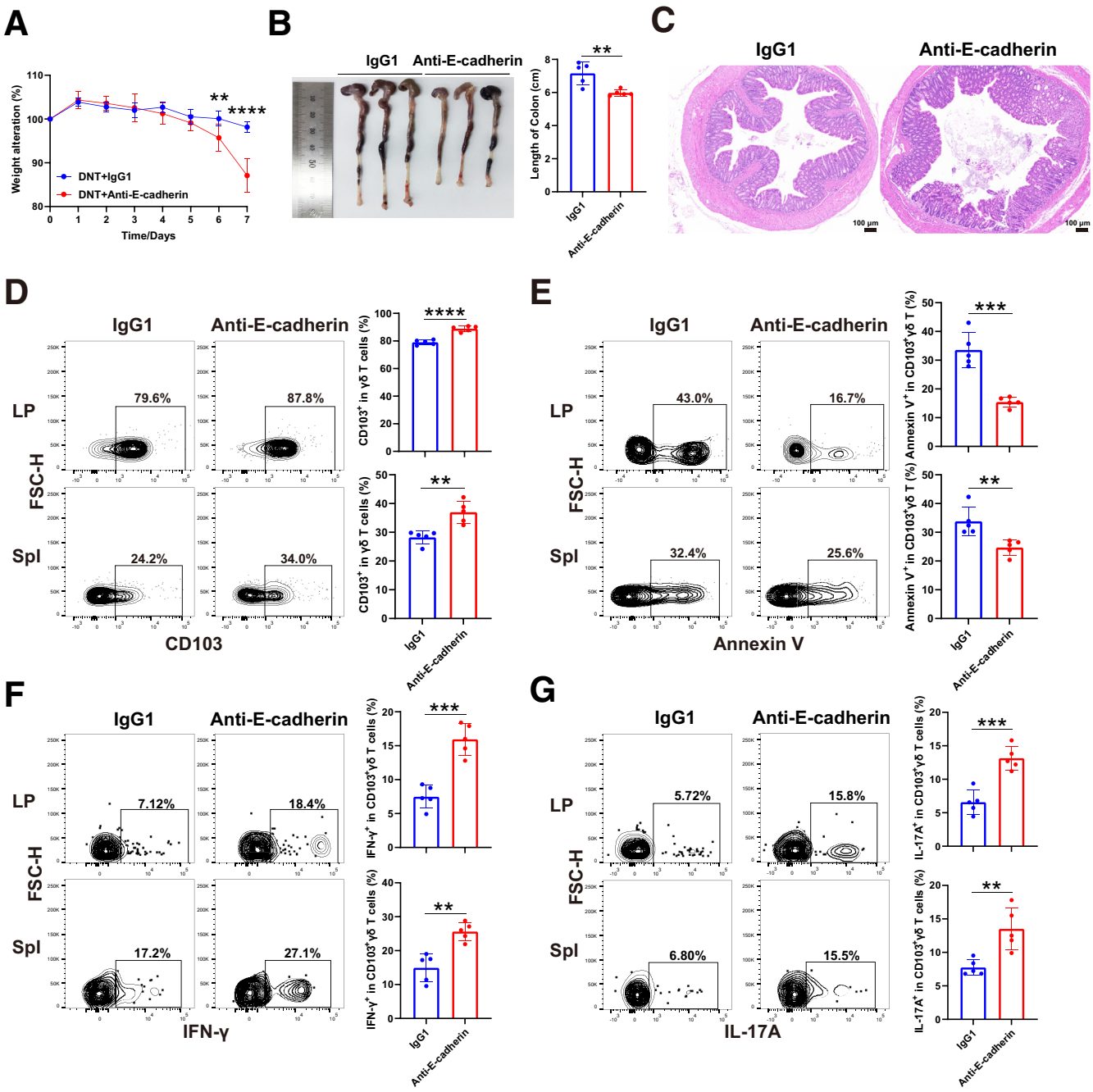


Figure 9. The blockade of E-cadherin impairs DNT cell-mediated suppression of CD103$^+$ γδ T cells. E-cadherin blockade was incorporated into the in vivo model in which DNT cells alleviated colitis. (A) Comparison of the weight alterations of mice. (*B*) Gross morphologic assessment of the colon. (*C*) Representative H&E staining of the colon sections. Comparisons of the percentages of CD103$^+$ cells in γδ T populations (*D*) and the apoptosis levels of CD103$^+$ γδ T cells (*E*) in the colonic LP and spleen. Flow cytometry analysis of IFN-γ (*F*) and IL17A (*G*) expression in CD103$^+$ γδ T populations from the colonic LP and spleen. At least 2 independent experiments were performed with 5 mice in each group. The 1-way analysis of variance with multiple comparisons and the *t* test were used to compare the differences described previously.

mice were subjected to DSS administration 12 hours post-transfer. To investigate the distribution of DNT cells in mice with colitis, DNT cells from GFP transgenic mice were expanded as described and transferred into both healthy mice and those slated to receive DSS. To elucidate the roles of E-cadherin and NKG2D in the suppressive effect of DNT cells on CD103$^+$ γδ T cells, DNT cells were preincubated with blocking antibodies targeting either E-cadherin or NKG2D, before adoptive transfer into mice. Following transfer, these mice were subjected to DSS treatment for 7 days. On Days 2 and 5, the mice were also intraperitoneally injected with 250 μg per dose of the corresponding

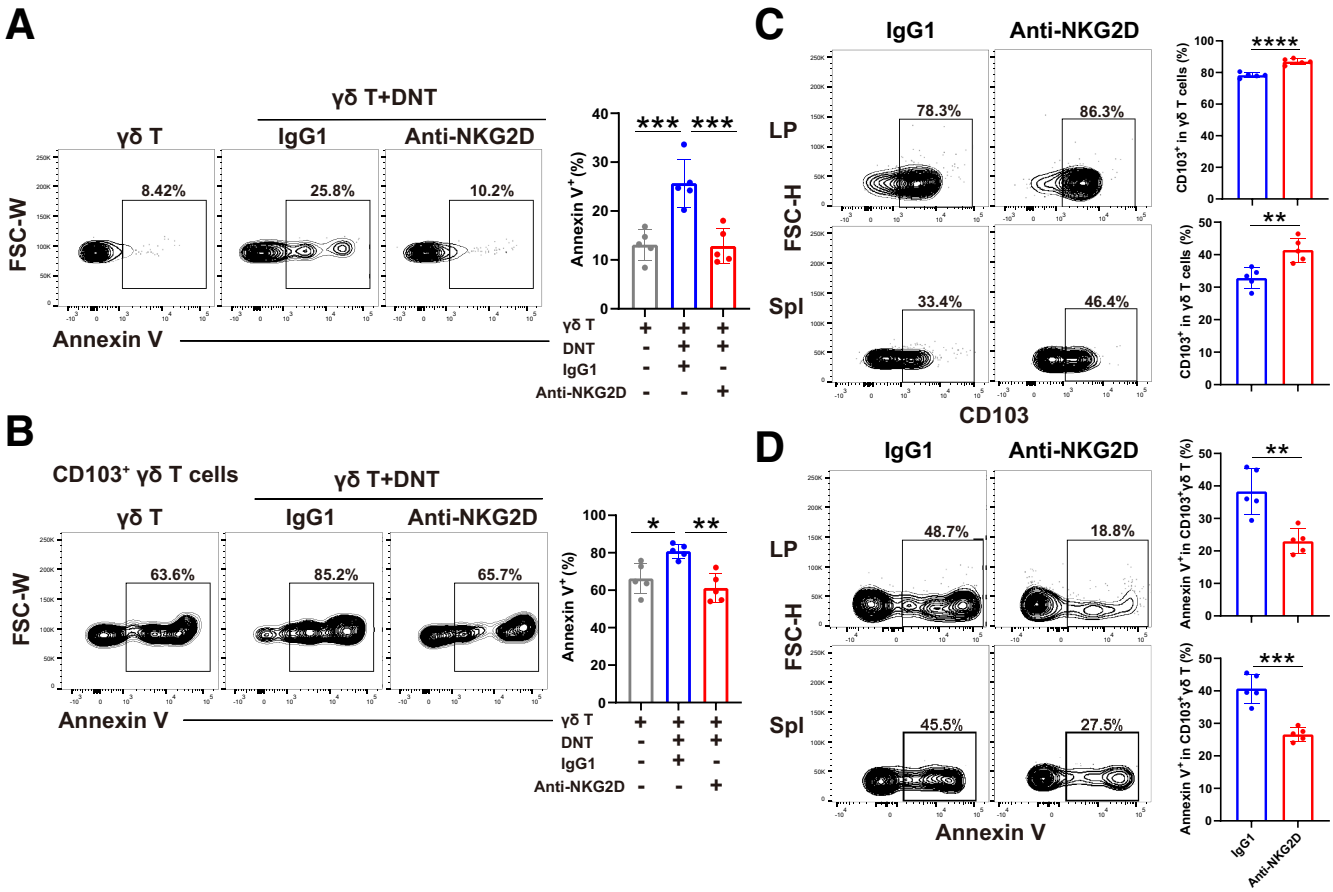


Figure 10. NKG2D blockade attenuates the suppressive effect of DNT cells on CD103$^+$ γδ T cells. (*A*, *B*) γδ T cells and DNT cells were cultured with anti-NKG2D or isotype IgG1. After 24 hours, the percentages of Annexin V$^+$ cells in γδ T cells (*A*) and in CD103$^+$ γδ T populations (*B*) were examined. (*C*, *D*) NKG2D blockade was introduced into the in vivo experimental model of DNT cells alleviating DSS-induced colitis. Flow cytometry analysis was performed to determine the percentages of CD103$^+$ cells in γδ T populations (*C*) and the apoptosis levels of CD103$^+$ γδ T cells (*D*). At least 2 independent experiments were performed with 5 mice in each group. The 1-way analysis of variance with multiple comparisons and the *t* test were used to compare the differences mentioned previously.

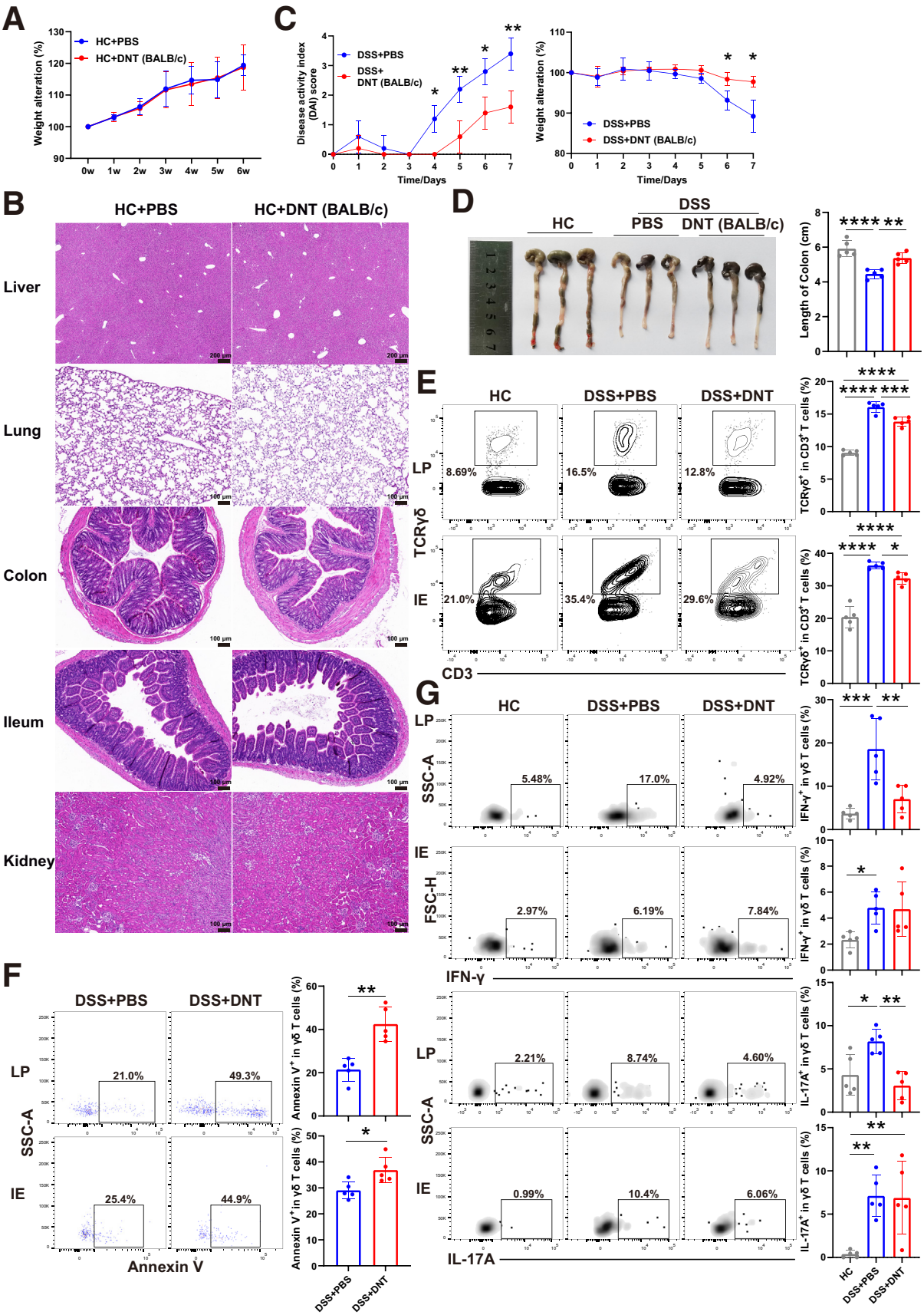
antibodies, respectively. The animals were subsequently sacrificed on Day 7 for further analysis. Daily monitoring was conducted to record the changes in body weight and stool characteristics. The disease activity index score was calculated based on the weight loss, stool consistency, and bleeding, with each parameter assigned a score ranging from 0 to 4. The total length of the colon was measured, and reductions therein served as indicators of colitis severity. In certain experiments, colons were collected, fixed in 4% paraformaldehyde, and subsequently embedded in paraffin for hematoxylin and eosin and alcian blue/periodic acid–Schiff staining.

Reagents

Antibodies against TCRγδ (GL3), TCRβ (H57-597), CD3 (17A2), Annexin V, IFN-γ (XMG1.2), NK1.1 (S17016D), IL17A (TC11-18H10), CD45 (A20), CXCR6 (SA051D1), CD4 (GK1.5), CD8α (53-6.7), Granzyme B (GB11), CD11b (M1/70), Ly6G (1A8), tumor necrosis factor-α (MP6-XT22), CD103 (2E7), Fas (SA367H8), FasL (MFL3), NKG2D (CX5), and E-cadherin (DECMA-1) were purchased from Biolegend. Anti-MULT-1 and anti-Rae-1 antibodies were obtained from R&D Systems. Before cell staining, Zombie Aqua and TruStain FcX PLUS (anti-mouse CD16/32) antibodies (Biolegend) were used to exclude dead cells and block nonspecific binding. For the intracellular staining of cytokines, cells were stimulated with Cell Activation Cocktail (with Brefeldin A) from Biolegend. After 6 hours, the cells were stained with antibodies against surface molecules and prepared according to the instructions of the Cyto-Fast Fix/Perm Buffer Set (Biolegend). Data acquisition was conducted using a FACS Aria II cell sorter (BD Bioscience).

Cell Isolations

Briefly, colons were meticulously sectioned into small pieces and incubated in the HBSS solution supplemented with 10 mM EDTA and 1 mM dithiothreitol on a shaker at 37°C for 20 minutes. The IE cells were dissociated into the supernatant of the culture system. Then, the remaining tissues were washed with PBS solutions and transferred to the


A
Weight alteration (%)
HC+PBS
HC+DNT (BALB/c)
0w 1w 2w 3w 4w 5w 6w
C
Disease activity index (DAI) score
DSS+PBS
DSS+ DNT (BALB/c)
Time/Days
Weight alteration (%)
DSS+PBS
DSS+DNT (BALB/c)
Time/Days
B
HC+PBS
HC+DNT (BALB/c)
Liver
Lung
Colon
Ileum
Kidney
D
HC
DSS
PBS
DNT (BALB/c)
Length of Colon (cm)
E
HC
DSS+PBS
DSS+DNT
LP
IE
8.69%
16.5%
12.8%
21.0%
35.4%
29.6%
TCRγδ
CD3
TCRγδ+ in CD3+ T cells (%)
G
HC
DSS+PBS
DSS+DNT
LP
IE
SSC-A
FSC-H
5.48%
17.0%
4.92%
2.97%
6.19%
7.84%
IFN-γ
IFN-γ+ in γδ T cells (%)
2.21%
8.74%
4.60%
0.99%
10.4%
6.06%
IL-17A
IL-17A+ in γδ T cells (%)
F
DSS+PBS
DSS+DNT
LP
IE
SSC-A
21.0%
49.3%
25.4%
44.9%
Annexin V
Annexin V+ in γδ T cells (%)
DSS+PBS
DSS+DNT

digestive solutions (RPMI 1640 solution with 5% fetal bovine serum, 10 mM HEPES, 0.5 mg/mL Collagenase D and DNaseI). After 30 minutes digestion, the cells were filtered through a mesh and suspended in a 35% Percoll solution for further purification via centrifugation. The resulting pellet was cells isolated from the colonic LP.

scRNA-seq Data Processing

In brief, colonic $CD45^+$ and $CD45^-$ live cells, sorted using flow cytometry, were mixed and sent to Annoroad Gene Technology (Beijing) for 10× Genomics scRNA-seq. *R* package Seurat (version 4.2.1) was used to process files derived from the Cell Ranger (version 7.0.0). Low-quality cells were removed based on the thresholds (gene counts <600, detected genes <500, and percentage of mitochondrial genes >20%). The Harmony algorithm was used to correct for batch effects, and uniform manifold approximation and projection was implemented via the RunUMAP function for dimensionality reduction and visualization. The FindClusters function (Seurat) was subsequently applied to uncover the cellular population structures. The identified cell clusters were preliminarily annotated as T, B, myeloid, and epithelial cells based on the expression of specific markers (*Cd19*, *Cd22*, *Cd79a*, *Cd3e*, *Cd3d*, *Cd3g*, *S100a9*, *S100a8*, *Cd14*, *Epcam*, *Krt8*, *Muc2*). DNT cells within the T-cell clusters were extracted based on the expression of *Cd4*, *Cd8*, *Klrb1c*, and TCR$\alpha\beta$ and TCR$\gamma\delta$-associated genes. A second round of unsupervised clustering was conducted to identify the refined DNT cell clusters using the previously mentioned methodology.

Cell-Cell Contact Analysis

To elucidate cell-cell interactions, the CellPhoneDB analysis was performed.[47] This analysis calculated the mean and significance ($P < .05$) based on the observed interactions and normalized cell matrix achieved through the Seurat normalization, enabling the identification of relevant interactions between different cell populations within the dataset.

Bulk RNA-sequencing

To examine the chemokine alterations in the inflamed colon, bulk RNA sequencing was conducted. RNA samples extracted from the entire colon of healthy and DSS-treated mice (7 days) were collected and sequenced using a standard Illumina protocol (Annoroad Gene Technology, Beijing). The sequencing reads were mapped to the mouse genome (Mm9) using HISAT2, and the gene counts were estimated via HTSeq. The *R* package DESeq2 was used to identify the differentially expressed genes (fold-change >2 and adjusted $P < .05$).

In Vitro Suppressive Assay

Expanded DNT cells isolated from the GFP transgenic mice (5×10^4 per well) were cocultured with $\gamma\delta$ T cells purified from the spleen and MLN of DSS-treated mice at a ratio of 1:1 for 24 hours in a round-bottom 96-well plate. To evaluate the impact of CD103-E-cadherin interactions on the suppression of $\gamma\delta$ T cells by DNT cells, 5 μg/mL anti-CD103, anti-E-cadherin, and isotype antibodies (IgG2a and IgG1) (Bio X cell) were added, respectively. Additionally, 5 μg/mL anti-NKG2D (BioLegend) was used to demonstrate the role of NKG2D in promoting the suppressive effect of DNT cells on $\gamma\delta$ T cells.

Statistical Analysis

GraphPad Prism 8 software was applied to perform the statistical analysis. Student *t* test was used to evaluate the significance of differences between 2 groups. For comparisons involving multiple groups, 1-way analysis of variance with multiple comparison test or 2-way analysis of variance was performed, as appropriate. Data are presented as mean ± standard deviation, with $P < .05$ considered significant ($^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$).

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Figure 11. (***See previous page***). **Regulatory DNT cells ameliorate allogeneic murine colitis.** BALB/c mice-derived DNT cells were transferred into healthy C57BL/6 mice (*A*, *B*) or into mice (C57BL/6) scheduled to undergo DSS treatment (*C*-*G*), respectively. (*A*) Comparison of weight changes between allogeneic DNT cells–treated and PBS-treated mice. (*B*) Representative H&E staining of the liver, lung, colon, ileum, and kidney sections. (*C*) Evaluations of disease activity index scores and weight changes in colitis mice. (*D*) Comparison of the colon length among the experimental groups. (*E*) Comparisons of the percentages of TCR$\gamma\delta^+$ cells in $CD3^+$ T cells isolated from the inflamed colon. (*F, G*) Flow cytometry analysis of Annexin V^+ (F), IFN-γ^+, and $IL17A^+$ (*G*) cell proportions in colonic $\gamma\delta$ T cells. At least 2 independent experiments were performed with 5 mice in each group. The analysis of variance with multiple comparisons and the *t* test were used to compare the differences mentioned previously.

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Conflicts of interest
These authors disclose the following: Dong Zhang and Dan Tian are inventors of a patent for the ex vivo generation of DNT cells (China). The remaining authors disclose no conflicts.

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