Running title: IL33 Remediation of dysbiosis potentiates the anti-toxin B antibody responses in a mouse model of CDI

IL33 protects from recurrent *C. difficile* infection by restoration of humoral immunity

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ABSTRACT

Clostridioides difficile infection (CDI) recurs in one of five patients. Monoclonal antibodies targeting the virulence factor TcdB reduce disease recurrence, suggesting that an inadequate anti-TcdB response to CDI leads to recurrence. In patients with CDI, we discovered that IL33 measured at diagnosis predicts future recurrence, leading us to test the role of IL33 signaling in the induction of humoral immunity during CDI. Using a mouse recurrence model, IL33 was demonstrated to be integral for anti-TcdB antibody production. IL33 acted via $ST2^+$ ILC2 cells, facilitating germinal center T follicular helper (GC-Tfh) cell generation of antibodies. IL33 protection from reinfection was antibody-dependent, as mMT KO mice and mice treated with anti-CD20 mAb were not protected. These findings demonstrate the critical role of IL33 in generating humoral immunity to prevent recurrent CDI.

KEYWORDS: *Clostridioides difficile*, IL33 signaling, ILC2s, dysbiosis, toxin-specific antibody,

TH17 cells, GC-TFH, recurrent *C. difficile* infection

Introduction

A unique and challenging aspect of *Clostridioides difficile* infection (CDI) is its tendency to recur in up to 25% of patients, a risk that increases with each subsequent recurrent episode(1, 2). Antibiotics targeting *C. difficile* are a double-edged sword in CDI, which despite treating the acute infection further disrupts the intestinal microbiome, predisposing patients to recurrence(3). The most effective therapy to prevent recurrent CDI (rCDI) is fecal microbiota transplantation (FMT) and newer microbiota therapeutics (e.g., SER109 and RBX2660); however, these are not 100% efficacious and have significant limitations (i.e., cost/logistical barriers and the risk of transmitting pathogens in the case of FMT)(4, 5).

Recurrent infection can partly be attributed to compromised adaptive immunity, suggesting a role for the immune system in preventing and managing repeated infections(6). *C. difficile* toxin B plays a key role in CDI pathogenesis(7-9), and exogenous IgG antibodies against toxin B are capable of averting rCDI(10, 11). Investigation into the role of immunoglobulins IgA, IgG, and IgM has been a consistent focus in studies involving human CDI patients(10). For example, reduced levels of antibodies (IgG, IgA) against TcdA and TcdB in the serum were linked to recurrence, whereas antibodies targeting cell surface antigens did not show any such correlation(12). Recent findings indicate that the presence of toxin B-specific IgG during acute CDI correlates with a delay in the onset of recurrence (11, 13).

Previous work has shown that IL33 prevents mortality and epithelial disruption by activating ILC2s in the acute mouse model of CDI(14). The microbiota influences IL33 expression, and dysregulated IL33 signaling predicts acute *C. difficile*-associated mortality in humans(14), emphasizing its crucial role in the defense against acute CDI. In this investigation, it is demonstrated that the type 2 alarmin IL33 serves as a biomarker of recurrence in humans. Additionally, utilizing a mouse model, the pivotal role of IL33 in antibody-dependent protection from recurrence is elucidated.

Results

IL33-induced toxin-specific antibodies in the *C. difficile* mouse model

Studies have shown that IL33 triggers the activation of ILC2s(14), which has the potential to enhance humoral immunity(15). Antibodies to *C. difficile* toxin B are known to prevent recurrence(10). We hypothesized that IL33 promotes ILC2-dependent anti-toxin antibody production. In the mouse model of acute (primary) CDI, antibiotics induce susceptibility by decreasing IL33 and subsequent IL33 activation of ILC2(14). The antibiotic-induced deficiency in IL33 in acute CDI could therefore predispose to recurrent infection by impairing the production of anti-toxin B antibody.

To investigate the role of IL33 in anti-toxin B antibody production, IL33 was first supplemented in the acute CDI mouse model. Prior to infection with the hypervirulent epidemic R20291 strain, mice were given antibiotics, and IL33 protein was administered daily for five days by intraperitoneal injection (0.75 μg/mouse) **(Fig. 1A).** IL33 treatment reconstituted the antibiotic-depleted IL33 protein level within the colon before infection (**Supplementary Fig. 1A).** As previously observed, acute CDI was less severe in IL33-treated mice as shown in the survival curve, weight loss, and clinical scores between the groups (**Figs. 1B, 1C and 1D**) (14). Toxin B-specific antibodies (IgG, IgM, and IgA) 15 days post-infection were higher in cecal contents and plasma of IL33-treated mice (**Figs. 1E, 1G, and 1H**). A similar IL33 induction of anti-toxin B antibody (IgG) was seen after infection with the classical *C. difficile* strain VPI 10463 (**Fig. 1F**). Survival, weight loss, and clinical scores for VPI strain infection were found similar to those of R20291 (data not shown). *C. difficile* burden at day 15 post-infection was unaltered by IL33 (**Supplementary Figs. 1B and 1C).** It is concluded that the administration of IL33 at the time of antibiotic pretreatment protected from acute CDI and enhanced the production of anti-toxin antibodies.

The ST2 receptor for IL33 is expressed on many immune cells including B cells(16). To test if the induction in antibody production was due to IL33 signaling via its receptor ST2, we compared antibody production in ST2 knockout and wild-type mice following IL33 administration **(Fig. 1I).** Cecal contents

and plasma were collected 15 days post-infection. As expected, IL33 did not induce the production of anti-TcdB antibodies (IgG, IgA, and IgM,) in ST2 KO mice **(Figs. 1J, 1K, 1L, and 1M).** As a negative control, we infected ST2 knockout mice without IL33 treatment and observed no significant difference in Toxin B-specific antibody production **(Supplementary Figs. 1D and 1E).** This led to the conclusion that IL33 exerts its effects through the ST2 receptor.

Decrease in severity of *C. difficile* reinfection by IL33

To test if restoration of IL33 during acute CDI could protect from reinfection, a murine model of reinfection was utilized (**Fig. 2**). C57BL/6J mice were infected on day 0 with *C. difficile* strain R20291 after pretreatment with antibiotics with or without exogenous IL33 administration. First, we established the mouse reinfection model of *C. difficile*. Of note, we did not find any difference in bacterial colonization between the groups throughout the infection trajectory (data not shown). On day 54, after recovery from the primary infection, the mice were treated with antibiotics before reinfection with 10^4 *C. difficile* spores from strain R20291 (**Fig. 2A**). Antibiotic re-treatment was important to clear the bacterial colonization, as *C. difficile* and toxin production were still detected even 100 days after primary infection, using a bacterial quantification kit (TechLab Inc., catalog #TL5025) and an ELISA kit to detect toxin B in the stool (TechLab Inc., catalog #T5015). Unlike the control mice, mice treated with IL33 during the primary acute CDI did not show clinical signs or lose weight upon reinfection (**Figs. 2B and 2C**). Further, IL33 treatment during acute CDI led to improved gut barrier function during reinfection (**Fig. 2D**). The treated group also experienced reduced submucosal edema and epithelial damage (**Figs. 2E and 2F**). It is concluded that IL33 restoration during primary CDI promoted gut integrity to protect from reinfection.

We then tested if IL33 could be used after a primary infection to prevent reinfection. IL33 was administered after the acute infection prior to rechallenge **(Supplemental Fig. 2A)**. The group who received IL33 before reinfection regained weight faster than the PBS recipient group **(Supplemental Fig. 2B)** and returned to a clinical score of zero faster than the PBS group **(Supplemental Fig 2C)**. The IL33-treated group also produced more IgM and IgG in the serum than the control group **(Supplemental**

Figs. 2D and 2E) suggesting that protection from reinfection may be achieved through increased toxin-specific antibody production. Of note, IL33 did not alter the colonization of the bacteria.

Importance of antibody production for IL33 mediated protection against reinfection

In order to determine if IL33-mediated protection against reinfection was mediated by antibody, mMT knockout mice that lack mature B-cells were pretreated with IL33 and infected with *C. difficile* (**Fig. 3A)** During the first ten days of the initial *C. difficile* infection, there was no difference in weight loss or clinical scores between wild-type (WT) and mMT knockout mice, consistent with prior work that showed no role of B cells and T cells in the acute phase of CDI (**Figs. 3B and 3C)**(14, 17). Interestingly, from day 11 onwards, WT mice gained significantly more weight than the mMT knockout mice, suggesting a role of antibodies in the sub-acute recovery phase of primary CDI. To confirm the absence of antibodies, plasma and stool IgG, IgM, and IgA were measured from the mMT knockout mice that did not produce toxin B-specific antibodies (**Supplementary Figs. 3A, 3B, 3C, 3D, 3E**) (18). Mice were retreated with antibiotics cocktails and reinfected on day 60 after the primary *C. difficile* infection. The mMT knockout mice lost more weight than the wild-type mice when given antibiotics and reinfection (**Fig. 3D**). Interestingly, mMT knockout mice had higher levels of toxin A/B in the stool (**Fig. 3E**), had increased gut permeability calculated by FITC-dextran gut permeability assay (**Fig. 3F**), and greater submucosal edema and epithelial damage (**Figs. 3G and 3H**). Samples were collected after the endpoint of the experiment, i.e., 11 days post-reinfection. Surprisingly, mMT knockout mice had a lower *C. difficile* bacterial burden as measured by a GDH ELISA kit (TechLab Inc., catalog #TL502) (**Supplementary Fig. 3F**).

Given the limitations of mMT knockout mice in producing IgE and IgG antibodies(19), we employed antiCD200 to deplete B cells (**Fig. 4A**). During acute CDI, the antiCD200 treated mice had slightly higher mortality (All but one of the mice were found dead) (**Fig. 4B**), but no difference in weight loss and clinical scores was found **(Figs. 4C and 4D).** Upon reinfection, the antiCD200 treated mice lost more weight (**Fig. 4E**), had higher clinical scores (**Fig. 4F**), and worse submucosal edema and epithelial damage (**Figs. 4G and 4H**). No toxin-specific antibodies (IgG, IgA) were detected at the end of

reinfection, as assessed from plasma and cecal tissue **(Figs. 4I, 4J, 4K, and 4L**). B cell depletion was confirmed in the colon and MLN **(Figs. 4M and 4N & 4V and 4W**). Interestingly, we observed that TH2 **(Figs. 4O& 4P)** and Treg cell populations **(Figs. 4S & 4U)** in the colon were significantly lower, and neutrophils were higher **(Figs. 4Q and 4R**) in the antiCD200 treated mice. This finding indicated a greater degree of inflammation in the colon of antiCD200 treated mice. The overall conclusion was drawn that antibody production was required for IL33-mediated protection from recurrent *C. difficile.*

The next question was whether IL33-mediated protection from recurrent *C. difficile* was strain-specific. The hypervirulent strain R20291 produces toxin B (TcdB2), which is antigenically distinct from the TcdB1 produced by the classical strain VPI 10463. The expectation was that if IL33 protection from recurrent R20291 infection was due to anti-TcdB2 antibody production, IL33 would not prevent recurrence from the classical VPI 10463 that produces TcdB1. 40 days after a primary infection with *C. difficile* strain R20291 with IL33 treatment, mice were reinfected with either R20291 or VPI 10463 (**Supplemental Fig. 4A**). Mice reinfected with a different strain (i.e., VPI 10463) than the strain used in a primary infection (i.e. R20291) lost more weight (**Supplemental Fig. 4B**) and had a more severe clinical score (**Supplemental Fig. 4C)** than the group that was reinfected with the same strain (i.e., R20291). It is concluded that the strain specificity of IL33 mediated protection against reinfection was consistent with its mediation by strain-specific anti-toxin B antibodies.

IL33-mediated increase in mucosal Type 2 immunity during primary *C. difficile* infection

To further understand cellular dynamics and the overall host response to the IL33 treatment in dysbiotic mice before and after the first *C. difficile* challenge, immunophenotyping was done on the host's innate and adaptive immune response. First, the immune population in infected and noninfected groups was checked after the recovery phase (16 days post-infection). Neutrophils and TH17 cells were significantly higher in the *C. difficile-infected* mice even 16 days after infection, indicating type 3 immunity dominates and persists beyond the resolution of CDI **(Supplemental Fig. 5A, 5B, 5C, 5D)**. During primary CDI, IL33 treatment led to an increase of ILC2s and a decrease in ILC1 and ILC3 populations decreased in the mesenteric lymph nodes (MLN) (**Figs. 5A, 5B, 5C, and 5D**) and colon

(Supplemental Figs. 6A, 6B, 6C and 6D). Additionally, IL33 remediation of dysbiosis led to an increase in TH2 and a decrease in TH1 cell populations in the MLN (**Figs. 5E, 5F, 5G, 5H, 5I, 5J, 5K, 5L, 5M, 5N, 5O, and 5P**) and the colon (**Supplemental Figs. 6E, 6F)** both before and after the *C. difficile* challenge. Flow cytometry at days 2 and 6 after primary CDI demonstrated an IL33-induced downregulation of TH17 cells and upregulation of Treg cells in MLN (**Figs. 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 6I**) and colon (**Supplemental Figs. 6G, 6H, and 6I**). In line with our previous study, IL33 increased colonic eosinophils and decreased inflammatory monocytes (Ly6C high populations) (**Supplemental Figs. 6J, 6K, 6L, and 6M**) (14). We concluded that IL33 treatment during acute CDI promoted a long-lasting innate and adaptive type 2 immune response in the intestine and MLN.

IL–33–mediated increase in activated mesenteric lymph node GC-TFH cells during primary *C. difficile* infection

CDI induces an inferior IgG response and is associated with a lack of T follicular helper cell (TFH) expansion (20). We hypothesized that IL33 protection from recurrent CDI was due to TFH expansion to promote anti-toxin B antibody. We chose to measure Tfh cells on days 0 and 6 as this is consistent with the expected time it takes for Tfh cells to differentiate in the germinal center (21, 22). In support of this hypothesis, we found that IL33 treatment increased activated GC-TFH by 1.3% to 4.6% of activated GC-TFH (**Figs. 6J, 6K, 6L, and 6M**). Gating strategies are described in **Supplementary Figure 7**.

Because IL33 could activate dendritic cells via the ST2 receptor, we tested activation markers on dendritic cells before (day 0) and at 2, and 5 days of infection **(Supplemental Figs. 8-8I).** There was a significant influx of CD11c-positive dendritic cells in MLN of the IL33-treated group prior to the infection; however, only a small number of these dendritic cells expressed activation markers such as CD86 **(Supplemental Figs. 8A, 8B, 8C)**. At day 2 post-infection, significantly more CD11c+ dendritic cells with higher expressing activation marker CD86 were found in MLN **(Supplemental Figs. 8D, 8E, 8F)**, but this difference was no longer observed by day 5 of infection **(Supplemental Figs. 8G, 8H, 8I)**.

We concluded that IL33 promoted anti-toxin B antibodies by TFH expansion and in part also by recruiting dendritic cells to the MLN.

Role of ILC2s in IL33-mediated protection from reinfection

We wanted to determine which primary or upstream cells responded to IL33 via the ST2 receptor. We hypothesized that ILC2s respond to IL33 to promote anti-toxin B antibodies and protect from reinfection, in part due to their known role in adaptive immunity(23) and due to the observed increase in ILC2s in the IL33 treated group after infection in the MLN and colon **(Figs. 5A, 5B, 5C, and 5D & Supplemental Figs. 6A and 6B)**.

To test the role of ILC2 in IL33-mediated anti-toxin B antibody production, $ST2^+$ ILC2s were isolated from the spleen, mesenteric lymph nodes, and colon of IL33-treated mice, expanded *in vitro*(24, 25), flow sort purified (S**upplemental Fig. 9)** and adoptively transferred into $ST2^{-/-}$ mice (**Fig. 7A**). Mice were infected with R20291 strain of *C.difficile* after a day of $ST2^+$ ILC2s adoptive transfer. During acute and reinfection, the $ST2^+$ ILC2 recipient group had slightly less mortality **(Supplemental Fig. 10A, and 10B)** but no difference in weight loss or clinical scores was found during the acute infection **(Supplemental Fig. 10C, and 10E)**. Whereas, upon reinfection, the $ST2^+$ ILC2 recipient group showed a modest effect on weight **(Supplemental Fig. 10D**), and clinical scores **(Supplemental Fig. 10F)**. $ST2^{-/-}$ mice that received $ST2^+$ ILC2s had increased plasma anti-toxin B IgG following primary challenge with *C. difficile* (**Figs. 7B**). The presence of donor $ST2^+$ ILC2s within the colon and MLN of recipient $ST2^{-/-}$ mice was confirmed (**Figs. 7C, 7D, and 7E**). $ST2^+$ ILC2 recipient mice had an increase in activated GC-TFH population (**Figs. 7F and 7G**). Upon reinfection, $ST2^+$ ILC2 recipient mice had improved gut permeability**(Supplemental Fig. 10G)**, significantly less epithelial damage (**Figs. 7H and 7I**). We did not find any difference in the bacterial load and toxin level in the cecal content **(Supplemental Fig. 10H, and 10I).**

To further validate the role of ILC2s in IL33-mediated anti-toxin B antibody production, we utilized ROSA26-DTR^{Nmur1} mice (26). These mice express improved Cre (iCre) recombinase and enhanced

GFP (eGFP) from the regulatory elements of Nmur1, allowing for selective Cre and reporter expression in ILC2s. The mice also have a loxP-flanked STOP cassette upstream of the open reading frame of the simian diphtheria toxin receptor (DTR) gene. Therefore, ILC2s selectively and constitutively express DTR, allowing for near-complete depletion of ILC2 upon administration of diphtheria toxin (DT). Employing repeated injection of diphtherial toxin (DT) in $Nmur1^{iCre\text{-}eGFP}ROSA26^{LSL\text{-}DTR}$ (n = 10) mice or $ROSA26^{LSL\text{-}DTR}$ littermates (n = 10) with IL33 treatment and *C. difficile* infection as shown in (**Fig. 7J**), we validated that ILC2s depletion at the endpoint of the experiment (**Fig. 7K**). We observed that depletion of ILC2s abrogated IL33 induced toxin B specific antibody production estimated in plasma and cecal content antibodies collected at 15 dpi. (**Fig. 7L**). ILC2-depleted mice exhibit a limited impact on mortality **(Supplemental Fig. 10J)** but increased morbidity based on weight loss **(Supplemental Fig. 10K)** and clinical scores **(Supplemental Fig. 10L)** measured during the acute infection. ILC2 depletion had no impact on the bacterial count **(Supplemental Fig. 10M)** or toxin A/B level **(Supplemental Fig. 10N)**.

Both experiments conclusively demonstrate that IL33 facilitates the production of toxin-specific antibodies through the activation of group 2 innate lymphoid cells (ILC2s). Moreover, findings from adoptive transfer experiments establish that these ILC2-mediated antibodies confer protective immunity against reinfection **(Graphical abstract).**

IL33 is a biomarker for recurrent *C. difficile* infection

Utilizing a commercial multiplex proximity extension assay, IL33 was measured in the blood of 56 hospitalized patients with CDI (within 48 hours of diagnosis) and 17 healthy controls. Patient details including demographics, comorbidities, etc. are described in **Supplementary Table. 1**. Among the *C. difficile*-infected patients, 12 developed recurrent infections, and 5 died within eight weeks. IL33 was elevated in uncomplicated CDI (median 0.309 pg/mL) compared to healthy controls (median 0.068 pg/mL; Wilcoxon $P<0.001$). Only 3 out of the 45 cytokines measured were significantly different between patients who developed recurrent infection and those who did not. These cytokines included IL33, C-X-C motif chemokine 10 (CXCL-10), C-C Motif Chemokine Ligand 3 (CCL3), and Tumor

Necrosis Factor (TNF) (**Supplemental Figs. 11A). Figure 11B** presents ROC curve analysis for univariate and multivariable logistic regression models predicting recurrent *C. difficile* infection within 8 weeks. The AUC value for IL33 was 0.72(95% Cl: 0.53-0.9) and for IL33 with CXCL10, CCL3, and TNF was 0.71(95% Cl: 0.5-0.91). The univariate model, which includes only IL33, performs similarly to the multivariable model that incorporates all four significant cytokines (IL33, CXCL-10, CCL3, and TNF). This suggests that while CXCL-10, CCL3 and TNF are altered in patients with recurrent infection, they do not significantly improve the predictive power of the IL33 model, which performs comparably in univariate and multivariable settings. IL33 was higher in patients who went on to develop recurrent infection (median 0.600 pg/mL; P=0.031) compared to uncomplicated infection (**Figs. 8A and 8B**). Immunohistochemical staining revealed abundant anti-IL33 staining of colonic epithelium from three non-recurrent and three recurrent human CDI patients (**Fig. 8C**). Different T cell populations, including Th1, Th2, and Th17 cells, were measured in peripheral blood samples from patients with non-recurrent [uncomplicated CDI (n=16)], recurrent CDI (n=10), and controls (n=15) **(Supplementary Figs. 12A and 12B)**. While the numbers of Th1, Th2, and Th17 cells were significantly lower in infected patients compared to controls, no significant differences were observed between non-recurrent and recurrent CDI patients. Additionally, the percentage of $CD4^+$ cells within these populations showed no significant variation across the groups. Based on these findings, we conclude that IL33 serves as a biomarker for recurrent CDI.

Discussion and Conclusion

C. difficile patients with the highest quartile serum IL33 levels (>0.641 pg/mL) measured at diagnosis were more than 2.5 times more likely to develop recurrent infection within the following 8 weeks compared to patients with lower IL33, suggesting IL33 could serve as an early biomarker for reinfection. The addition of CXCL-10 and TNF does not significantly improve the performance of a univariate IL33 predictive model for recurrence. We employed a mouse model of reinfection to elucidate the role of IL33 in rCDI. Our investigation revealed that IL33 exerts a protective effect against

CDI reinfection by activating group 2 innate lymphoid (ILC2) cells. ILC2 activation promoted humoral immunity against *C. difficile.* The transfer of $ST2^{+}$ ILC2s to $ST2^{-/-}$ mice alone stimulated the production of toxin B-specific antibodies and promoted the formation of T follicular helper (TFH) cell populations, which is consistent with earlier studies that ILC2 contributed to the development of T follicular helper (TFH) cells and antibody production(27).

The IL33 signaling pathway plays a crucial role in promoting the humoral immune response and protecting against infection and reinfection by *C. difficile* colitis via the action of ILC2s. We observed that post-antibiotic dysbiosis, the administration of IL33 resulted in elevated toxin B-specific antibody production, thereby conferring protection against toxin-induced epithelial damage and morbidity upon reinfection. To check whether the protection during reinfection is due to the IL33-induced anti-toxinB antibodies, we utilized the μMT knock-out mouse model where B cells are depleted. Considering the limitations of this model as these mice could produce IgG and IgE antibodies(19), the antiCD200 treatment mice model was used to reconfirm the results by depleteing *C. difficile* specific B cells. An important note is that antiCD200 does not deplete terminally differentiated antibody-producing B220+ plasmablast and plasma cells that do not express CD20(28). These plasma cells could confer non-specific B cell antibody-mediated protection. Combining both results, it was shown that IL33-mediated protection during reinfection was due to toxin-specific antibodies. Our investigation corroborates earlier work showing that IL33 facilitates the generation of IgA, contributing to the preservation of gut microbial homeostasis while mitigating IL-1α–induced colitis and colitis-associated cancer(29).

A likely target of humoral immunity is TcdB, which is recognized as the principal virulence determinant of *C. difficile.* TcdB has undergone expedited evolutionary changes, presumably in part to evade antibody neutralization. Clade 2 hypervirulent strains such as R20291 produce TcdB2, while VPI 10463 strains produce TcdB1(2). The disparities in the sequences of the two TcdB forms result in modifications to antigenic epitopes, diminishing cross-neutralization effectiveness by antibodies directed toward the C-terminal domain(30). Consistent with IL33 acting to prevent recurrence by

promotion of anti-TcdB antibodies, IL33 administration did not provide cross-protection from a different TcdB type.

The interaction between antigen-stimulated B cells and the T follicular helper (TFH) cell subset determines the subsequent course of immune activity, including antibody production. TFH cells play a pivotal role in supporting activated B cells through cognate interactions, which involve antigen-specific binding, and the secretion of functionally significant cytokines, thereby coordinating and enhancing the humoral immune response (20). GC-TFH cells orchestrate B cells in germinal centers, facilitating somatic hypermutation and class-switch recombination, resulting in high-affinity antibodies. GC-TFH cells express specific molecules including CXCR5 and PD1, which enable their precise modulation of B-cell interactions. Cytokines released by TFH augment B-cell differentiation into plasma cells, ultimately amplifying antibody secretion(31). Research has also shown that IL33 has the potential to boost humoral immunity through its interactions with TFH cells(32). We found an IL33-dependent increase in antibody production through increased GC-TFH activity.

There are several important limitations to this study. IL33 was upregulated in patients at the highest risk for recurrent CDI, suggesting that IL33 could predispose to recurrent infection. In addition, hospitalized patients with *C. difficile* infection were compared with healthy controls but were not adjusted for age, comorbidities, etc. The absence of a non-*C. difficile* hospitalized patient comparator group limits our ability to contextualize the role of IL33 outside of *C. difficile* infection; it remains unclear whether patient IL33 levels were specific to this infection or if similar or higher levels may be present in hospitalized patients without *C. difficile.* Also, our mouse data suggest that increased endogenous IL33 is more likely a physiologic response to high-risk infection, given the protective role of exogenous IL33 in stimulating protective humoral immunity.

We have shown an additional role for IL33 signaling in mitigating dysbiosis by downregulating TH17 and TH1 cells to create an environment inadequate for *C. difficile* disease. TH17 cells are crucial in elevating the risk of severe CDI by serving as a significant source of IL17A(33). The sole adoptive transfer of TH17 cells has the potential to heighten the severity of CDI(33).

Understanding the role of IL33 signaling in countering recurrent *C. difficile* infection (rCDI) through the ILC2-TFH axis is crucial for crafting potent CDI vaccines. A significant hurdle is addressing antigenic variation, notably in TcdB, demanding a nuanced approach for broad-spectrum defense. A successful vaccine must navigate these complexities to ensure comprehensive protection. IL33-associated mechanisms present a promising avenue for advancing CDI vaccine strategies, fostering optimism for more effective preventive measures. A proposition arises to enhance vaccine effectiveness by supplementing the antigen with a type 2-skewed adjuvant, amplifying IL33 signaling and fortifying protection against CDI recurrence.

In summary, our investigation reveals that IL33 prompts the expansion of ILC2s, which in turn, either directly or indirectly, amplify TFH cells, supporting B cells in antibody production. The resulting toxin-specific antibodies are essential in mitigating clinical illness due to reinfection**.**

Materials And Methods

Sex as a biological variable Our study examined male and female animals, and similar findings were reported for both sexes.

Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia (IACUC). C57BL/6J and mMT mice were purchased from the Jackson Laboratory, $ST2^{-/-}$ mice were obtained from Dr. Andrew McKenzie (Laboratory of Molecular Biology, Cambridge University, Cambridge, UK), $ROSA26^{LSL\text{-}DTR}$ (ROSA26iDTR; Jax 007900), transgenic Nmur1iCre-eGFP reporter mouse was gifted by David Artis group (26, 34). Sex-matched 8-to-12-week male or female mice were used in experiments. Animals were housed in a specific pathogen-free environment at the University of Virginia's animal facility. The bedding was exchanged every 2 days for a minimum of 3 weeks to equilibrate their microbiota. Mice were infected with *C. difficile* as previously described (14). In short, for 3 consecutive days, mice received an antibiotic cocktail in

drinking water consisting of 215 mg/L metronidazole (Hospira), 35 mg/L colistin (Sigma), 45 mg/L vancomycin (Mylan), and 35 mg/L gentamicin (Sigma), starting 6 days before infection. For the 3 days leading up to infection, regular drinking water was provided to the mice. One day before infection, clindamycin 0.016 mg/g (Hospira) was administered i.p. After infection, mice were monitored twice daily to evaluate clinical scoring parameters and weight loss over the course of infection and reinfection. The scoring criteria included weight loss, coat condition, eye condition, activity level, diarrhea, and posture (14). If mice reached a clinical score of 14 or lost more than 25% of weight, they were humanely euthanized. We defined the acute phase as when the mice have active diarrhea and lose weight. We defined recovery as when the mice started regaining weight.

Bacterial strains and culture

For the first infection, mice were infected with 5×10^4 CFU/mL vegetative cells of either R20291 or VPI 10643 (ATCC 43244) strain. For reinfection, 10^6 spores/ml of the R20291 strain or 5.2×10^4 spores/ml of the VPI 10643 strain were used. *C. difficile* strains were plated on BHI agar from glycerol stocks and incubated overnight at 37° C in an anaerobic chamber (35). Columbia, clospore, and BHI broth were reduced for at least 24 hours.

To generate spore stocks

A single colony was inoculated into 15 ml of Columbia broth overnight at 37 °C, and then 5 ml of this culture was added to 45 ml of Clospore broth anaerobically and left for 7 days at 37 °C (36). The culture was washed with cold sterile water at least 5 times and resuspended in 1 ml of sterile water. The spores were stored in a 1.5 ml twist cap tube at 4 °C (Corning # 4309309).

For vegetative infection

A single colony was inoculated in BHI medium overnight at 37 °C. The next day, cultures were washed twice with anaerobic PBS. The concentrations were measured by optical density for R20291 infection. For infection with the VPI 10643 (ATCC 43244) strain, the overnight culture was subcultured for 5 h

before optical density measurement. The needed concentrations of vegetative cells were prepared and loaded into a syringe with a gavage needle inside the anaerobic chamber. Each mouse received 100 µl (5×10^3 CFU for R20291 and 5×10^3 CFU for VPI 10643) of inoculum by oral gavage. The actual inoculum was further verified by plating on BHI agar supplemented with 0.032 mg/mL cefoxitin, 1 mg/mL D-cycloserine, and 1% sodium taurocholate (Sigma), and incubating anaerobically at 37 °C overnight. *C. difficile* burden was measured either by toxin A and toxin B specific qPCR on the DNA isolated from the stool or cecal content using a QIAamp Fast DNA Stool Mini Kit according to the manufacturer's instructions or by using an ELISA kit (TechLab Inc., catalog #TL5025) according to the manufacturer's instructions to quantify bacterial count, normalized to stool or cecal content weight. The TOX A/B II ELISA kit (TechLab Inc., catalog #T5015) was used per the manufacturer's instructions to quantify Toxin A/B, normalized to stool or cecal content weight.

Antibodies

Horseradish peroxidase (HRP)-conjugated anti-mouse IgM, IgG, IgG1, and IgA were purchased from Southern Biotech (Birmingham, AL), and goat anti-human IgG and Fcg fragment specific antibodies from the Jackson Laboratory (109035-098).

Tissue transcript and protein analysis

Tissue lysates were obtained by washing the ceca with 1X PBS and then homogenizing them for 1 minute in 300 µl of lysis buffer I, which contained 5 mM HEPES and 1X HALT protease inhibitor (Pierce). The tubes were then incubated on ice for 30 min after adding 300 µl of buffer II, containing 5 mM HEPES, 1X HALT protease inhibitor, and 2% Triton X-100. The supernatant was collected by centrifuging at 13,000 × g at 4° C, and the total protein concentration was measured using a BCA assay, following the manufacturer's instructions (Pierce). The mouse Duoset sandwich ELISA kit (R&D) was used to detect the IL33 in the cecal tissue lysates, according to the manufacturer's instructions.

For IL33 mRNA transcript analysis, the RNeasy Mini Kit from Qiagen and DNase digestion (TURBO DNA-free™ Kit, Invitrogen™) were used according to the manufacturer's instructions. RNA from cecal tissue was stored in RNAlater at -80 °C. Tetro cDNA Synthesis Kit (Bioline) was used to prepare the cDNA, and amplification of IL33 was accomplished by the Taqman IL33 Primer/Probe Set (Applied Biosciences, Mouse Assay ID: Mm00505403_m1). Normalization of gene expression was completed using HPRT and GAPDH housekeeping genes.

Flow cytometry

The colon was rinsed in a buffer containing HBSS, 25 mM HEPES, and 5% FBS. Dissociation buffer (HBSS with 15 mM HEPES, 5 mM EDTA, 10% FBS, and 1 mM DTT) was used to remove epithelial cells from the isolated colon for 40 minutes at 37 °C with 122 rpm agitation. Digestion buffer (RPMI 1640 containing 0.17 mg/mL Liberase TL (Roche) and 30 µg/mL DNase (Sigma)) was used to digest manually diced lamina propria for 40 minutes at 37 °C with 122 rpm agitation. After digestion, a 100 µM cell strainer followed by a 40 µM cell strainer (Fisher Scientific) was used to obtain single-cell suspensions. Extracellular staining was done with BB515-CD19 (BD 564509, dilution 1/25), PerCP-Cy5.5-CD5 (100624, dilution 1/100), PerCP-Cy5.5-CD3 (100218, dilution 1/100), PerCP-Cy5.5-FcεRIα (134320, dilution 1/100), BV510-CD90 (140319, dilution 1/25), BUV805-CD11b (368-011282, dilution 1/400), PE-CY5-CD64 (139332, dilution 1/75), APC-Fire 810-Ly6C (128055, dilution 1/400), BV785-CD45 (103149, dilution 1/200), NovaFluor Blue 610-70s-CD8a (M003T02B06, dilution 1/200), AF700-CD4 (100430, dilution 1/400), FITC-TCRbeta (159706, dilution 1/50), BV605-TCRgd (118129, dilution 1/200), BV650-CD11c(117339, dilution 1/100), APC Cy7-CD103 (121432, dilution 1/30), Pacific blue-CD40 (124626, dilution 1/50), AF647-CD80 (104718, dilution 1/100), PE-CD86 (105008, dilution 1/50), BUV 737-Ly6G (367-9668-82, dilution 1.25/100), Spark UV 387-MHCII (107670, dilution 1/400), BUV 563-SiglecF (365-1702-82, dilution 1/400), PE-Dazzle 594-CD127 (135032, dilution 1/100), PE Dazzle 594-CXCR5 (145522, dilution 1/100), FITC-CD44 (103005, dilution 1/100), BV510-PD1 (135241, dilution 1/100). Intracellular staining was done with BV421-Tbet (5563318, dilution 1/20), APC-RorgT (1769818, dilution 1/33), BV711-GATA3 (565449,

dilution 1/50), PerCP-eFluor 710-FOXP3 (46577382, dilution 1/100), and PE-fire 700-CD206 (141741, dilution 1/75). For surface staining, 1×106 cells/sample were Fc-blocked with TruStain fcX (BioLegend, #101320, dilution 1/200) for ten minutes at room temperature followed by the addition of LIVE/DEAD blue (Thermoscientific L34962) for 30 minutes at 4 °C. Cells were washed twice in FACS buffer (PBS + 2% FBS) and stained with fluorochrome-conjugated antibodies for 30 minutes at 4 °C. Cells were washed and resuspended in Foxp3 Fix/Perm WorkinFg Solution (ebiosciences, #00-5523-00) and incubated overnight at 4 °C. Cells were washed twice with permeabilization buffer and stained for 30 minutes at room temperature. Flow cytometry was performed on a Cytek Aurora (5-Laser) Spectral Flow Cytometer and analysis was done on Omiq software. All cell counts were normalized based on 80,000 live cell counts. SpectroFlo QC beads (SKU B7-10001) were used for routine performance tracking of the Cytek Aurora (5-Laser) Spectral Flow Cytometer. Unmixing was performed using single stains prepared on either cells or UltraComp eBeads™ Plus Compensation Beads (01-3333-42). All the gating strategies used in the article are presented in Supplementary Figure 10.

IL33 and antiCD20antiCD20 Treatment

Carrier-free recombinant mouse IL33 (Biolegend; Catalog #: 580504) was diluted with sterile PBS to prepare a 7.5 µg/ml solution. 100 µl was injected intraperitoneally daily for 5 days prior to the first infection or reinfection. antiCD20antiCD20 was a generous gift from Genentech. 250 µg of antiCD20antiCD20 per mouse was injected intraperitoneally on days -7, 3, 18, 33, 48, 63.

ELISA

96 well half-area assay plates (Corning) were used to detect toxin-B-specific antibodies. Plates were coated with 2 µg/ ml of toxin B in carbonate buffer (Sigma), a generous gift from Techlab, and kept at 4 °C overnight. The next day, plates were blocked with 1% bovine serum albumin (BSA) in PBS and 0.05% Tween 20 at 37 °C for 1 hour. Samples were added by diluting mouse sera, fecal supernatant, or cecal content in PBS-T, and plates were incubated for another 1 hour at 37 °C. *C. difficile* toxin B

monoclonal antibody, clone: A13I, from Invitrogen, was used as a positive control. Horseradish peroxidase (HRP)-conjugated IgG (1:5000), IgG1 (1:5000), IgM (1:1,000), or IgA (1:1000) was added after washing three times with PBS-T. Wells were developed by either 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate (KPL, Gaithersburg, MD) or Ultra TMB-ELISA substrate solution (Thermo Scientific) at room temperature for 10 minutes and stopped by either 1% SDS or 2 M H2SO4. Optical density for ABTS substrate was read at 410 nm with a background at 650 nm. The optical density for the TMB substrate was read at 450 nm with a background at 630 nm. A well-containing control plasma was used as the negative control.

ILC2 Adoptive Transfer Studies

Mesenteric lymph nodes (MLN), spleen, colon, and cecum were extracted from wild-type C57BL/6J mice that had received 5 daily doses of IL33 (0.75 µg). A single-cell suspension was prepared from the colon and cecum, as described above. For MLN and spleen, a 40 µM cell strainer (Fisher Scientific) was used to prepare a single-cell suspension. After passing through the 40 µM cell strainer, 1 ml of 1X red blood cell (RBC) lysis buffer (Thermo Scientific) was added for 1 minute. After centrifugation at 1600 rpm for 6 minutes, single cells were prepared in FACS buffer containing 2% heat-inactivated fetal bovine serum (FBS) in PBS. The single cells obtained from MLN, spleen, cecal, and colon were subjected to lineage-positive cell depletion by magnetic bead purification of lineage-negative (Lin^-) populations (bulk ILCs) (Miltenyi Lineage Cell Depletion Kit: 130-110-470). The lin^- cells were expanded *in vitro* in complete RPMI 1640 media containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml of IL33, and 10 ng/ml of IL-2 and IL-7 for 4 days. Cells were flow sorted on the Influx Cell Sorter (BD Biosciences) based on Lin^- (CD11c, CD3, CD5, CD11b, CD19, Fc epsilon R1 alpha with PECy7 fluorochrome) CD45+ CD90.1+ CD127+ CD25+ ST2+ expression after cell surface staining. Approximately 3^x10^5 ILC2s were transferred into each $ST2^{-/-}$ mouse.

Sex as a biological variable

Our study examined male and female sex for studies on human serum and PBMCs.

Detection of Human IL33 in serum

Male and female hospitalized patients between 18-90 years old with diarrhea and a positive CDI PCR test (nucleic acid amplification test (NAAT) GeneXpertSerum) were approached to be in the study. Patient demographics and clinical data were collected, and a follow-up was completed on all subjects to determine CDI recurrence and mortality after enrollment. Consented and enrolled subjects had 20 ml of blood drawn in EDTA tubes within 24 hours of CDI diagnosis. The blood was spun down at 2000xg for 15 minutes at room temperature and plasma was stored in aliquots at -80°C until use, at which point they were thawed on ice. Plasma was collected from 17 healthy donors without *C. difficile* infection, and 58 prospectively enrolled hospitalized patients within 48 hours of diagnosing *C. difficile* infection. Among *C. difficile*-infected patients, clinical outcomes (recurrent infection or death) were measured over an 8-week follow-up period. Recurrent *C. difficile* infection was defined as symptom relapse following completion of treatment for the index episode, requiring re-treatment. Serum cytokine concentrations were measured using a commercial multiplex proximity extension assay (Olink Proteomics; Watertown, MA). The lower limit of detection for our assay is 0.24 pg/mL. Any values below this threshold are reported as zero, which may influence our data interpretation. Concentrations of IL33 were compared between healthy controls, patients who survived the follow-up period without recurrent *C. difficile* infection, and those who developed a complication (recurrence or death). The collection and analyses of patient samples and healthy controls were approved by the University of Virginia Institutional Review Board (IBR-HSR18782 and HSR220013).

Mouse and human histology and immunohistochemistry

Proximal colonic sections were fixed in Bouin's solution and transferred to 70% ethanol after 24 h. Staining was done with either hematoxylin and eosin (H&E) or Periodic Acid Schiff (PAS) after preparing paraffin-embedded sections by the University of Virginia Research Histology Core. Two blinded observers scored histopathology using a scale from 0 to 3 for submucosal edema: 0 = none, 1

= mild, 2 = moderate, and 3 = intense/severe damage. Epithelial disruption and immune cell infiltration were scored using the same scale. Hemorrhage was scored as 1 = yes, 0 = no (37). Goblet cells were identified as PAS^+ and their number normalized to the number of crypts. Human biopsies sourced from the University of Virginia Biorepository and Tissue Research Facility were utilized. Researchers were kept blind to patient identities. The University of Virginia Biorepository Core conducted staining of human colon biopsy sections using a primary antibody targeting IL33 (R&D, AF3625, diluted at 1/80,000).

Flow cytometry of human PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by centrifugation to remove plasma, followed by Ficoll (Cytiva) density gradient centrifugation in SepMate tubes (Stemcell Technologies). The isolated PBMCs were counted, and 2 million cells were cryopreserved in liquid nitrogen until staining. Antibodies used for staining are listed in **Table 1**. Controls included unstained, single-stained, and fluorescence-minus-one (FMO)--stained PBMCs. A fixed viability stain (Live/Dead Blue; Thermo Scientific L23105) was applied to each sample. Samples were analyzed on a five-laser Cytek Aurora Borealis flow cytometer, with 130,000 cells collected per sample. Fluorescence data were analyzed using OMIQ to phenotype T cell subsets through traditional gating **(Supplementary Figs. 12C)**. Spectral deconvolution and gating were based on single-stained and FMO-stained PBMC control samples.

FITC-Dextran Gut Permeability Assay

To assess intestinal permeability, mice were orally administered a fluorescein isothiocyanate (FITC)-dextran solution (Sigma-Aldrich, #46944-500MG-F) at a dosage of 44 mg per 100 g body weight. Four hours post-administration, mice were euthanized, and serum samples were collected. The concentration of FITC-dextran in the serum was measured using a spectrophotometer set at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Statistical analyses

The Kaplan–Meier method was used to measure recurrence-free survival curves and to evaluate the effects of IL33 on risk for recurrent *C. difficile* infection. To account for competing risk against recurrent infection, death within the 8-week follow-up period was treated as a censoring event. A two-tailed t-test for normally distributed data,a Mann-Whitney test for non-normally distributed data (serum IL33), H, Šídák's for multiple comparisons were used to determine the statistical significance between groups. $P = 0.05$ is considered as the significant value. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) or R version 4.2.0 (R Core Team, Vienna, Austria).

Study approval

The collection and analyses of patient samples and healthy controls were approved by the University of Virginia Institutional Review Board (IBR-HSR18782 and HSR220013) Virginia. Subjects provided written informed consent prior to participation in the study. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia (IACUC) Virginia.

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Author Contributions

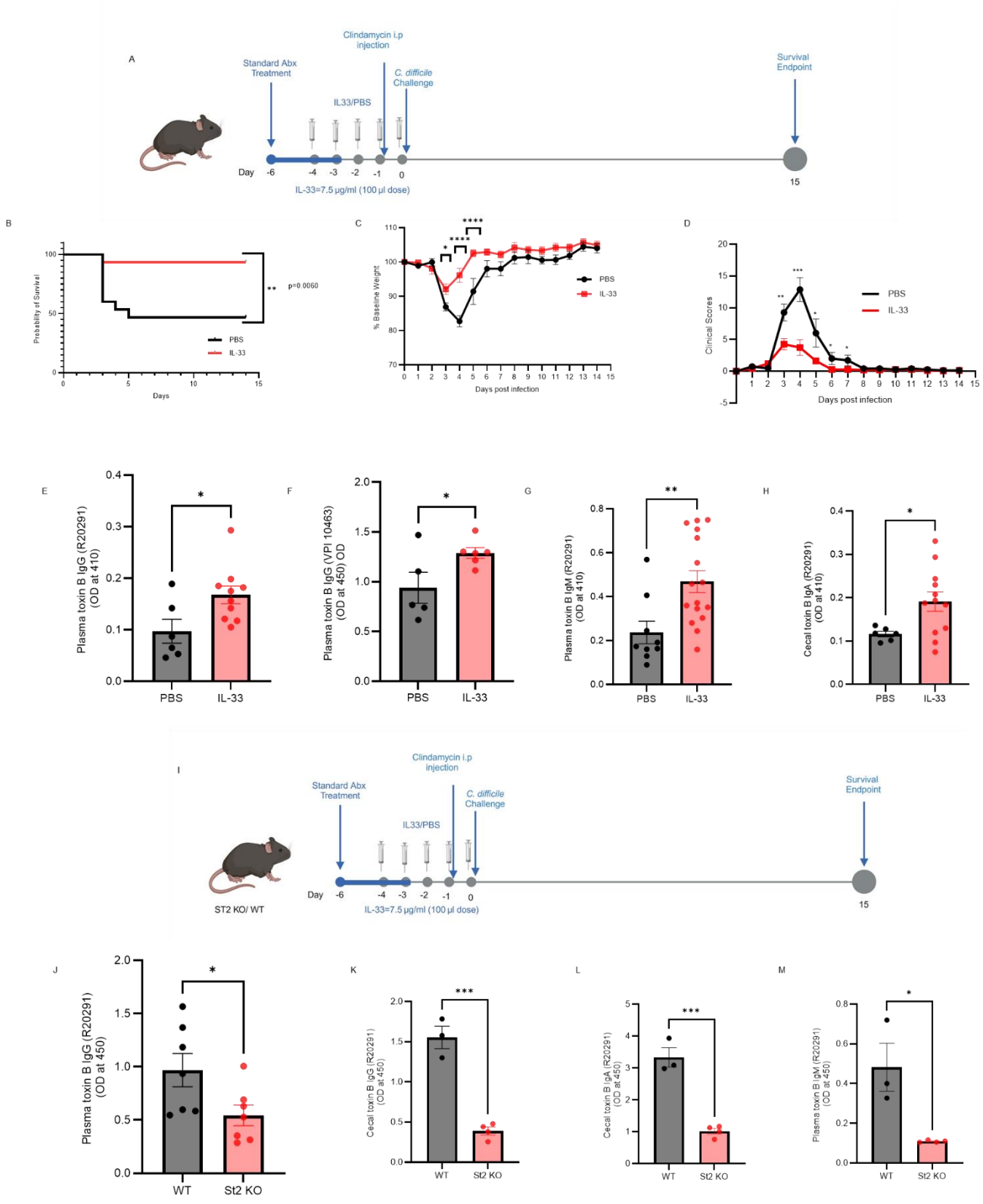
F.N. and W. A. P. designed all of the experiments. F. N. performed the experiments, analyzed and interpreted data, and wrote the manuscript. J. U. and N. H. helped with tissue extraction and processing. R. H. B. and A.B. helped with mouse tissue extraction. D.T. helped in tissue biopsies scoring. M.K.Y., G. R.; I. R.; and G. R. M. helped in human studies. C. F. helps in ILC2s knock-out mouse breeding. G. R. M., G. R., D.T., and W. A. P. edited the manuscript. W. A. P. supported all aspects of the work.

Competing interests

W.A.P. is a consultant for TechLab, Inc., which manufactures diagnostic tests for CDI. The authors declare no other competing interests.

Data availability Supporting data values for all the generated data is available in the XLS file for reference. Further information and requests for reagents will be fulfilled by the corresponding author William Petri (wap3g@virginia.edu) or Farha Naz (Ymw4xw@virginia.edu).

Figures



564

565 **1: IL33 increases toxin-specific antibody in mice after first infection with *C. difficile***

IL33 (0.75 µg) was administered i.p. on days -4 to 0 to C57BL/6J mice (A–H) and/or ST2−/− mice (I-M). Mice were infected with *C. difficile* strain R20291 (A-E and G-M) or VPI 10463 (F). On post-infection day 15 antibodies were measured in plasma and cecal content. (A) Schematic diagram showing infection and treatment timeline; (B) survival curves; (C) weight loss; (D) clinical scores; (E) plasma toxin B specific IgG from mice infected with *C. difficile* strain R20291 ; (F) plasma toxin B specific IgG from mice infected with *C. difficile* strain VPI 10463; (G) plasma toxin B specific IgM from mice infected with *C. difficile* strain R20291 ; (H) cecal content toxin B specific IgA from mice infected with *C. difficile* strain R20291. (I-M) WT vs ST2-/- mice infected with *C. difficile* strain R20291 : (I) Schematic diagram showing infection and treatment timeline; (J) plasma IgG; (K) cecal IgG; (L) cecal IgA; and (M) plasma IgM. B, Comparison made by log-rank test (n = 30). C, D, Comparison made by two-tailed Student's t-test (C, D n = 30). E (n=16), F (n=11), G (n=25), H (n=18), J (n=14), K (n=7), L (n=7), M (n=7), A two-tailed t-test for normally distributed data and a Mann-Whitney test for non-normally distributed data were used. *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM.

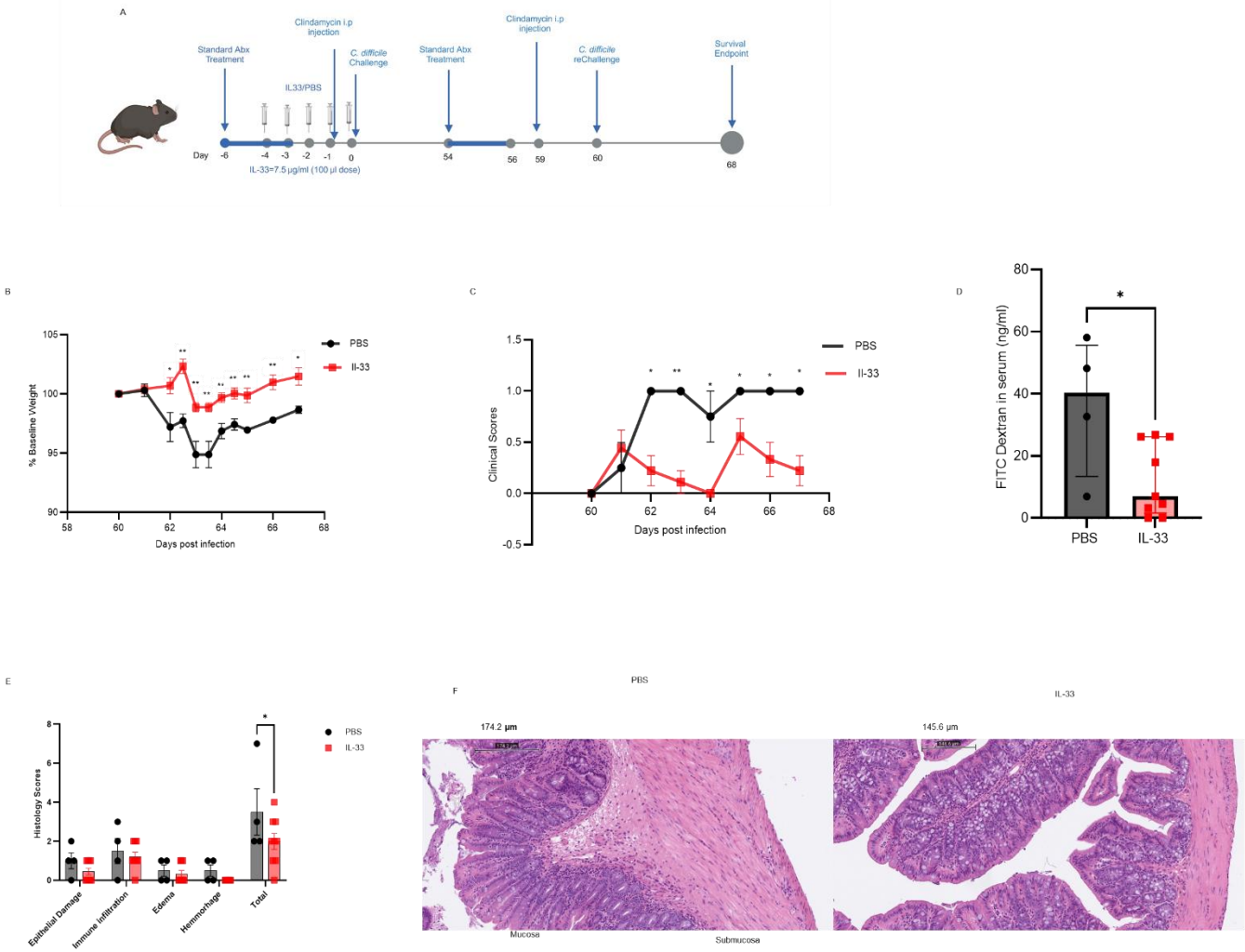
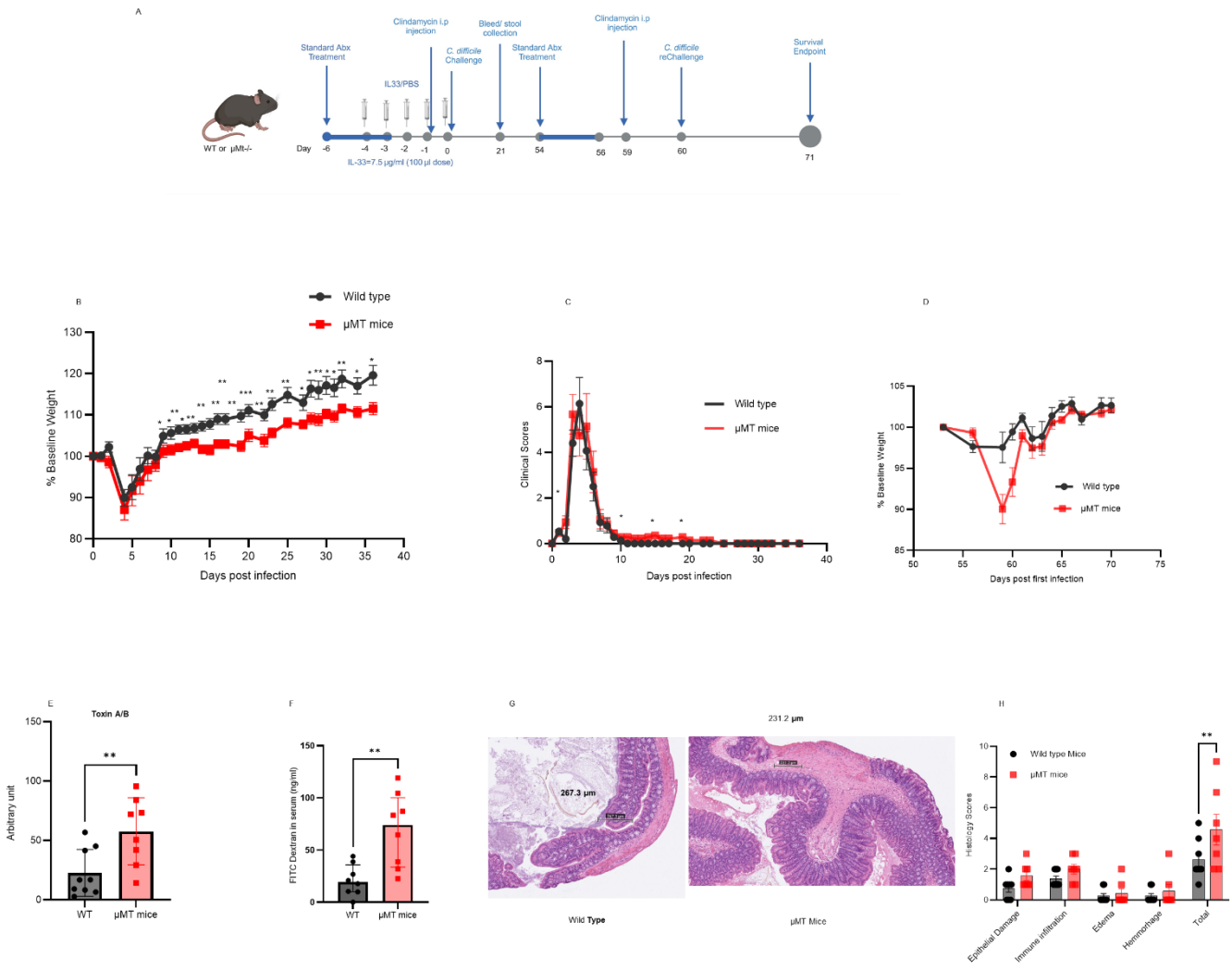


Fig 2: IL33 protects from a 2nd *C. difficile* infection

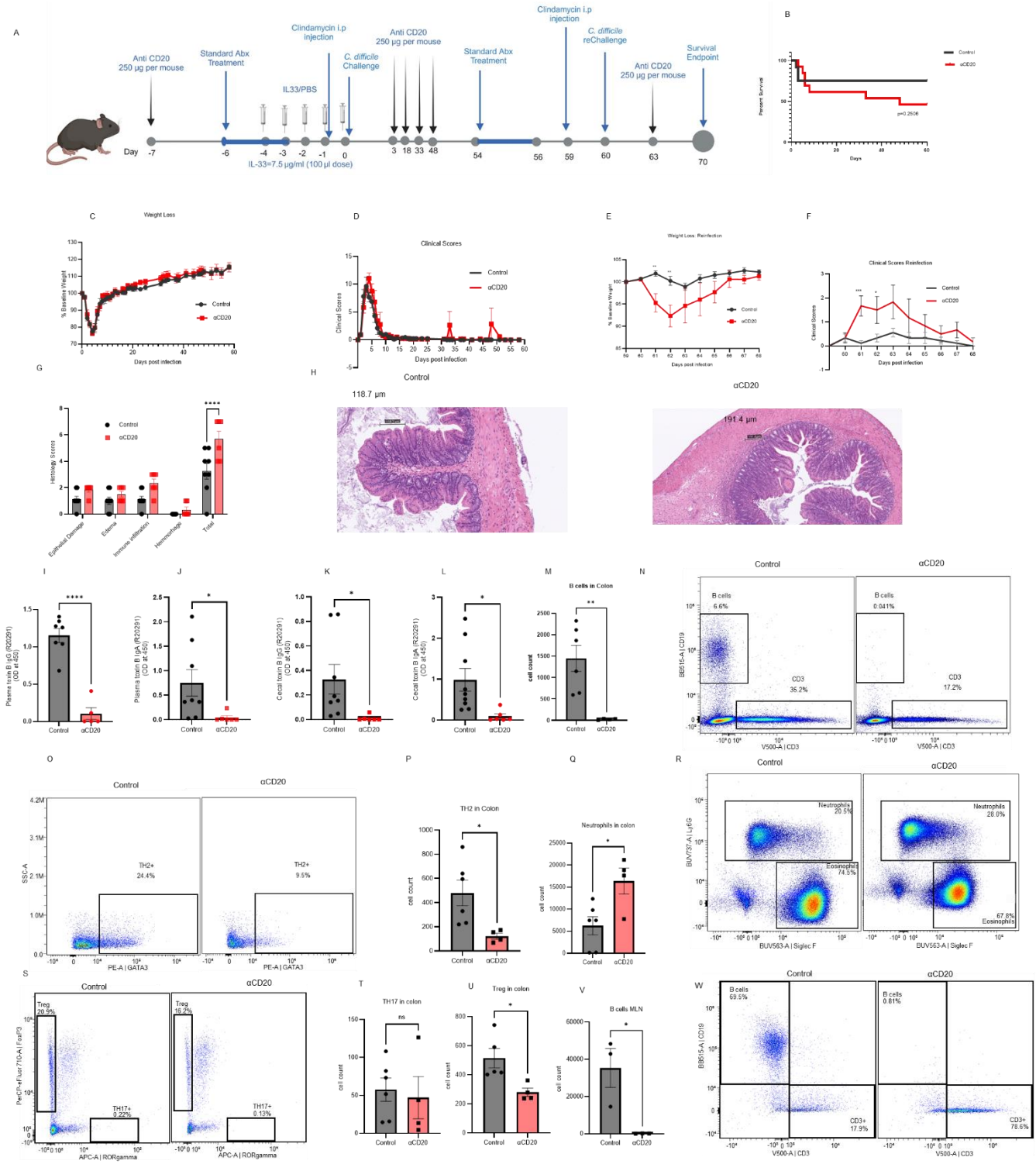
IL33 (0.75 µg) was administered i.p. on days -4 to 0 and wild-type mice were infected on day 0 and again on day 60 with *C. difficile* strain R20291. (A) Experimental design for 2nd infection; (B) 2nd infection weight loss (n=13); (C) clinical scores (n=13); (D) FITC-dextran gut permeability test (n=13); (E) epithelial damage scoring (n=13); (F) representative H&E stain of the colon. B, C, Comparison made by two-tailed Student's t-test. D Mann-Whitney test for non-normally distributed data was used E, Šídák's multiple comparisons test was used to determine the statistical significance between groups. Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM in B, D, and E but D indicates the median with interquartile range.



592

593 **Fig 3: IL33 protection from a 2nd *C. difficile* infection is antibody-dependent**

594 WT (n=15) and mMT KO (n=15) mice were administered IL33 (0.75 µg) i.p. on days -4 to 0 and mice
595 infected on day 0 and reinfected on day 60 with *C. difficile* strain R20291. (A) Experimental design;
596 (B) 1st infection weight loss; (C) clinical scores. WT and mMT KO mice were reinfected with *C.*
597 *difficile* R20291 60 days after the first infection. Reinfection (D) weight loss; (E) Stool *C. difficile* toxin
598 A and B measured by ELISA kit (Techlab); (F) FITC-dextran gut permeability assay; (G) H&E stain;
599 (I) epithelial damage scoring. B, C, D, Comparison made by two-tailed Student's t-test (D n=18). E, a
600 two-tailed t-test was used and the error bar indicates SEM. F, a Mann-Whitney test was used and the
601 error bar indicates the median with interquartile range. H, Šídák's multiple comparisons test was used.
602 Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001.



603

604 **Fig 4: Antibody deficient mice (antiCD20 treated) lost IL33 mediated protection from a 2nd *C.***

605 ***difficile* infection**

antiCD20 was administered to deplete B cells on days -7, 3, 18, 33, 48, and 63, and mice infected on day 0 and 2nd time infected on day 60 with *C. difficile* strain R20291. (A) Experimental design; (B) survival curve; (C) 1st infection weight loss; (D) 1st infection clinical scores; (E) reinfection weight loss ; (F) reinfection clinical scores (G) epithelial damage scoring; (H) H&E stain. (I) toxin B specific plasma IgG; (J) IgA; (K) cecal IgG; and (L) cecal IgA measured on day 10 post 2nd infection. MLN and colon were harvested on day 10 post-reinfection. Colonic (M, N) B cells (CD45+ CD3- CD19+); (O, P) TH2 cells (CD45+CD3+ CD4+ GATA3+); (Q, R) neutrophils; (S, T, U) Treg (CD45+CD3+ CD4+ FOXP3+)and TH17(CD45+CD3+ CD4+ RORγt+) cells; and (V, W) MLN B cells. B, Comparison made by log-rank test (n = 26 in both groups). C, D, E, F Comparison made by two-tailed Student's t-test (C, D n = 26, E, F n=15). G, Šídák's multiple comparisons test was used. I, J, K, L, M, P, Q, T, U, V A two-tailed t-test for normally distributed data and a Mann-Whitney test for non-normally distributed data were used. Statistical significance is demarked as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < <0.0001. The error bar indicates SEM.

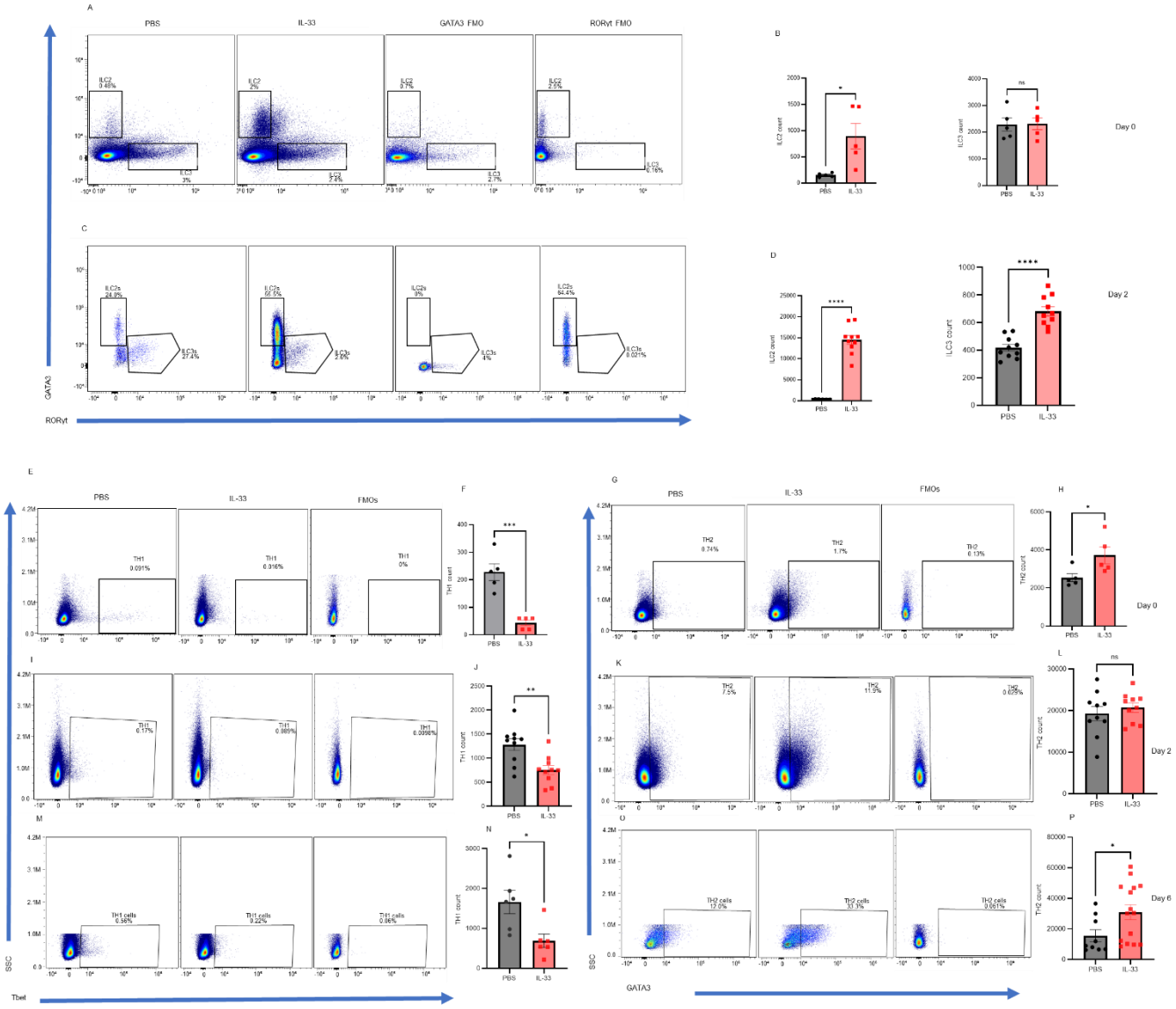


Fig 5: IL33 increased mesenteric lymph node ILC2 and decreased TH1 cells during 1st *C. difficile* infection

IL33 (0.75 µg) was administered i.p. on days -4 to 0 and mice infected on day 0. (A, B) ILC populations on day 0, prior to infection, and (C, D) at 2 days post-infection. TH1, TH2 populations (E-H) before infection, day 0; (I-L) day 2; and (M-P) day 6 post-infection. A two-tailed t-test for normally distributed data and a Mann-Whitney test (B, F, H n=10, D, J, L n=20) for non-normally distributed data were

used. Statistical significance is demarked as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < <0.0001. The error bar indicates SEM.

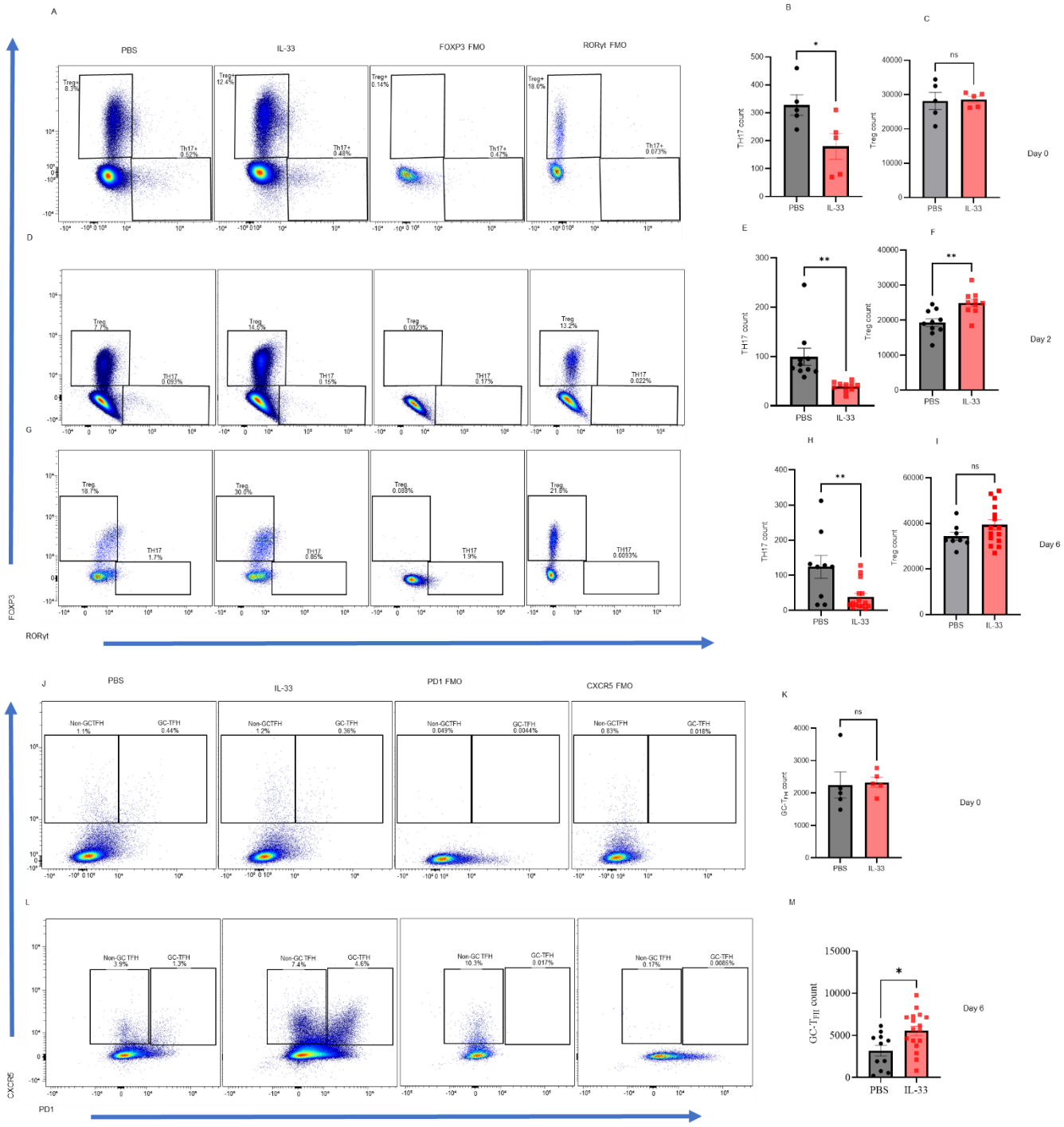
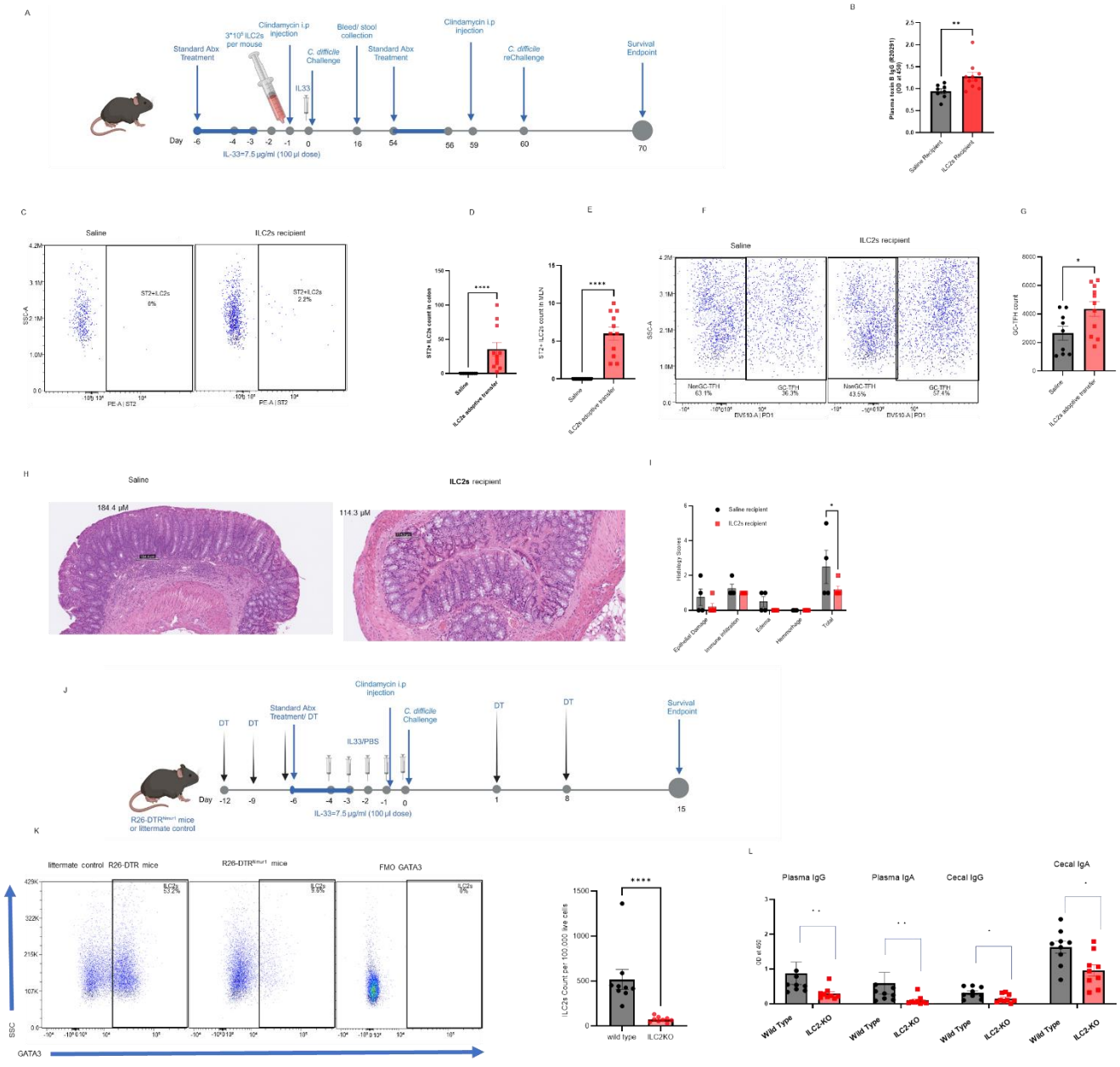


Fig 6: IL33 increased mesenteric lymph node GC-TFH, Tregs, and decreased TH17 cells during a primary *C. difficile* infection

IL33 (0.75 µg) was administered i.p. on days -4 to 0 and mice infected on day 0. Mesenteric lymph nodes were harvested to analyze T helper cells before infection, day 2, and day 6 post-first infection. (A-C) TH17 cells (CD45+, CD3+, CD4+, RORgt+) and Treg cells (CD45+, CD3+, CD4+, FOXP3+) on day 0 prior before infection (n=10); (D-F) on day 2(n=20); (G-I) on day 6 post-infection (n=24). (J-M) TFH cells were defined as germinal center (GC) TFH (CD45+ CD3+ CD4+ CD44+ PD1 high CXCR5+) and non-GC TFH cells (CD45+ CD3+ CD4+ CD44+ PD1 low CXCR5+) by flow cytometry. (J-K) TFH subsets on day 0 prior to infection (n=10); (L-M) TFH subsets on day 6 post-infection(n=28). A two-tailed t-test for normally distributed data and a Mann-Whitney test for non-normally distributed data were used. Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM.

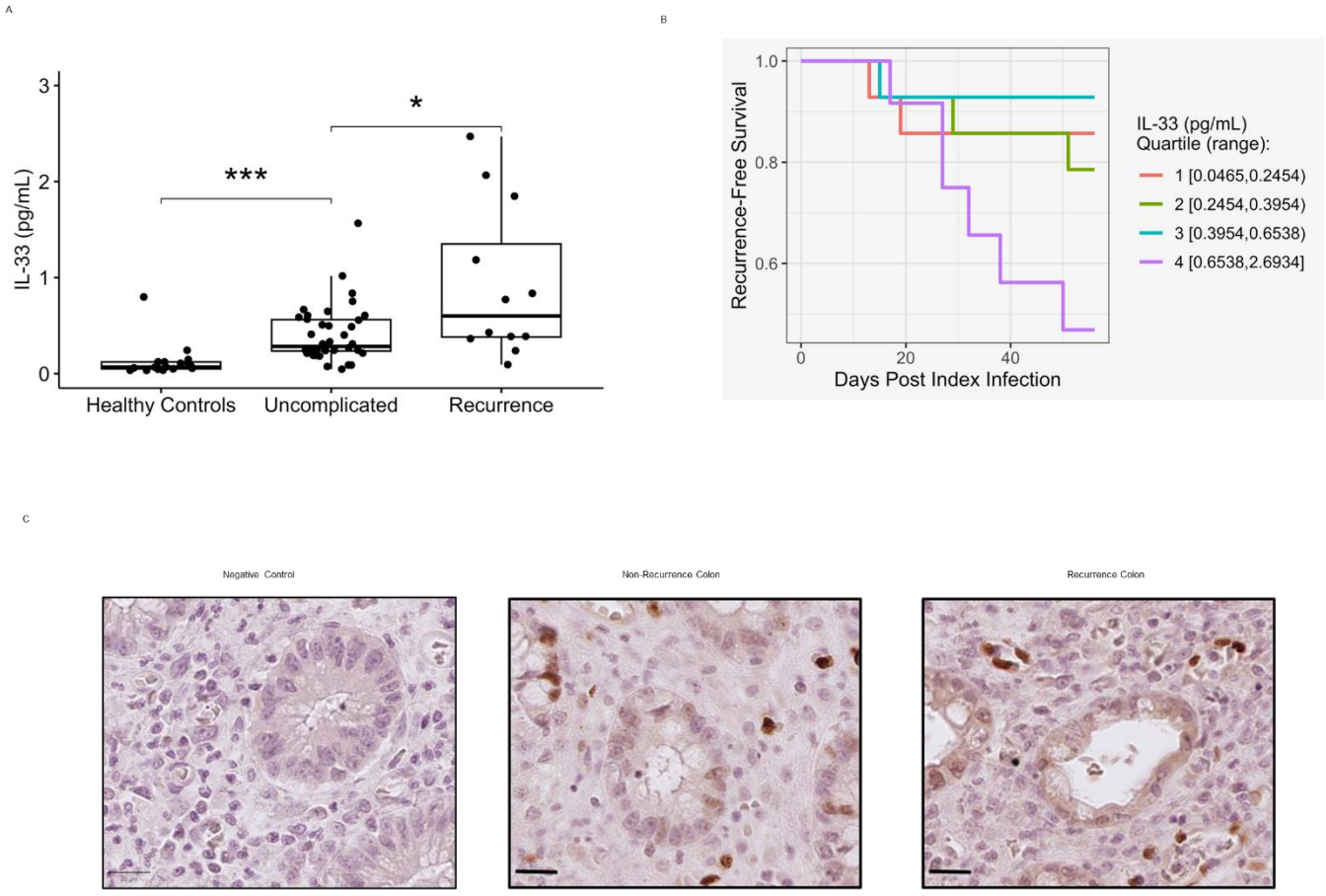


642

643 **Fig 7: ILC2s mediated production of Toxin B-specific antibodies, protecting against CDI**
644 **reinfection**

645 ST2^{+} ILC2s (from uninfected IL33 treated mice) were *ex-vivo* expanded, purified by flow-sorting, and
646 adoptively transferred into ST2 KO mice. (A-B) Mice were pretreated with antibiotics and injected with
647 0.75µg per dose per mouse of IL33 in the gut one day after the adoptive transfer of $3^{x}10^{5}$ ILC2s (n=12)
648 or Saline (n=12) per mouse. At 16 days post-primary infection, plasma toxin B-specific (B) IgG was
649 measured in plasma. (C-G) Mice were rechallenged with *C. difficile* on day 60. On day 70 (10 days

post-2nd infection), (C-E), ILC2 was measured in the colon and mesenteric lymph nodes. (F-G) GC-TFH measured in the MLN (H) Day 70 representative epithelial damage (H&E) of treatment groups and (I) assessed by blinded scoring of infected tissue. **Depletion of ILC2 decreases toxin-specific antibodies in CDI;** IL33 (0.75 µg) was administered intraperitoneally from days -4 to 0 to R26-DTR^{Nmur1} mice (n=9) or littermate control R26-DTR mice (n=9) (in which ILC2s lack DTR). Mice were then given intraperitoneal (i.p.) injections with diphtheria toxin (DT) on days -12, -9, -6, 1, and 8 dpi and infection was done with *C. difficile* strain R20291. (J) Experimental design; On post-infection day 15, ILC2 abundance and anti-TcdB antibodies were measured. (K) Density plot to show the depletion of ILC2s from the colon (L) Toxin-specific antibodies were measured in plasma and cecal content. A two-tailed t-test for normally distributed data and a Mann-Whitney test for non-normally distributed data were used. J, Šídák's multiple comparisons test was used. Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM.

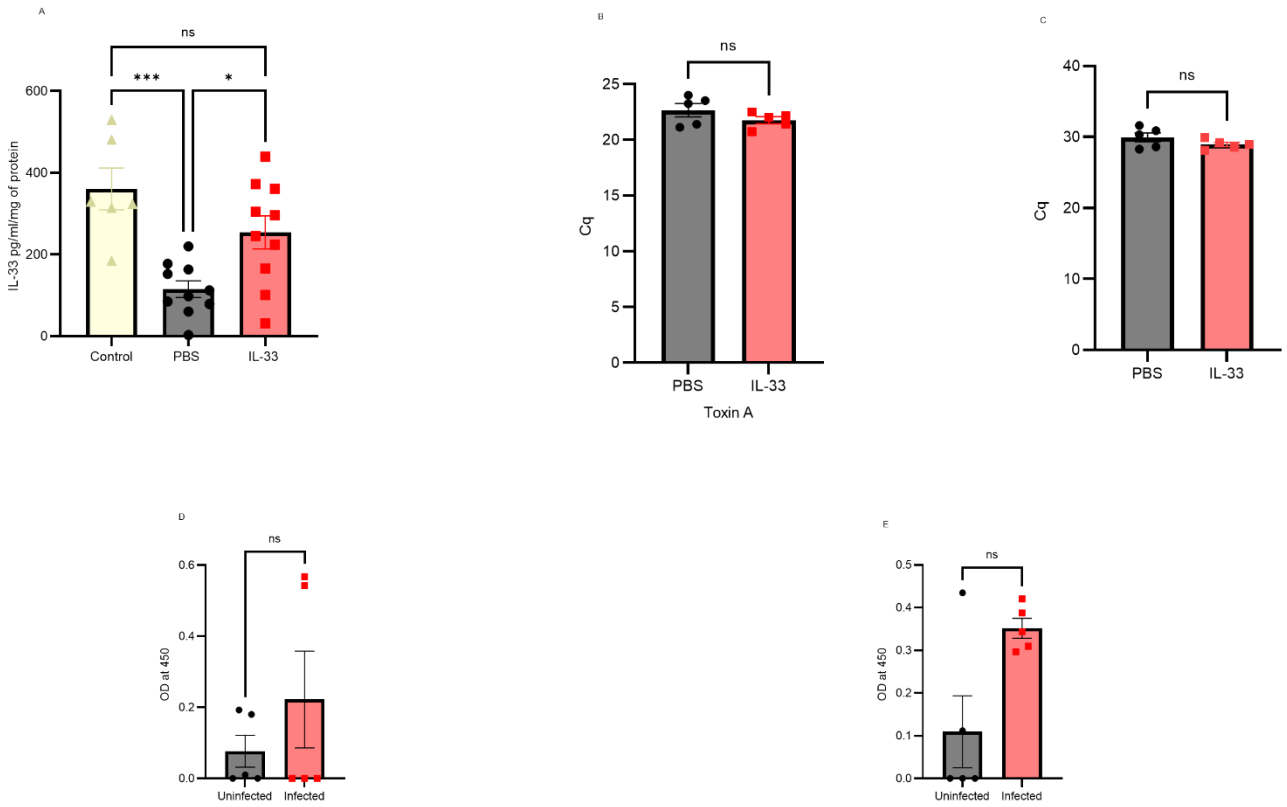


663

664 **Fig 8: IL33 is a biomarker for recurrent *C. difficile* infection in humans**

665 (A) Plasma IL33 was measured in healthy controls (n = 17), patients with uncomplicated CDI (n=39),
666 and recurrent CDI (n=12) (excluding 5 patients who died) within 8 weeks of diagnosis. (B) Recurrence-
667 free survival among the patients with C. difficile infection (1A), grouped by serum IL33 quartile
668 (Wilcoxon P=0.002). (C) Immunohistochemical staining of IL33 from colon tissue biopsies of patients
669 without or with recurrence. The Kaplan–Meier method was used to measure recurrence-free survival
670 curves and to evaluate the effects of IL33 on risk for recurrent *C. difficile* infection. To account for
671 competing risk against recurrent infection, death within the 8-week follow-up period was treated as a
672 censoring event.

673



674

675

676 **Supplementary Fig 1: IL33 treatment restores depleted IL33 levels caused by antibiotics without**

677 **altering *C. difficile* burden**

678 In a mice model of CDI, IL33 (0.75 µg) was administered i.e. on days -4 to 0 to C57BL/6J mice. (A)

679 Proteins were isolated from the cecal tissue and IL33 concentrations were determined with ELISA prior

680 to infection (n=26); (B-C) DNA was isolated from cecal content after 15 days of infection (n=10); and

681 qPCR was performed to check the bacterial load based on (B) Toxin A; and (C) for Toxin B. ST2$^{-/-}$

682 mice produces nonsignificant toxin-specific antibodies after first infection with *C. difficile* without IL33

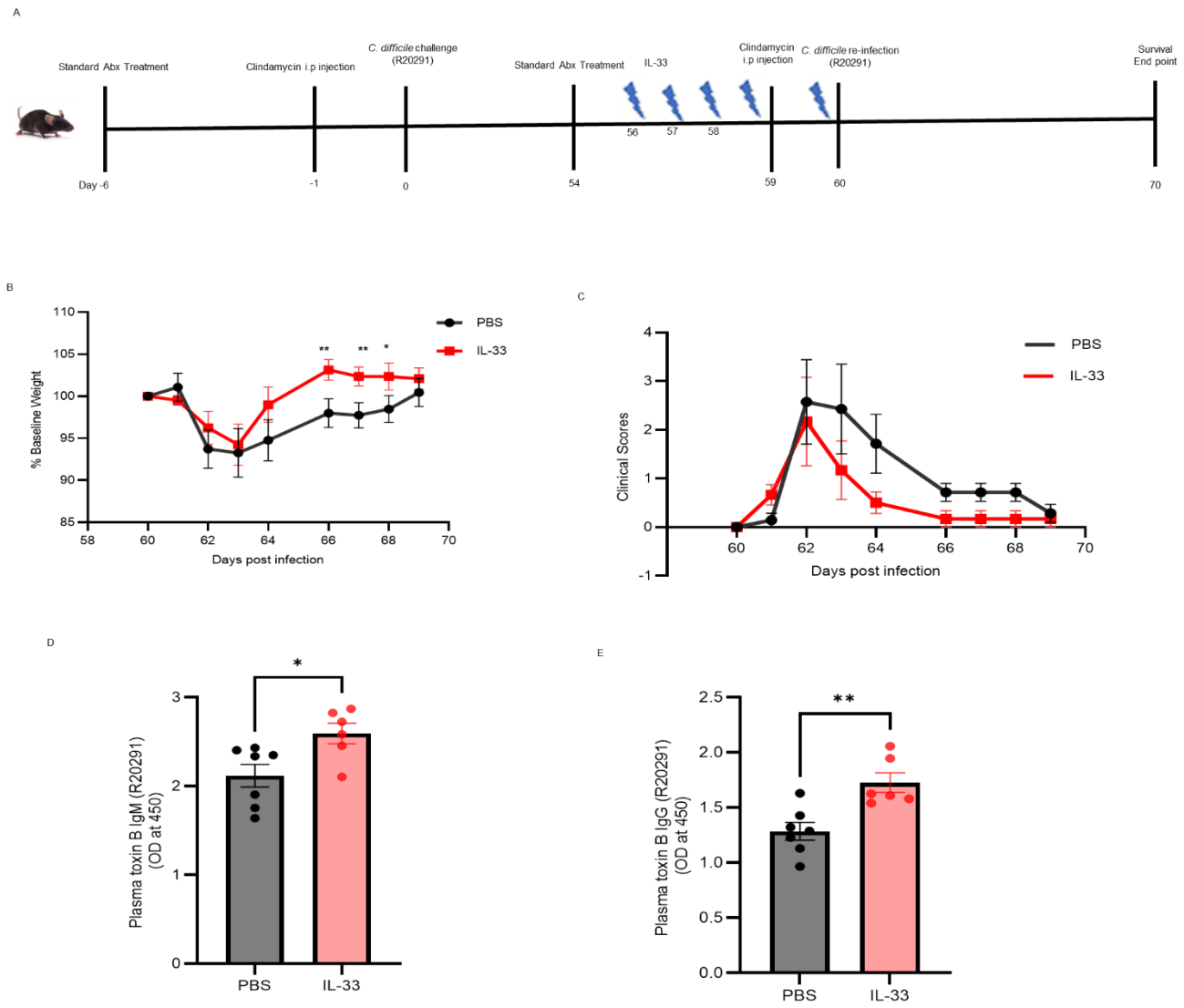
683 administration (D-E). On post-infection day 15, antibodies were measured in plasma. (D) plasma toxin

684 B specific IgG from ST2 $^{-/-}$ mice uninfected and infected with *C. difficile* (E) plasma toxin B specific

685 IgM from ST2 $^{-/-}$ mice uninfected and infected with *C. difficile* strain (n=10);. A two-tailed t-test was

686 used. Statistical significance is demarked as ns (non-significant), *P < 0.05, **P < 0.01, and ***P <

687 0.001. The error bar indicates SEM.



688

689 **Supplementary Fig2: IL33 decreases the severity of *C. difficile* reinfection when given after the**

690 **first infection**

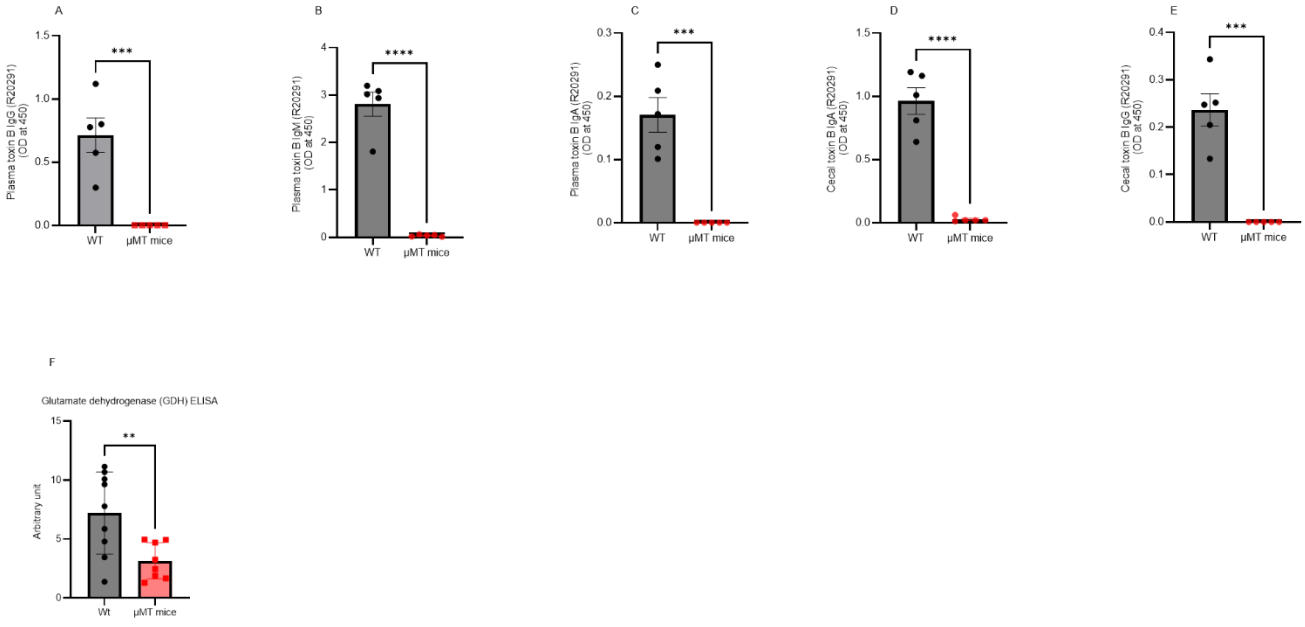
691 IL33 (0.75 µg) was administered i.p. on days 56 to 60 after the first infection and infected again on day

692 60 with the same *C. difficile* strain R20291. (A) Experimental design for IL33 treatment and reinfection;

693 (B) Reinfection weight loss (n=13); (C) clinical scores (n=13); (D) plasma toxin B specific IgM (n=13);

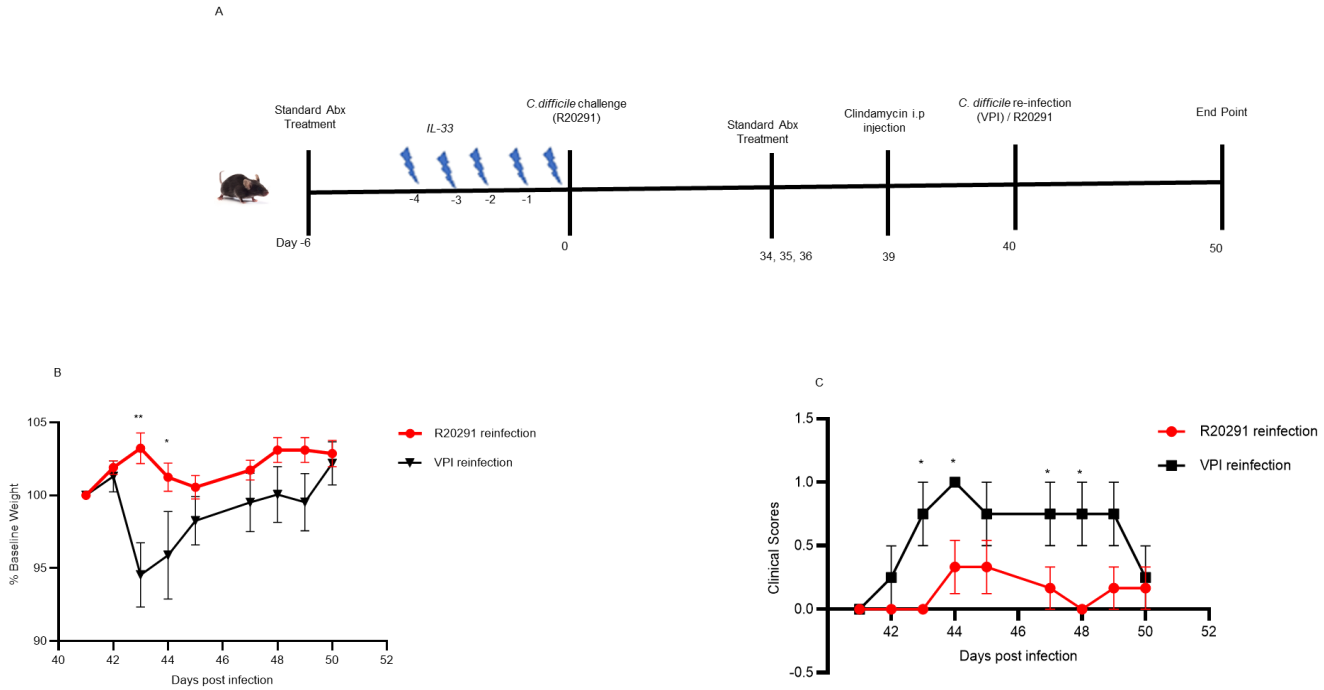
694 (E) plasma toxin B specific IgG (n=13). Comparison made by two-tailed Student's t-test. *P < 0.05,

695 **P < 0.01, and ***P < 0.001. The error bar indicates SEM.



Supplementary Fig 3: mMT KO mice are not able to produce toxin-specific antibodies

WT and mMT KO mice were administered IL33 (0.75 µg) i.p. on days -4 to 0 and mice infected on day 0 with *C. difficile* strain R20291. (A) Toxin B specific plasma IgG (n=10); (B) IgM (n=10); (C) IgA (n=10); (D) stool IgA (n=10); and (E) stool IgG measured on day 23 post-first infection (n=10). WT and mMT KO mice were reinfected with *C. difficile* R20291 60 days after primary infection. (F) Stool *C. difficile* burden measured by glutamate dehydrogenase ELISA (n=17). Comparison made by two-tailed Student's t-test. *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM.



704

705 **Supplementary Fig4: Lack of cross-protection by IL33 with a first infection with TcdB2 R20291**

706 **strain and second with TcdB1 VPI strain**

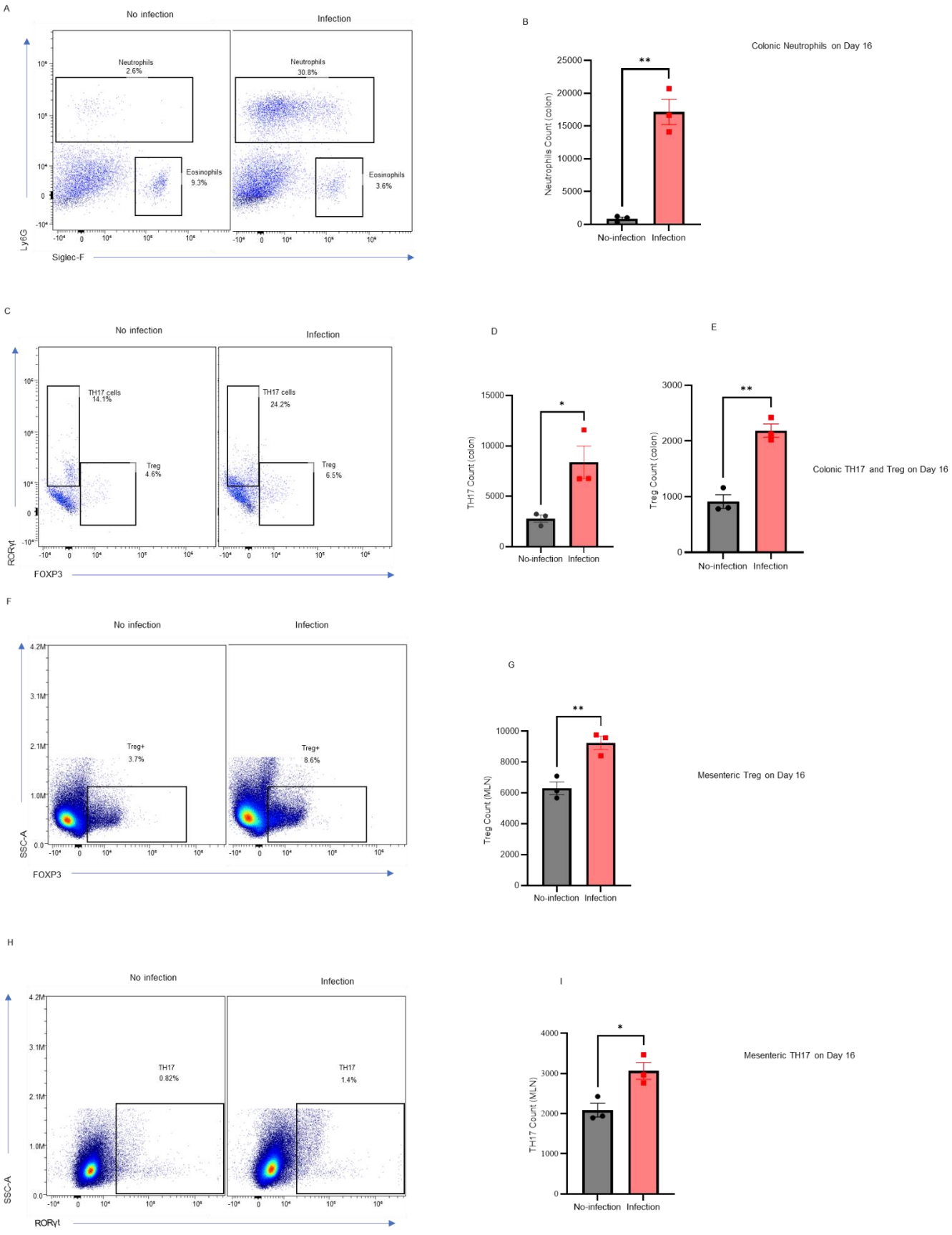
707 Mice were administered IL33 (0.75 µg) i.p. on days -4 to 0 and infected on day 0 with *C. difficile* strain

708 R20291 and again on day 40 with either *C. difficile* strain R20291 or the VPI strain. (A) Experimental

709 design; (B) reinfection weight loss (n=10); (C) clinical scores (n=10). Comparison made by two-tailed

710 Student's t-test. Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001. The

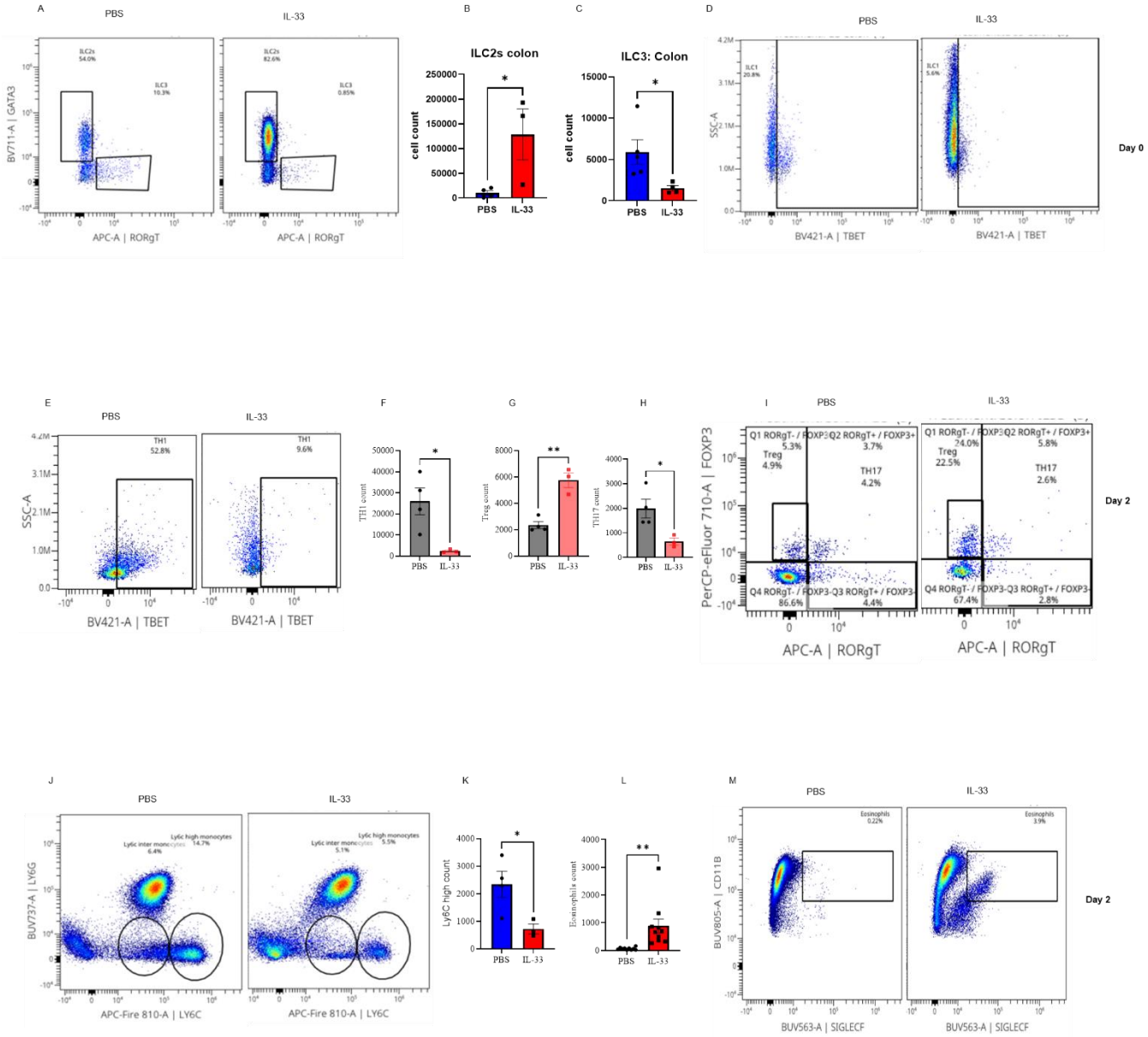
711 error bar indicates SEM.



712

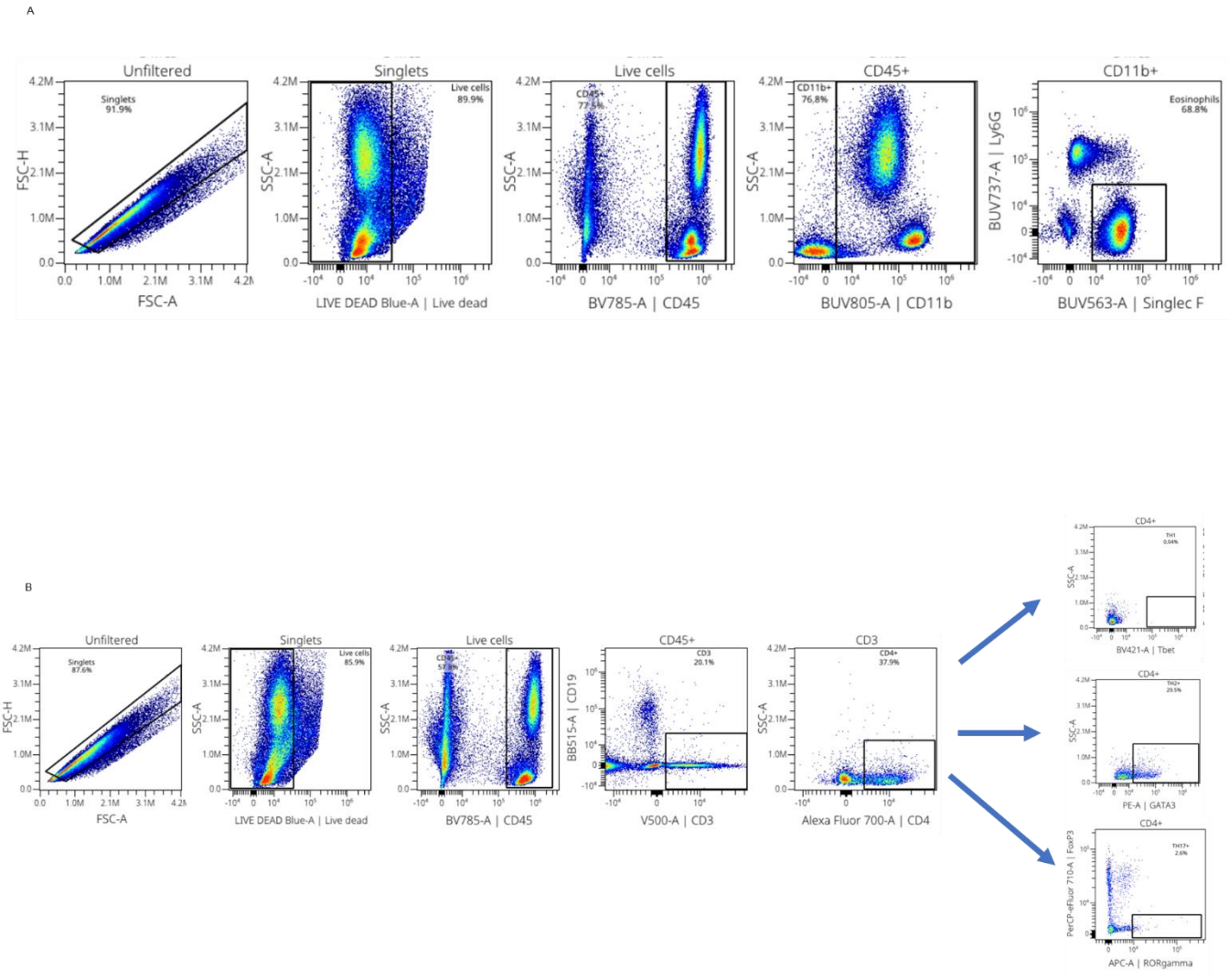
713 **Supplementary Fig 5: Th17 cells persist beyond the resolution of the disease**

Mesenteric lymph nodes and colon were harvested for analysis of neutrophils and T helper cells on day 16 post-first infection (n=6). Colonic (A-B) neutrophils; (C-E) TH17 (CD45+, CD3+, CD4+, RORgt+) and Tregs (CD45+, CD3+, CD4+, FOXP3+). MLN (F-G) Treg and (H-I) TH17 cells. Comparison made by two-tailed Student's t-test. Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM.

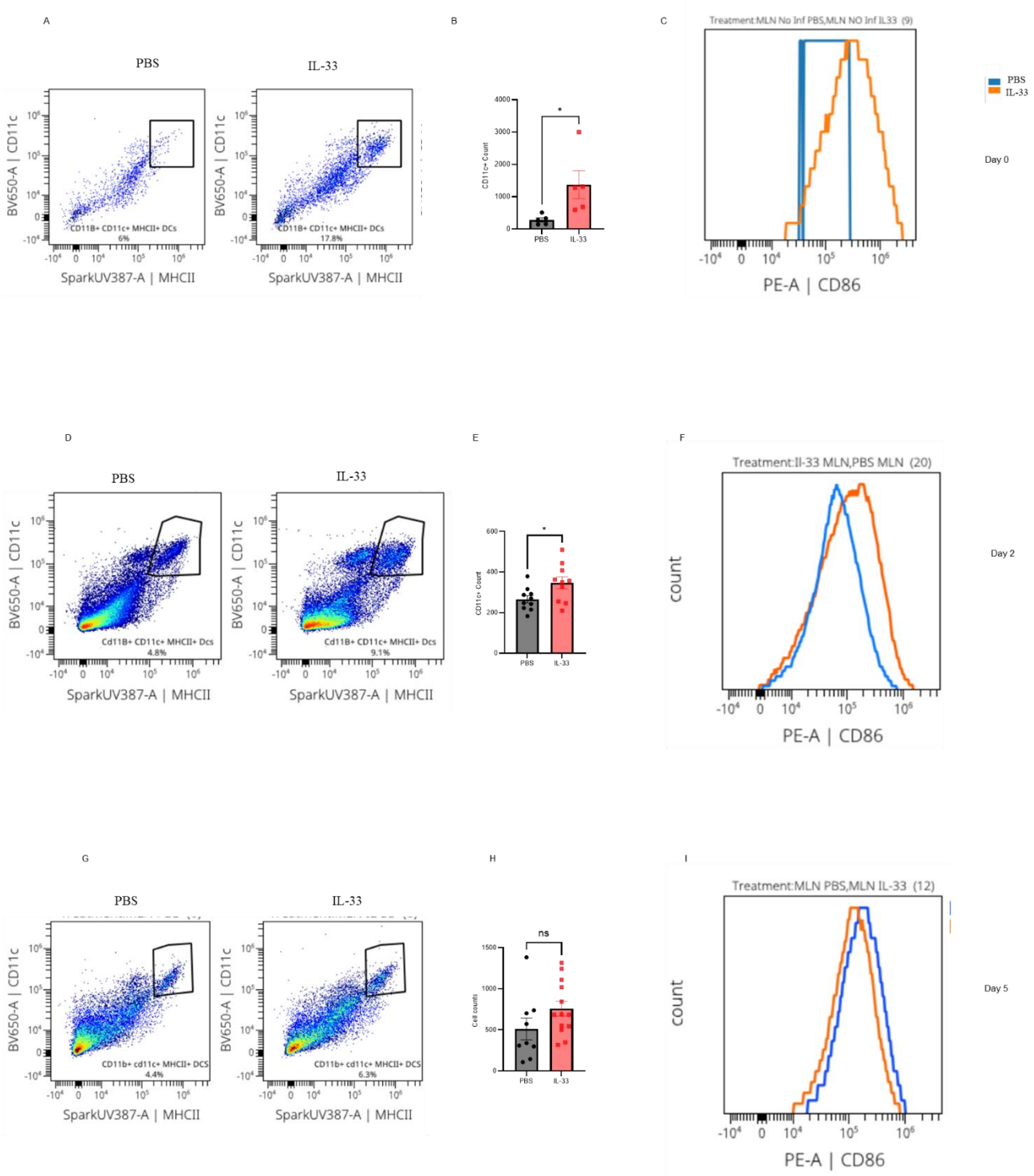


Supplementary Fig 6: IL33 increases colonic eosinophils, ILC2, Treg and decreased inflammatory monocytes, TH1, and TH17 cells during 1st *C. difficile* infection

IL33 (0.75 µg) was administered i.p. on days -4 to 0 and mice infected on day 0. Innate lymphoid cells (ILC), myeloid cells, and T cells from colonic lamina propria were evaluated on days 0 and 2 post-first infection. (A-D) ILCs (n=7); (E-F) TH1 cells (n=7); (G, I) Treg and TH17 cells on day 0 prior to infection (n=7). (J-K) inflammatory monocytes (n=7); and (L-M) Eosinophils (n=20) day 2 post-infection. Comparison made by two-tailed Student's t-test. Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM.



Supplementary Fig 7: Gating strategy used for (A) Eosinophils and neutrophils; (B) TH1, TH2, TH17, and Treg cells.



731

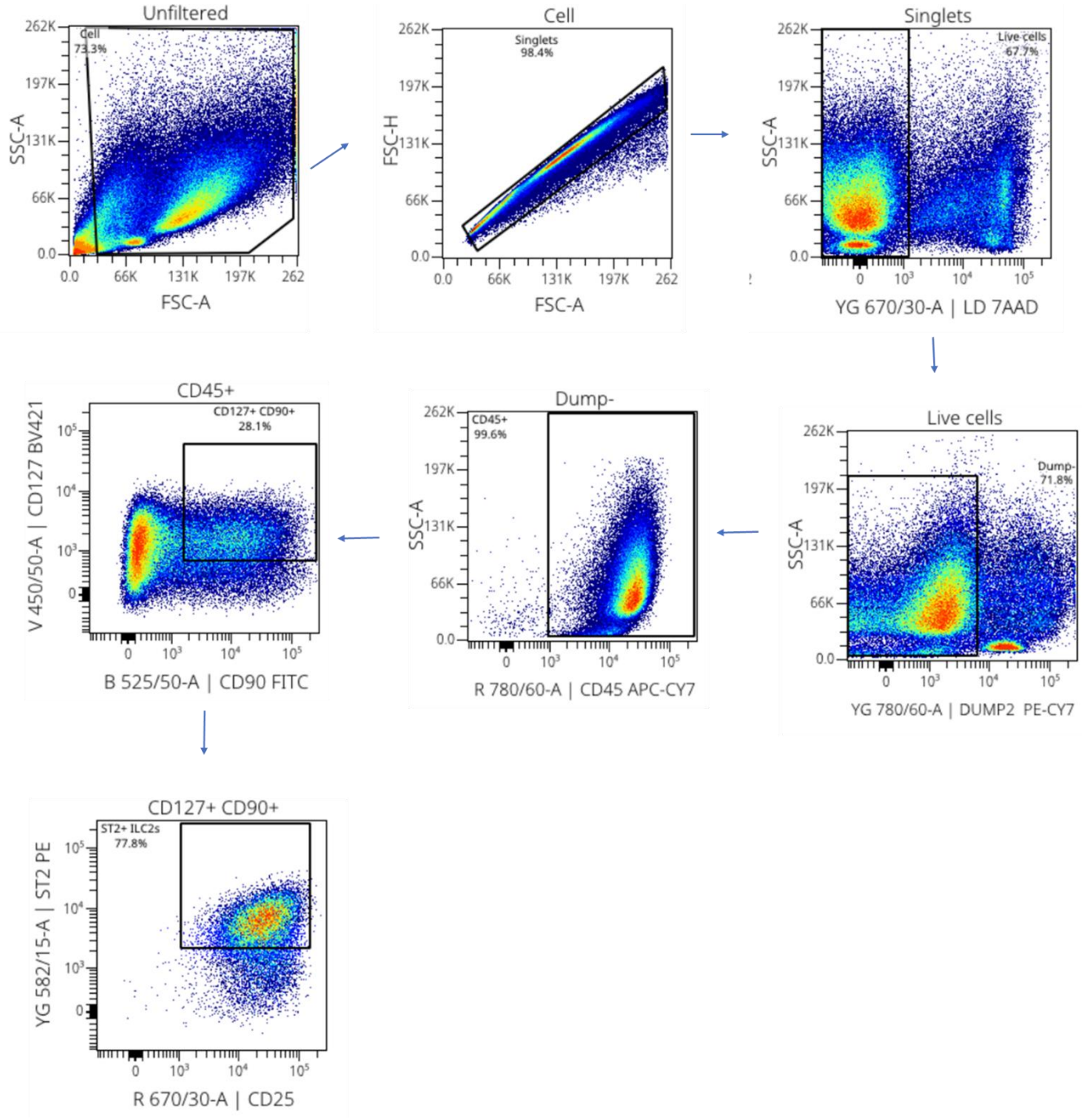
732 **Supplementary Fig 8: IL33 increases the number and activation marker of dendritic cells during**

733 **a primary *C. difficile* infection**

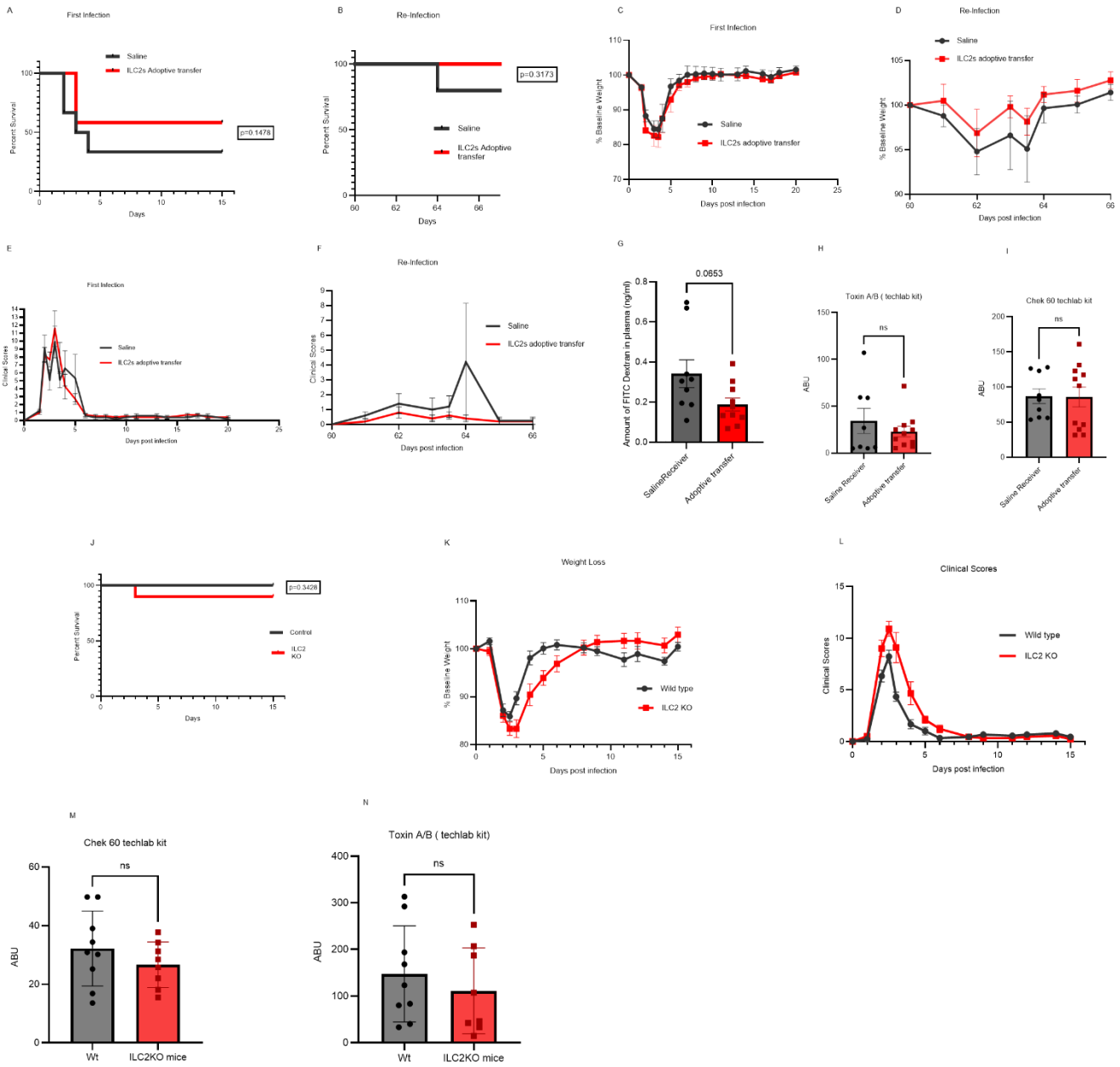
734 IL33 (0.75 µg) was administered i.p. on days -4 to 0 and mice infected on day 0. Mesenteric lymph

735 nodes were harvested for analysis of dendritic cells by flow cytometry before infection, day 2, and day

5 post-first infection. Dendritic cells (CD45+ CD11b+ MHCII+ CD11C+) (A-C) on day 0 prior infection (n=10); (D-F) on day 2 (n=20); (G-I) on day 5 post-infection (n=22) and MFI of activation marker, CD86 on dendritic cells (C, F, I) were analyzed. Comparison made by two-tailed Student's t-test. Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM.



Supplementary Fig 9: Gating strategy used for ST2^{+} ILC2s flow sorting for adoptive transfer



743

744 **Supplementary Figure 10: ILC2s mediated production of Toxin B-specific antibodies, protecting**

745 **against CDI**

746 ST2^{+} ILC2s (from uninfected IL33 treated mice) were *ex-vivo* expanded, purified by flow-sorting, and

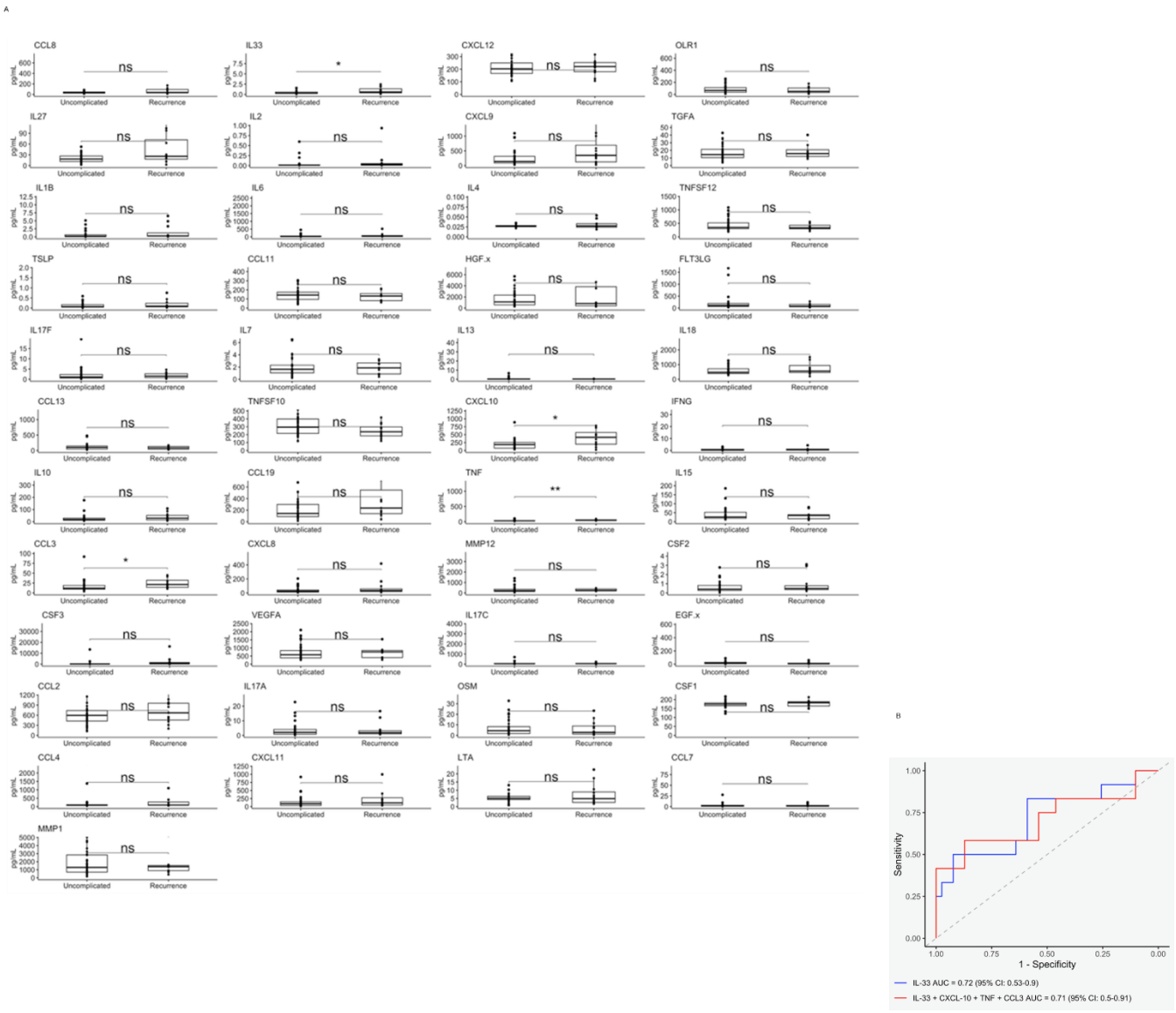
747 adoptively transferred into ST2 KO mice. (A-I) Mice were pretreated with antibiotics and injected with

748 0.75µg per dose per mouse of IL33 in the gut one day after the adoptive transfer of $3^{x}10^{5}$ ILC2s per

749 mouse. At 60 days post-primary infection, reinfection was done after the antibiotics treatment. (A, B)

750 survival curves during the first and reinfection (n=24, 10); (C, D) weight loss during the first and

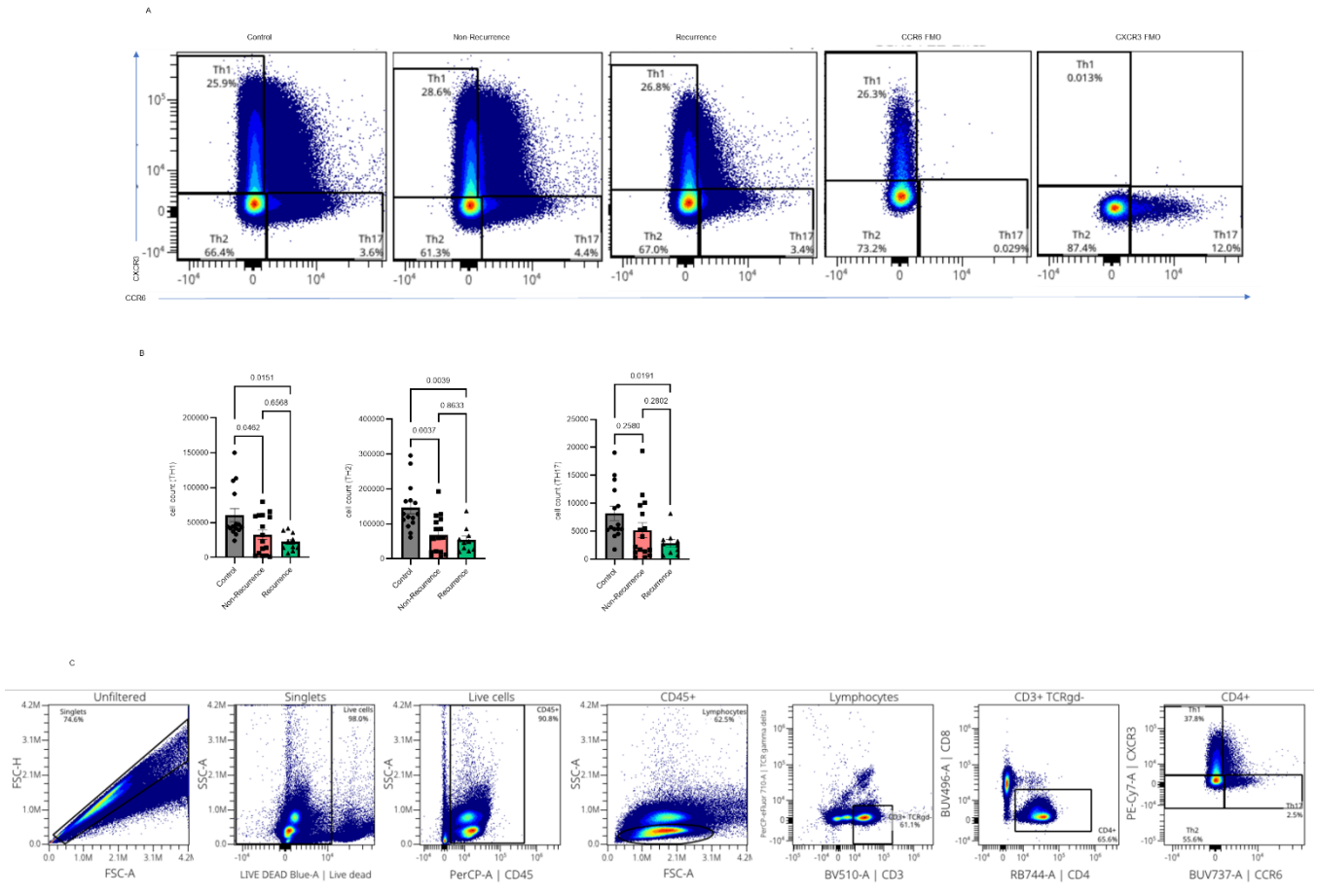
reinfection (n=24, 10); (E, F) clinical scores during the first and reinfection (n=24, 10); (G) FITC-dextran gut permeability test in plasma collected after 7 days of reinfection (n=19); (H) Stool *C. difficile* toxin A and B measured by ELISA kit (Techlab) (n=19); (I) Stool C. difficile burden measured by glutamate dehydrogenase ELISA (n=19). **Depletion of ILC2 increases the morbidity and mortality during the CDI**; IL33 (0.75 µg) was administered intraperitoneally from days -4 to 0 to R26-DTR^{Nmur1} mice or littermate control R26-DTR mice (in which ILC2s lack DTR). Mice were then given intraperitoneal (i.p.) injections with diphtheria toxin (DT) on days -12, -9, -6, 1, and 8 dpi and infection was done with *C. difficile* strain R20291. (J) survival curves during the first infection (n=19); (K) weight loss during the first infection (n=19); (L) clinical scores during the first infection (n=19); (M) Stool *C. difficile* burden measured by glutamate dehydrogenase ELISA (Techlab) (n=17). (N) Stool *C. difficile* toxins A and B were measured by ELISA kit (Techlab) (n=17); A two-tailed t-test for normally distributed data and a Mann-Whitney test for non-normally distributed data were used. J, Šídák's multiple comparisons test was used. Statistical significance is demarked as *P < 0.05, **P < 0.01, and ***P < 0.001. The error bar indicates SEM.



765

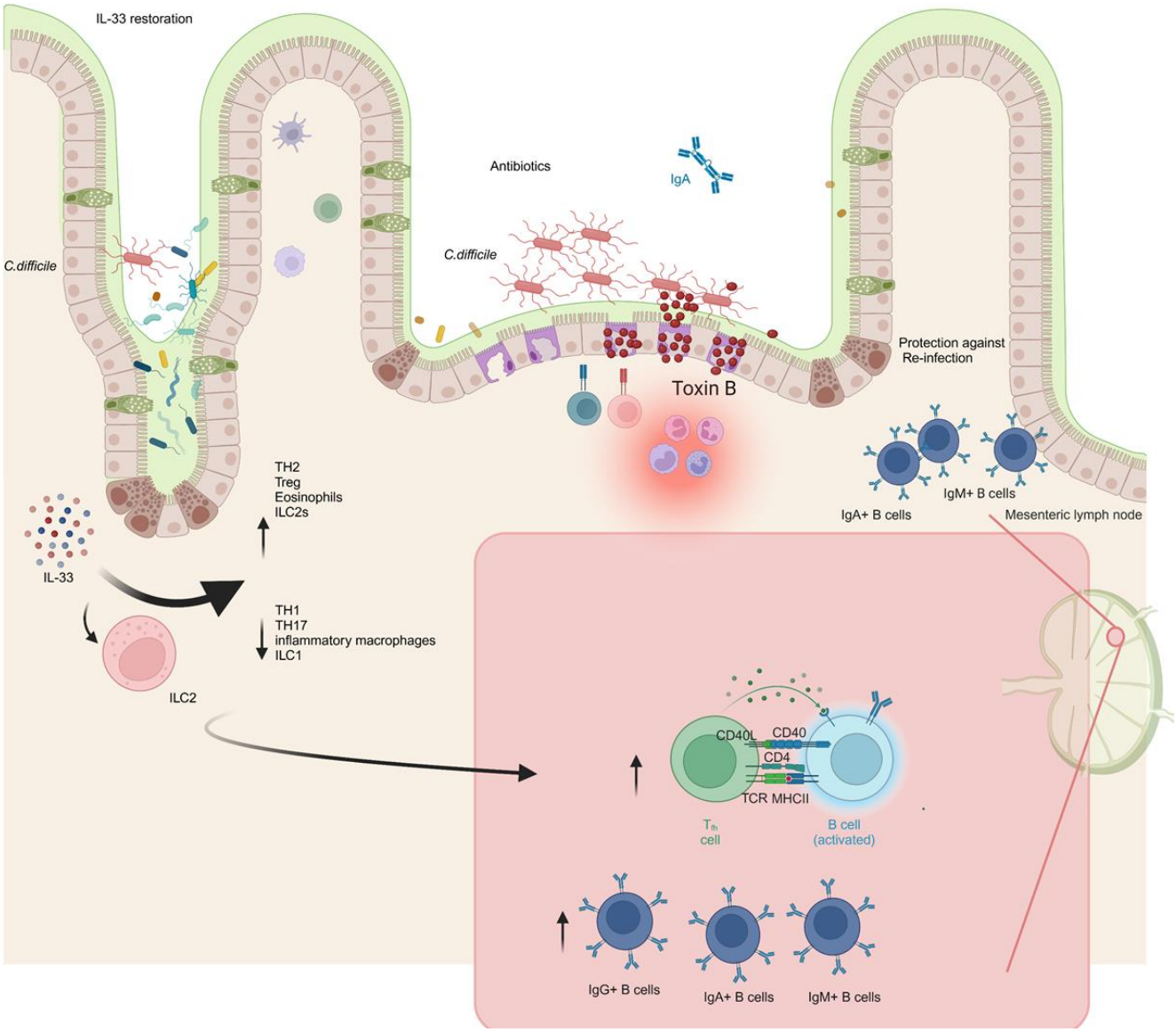
766 **Supplementary Fig 11: O-link Cytokine Measurements (at index *C. difficile* infection**
767 **diagnosis) Compared by Subsequent Uncomplicated versus Recurrent Infection**
768 **Outcomes.** Cytokines were measured in uncomplicated CDI (n=39), and recurrent CDI (n=12)
769 (excluding 5 patients who died) within 8 weeks of diagnosis. (A)Two-sided Wilcoxon Rank
770 Sum tests were performed comparing cytokines measurements taken within 48 hours of *C.*
771 *difficile* diagnosis and compared between patients with an uncomplicated infection (defined as
772 recurrence-free survival by 8 weeks) versus patients who developed a recurrent infection within
773 8 weeks. (B) ROC Curve Analysis of Univariable and Multivariable Logistic Regression
774 Models for Predicting Recurrent *C. difficile* infection within 8 weeks. The univariate model

shown in blue includes only IL33 as a predictor, while the multivariable model includes all four cytokines that were significantly altered between patients who did versus did not have recurrence (IL33, CCL3, CXCL-10, and TNF). Area under the curve (AUC) values were calculated using the DeLong method with 95% confidence intervals. **, *, ns (non-significant) correspond with P values <0.01, <0.05, and ≥0.05, respectively.



Supplementary Fig 12: Peripheral Th1, Th2, and 17 cells were decreased after CDI: Various peripheral T cell subsets were quantified from PBMCs of healthy controls (n = 15), patients with uncomplicated CDI (n=16), and recurrent CDI (n=10). (A) Density plot showing Control, Non-Recurrence, and Recurrence patients; (B) TH1, TH2, and TH17 cell counts; (C) Gating strategies used to analyze T cell populations. A significant decrease in the count of peripheral Th1, Th2, and Th17 cells was observed for CDI patients but no significant difference

was found between recurrence and non-recurrence patients. No significant differences were found in the groups of % of CD4 cells. The comparison was made by 2-way ANOVA (Tukey's multiple comparisons test).



Graphical Abstract: IL33 restoration induces toxin-B-specific antibody production via the ILC2-TFH axis. In the left panel, IL33 remediation increases ILC2s, subsequently inducing TFH directly or indirectly. TFH cell induction is pivotal for the production of antibodies. IL33 also downregulates type 1 and type 3 immunity, favoring type 2 immunity to enhance host survival and reduce morbidity.

The middle panel illustrates antibiotic-induced dysbiosis, resulting in decreased IL33 levels and reduced antibody production.

The right panel demonstrates the protective effect in reinfection, attributed to toxin-specific antibodies generated by IL33 remediation.

Table 1: Details of Antibodies Used for Flow Cytometry Analysis of Human PBMCs.

	SPECIFICITY	FLUOROCHROME	Company	Catalog no.	Clone
	CD45	PerCP	Bio Legend	368506	2D1
	CD3	BV510	Bio Legend	344828	SK7
	TCR γδ	PerCP-eFluor 710	Thermo-Fisher	46-9959-42	B1.1
	CD4	RB744	BD	570466	SK3
	CD8	BUV496	BD	741199	SK1
	CCR6	BUV737	BD Biosciences	612780	11A9
	CXCR3	PE-Cy7	Bio Legend	353720	G025H7
	Viability	Live Dead UV Blue	Thermo Fisher	L34962	

Supplementary Table 1: Basic patient characteristics.

	Uncomplicated CDI (n=39)	**Recurrent CDI within 90 days (n=12)**	**Death within 90 days (n=5)**	**P value**
Female Sex	20 (51.3%)	7 (58.3%)	4 (80%)	0.14
Age in a year, Mean (IQR)	56.5 (46-67)	59.3 (52-71.5)	72.4 (63-80.5)	**0.02**
Race/ethnicity:				0.07
White/Caucasian	30 (76.9%)	10 (83.3%)	3 (60%)	**0.05**
Black/African-American	7 (17.9%)	2 (16.7%)	2 (40%)	**0.03**
Hispanic/Latino	1 (2.6%)	0 (0%)	0 (0%)	0.51
Asian/Other	1 (2.6%)	0 (0%)	0 (0%)	0.51
Peak WBC, Mean (IQR)	12.6 (6.1-16.3)	12.2 (3.0-19.2)	14.1 (6.2-22.6)	0.82
Charlson Comorbidity Index, Mean (IQR)	4.3 (2-6)	4.8 (2.5-7)	8 (6-10)	**0.001**

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