Supplementary Material for

The binary toxin CDT enhances *Clostridium difficile* virulence by suppressing protective colonic eosinophilia

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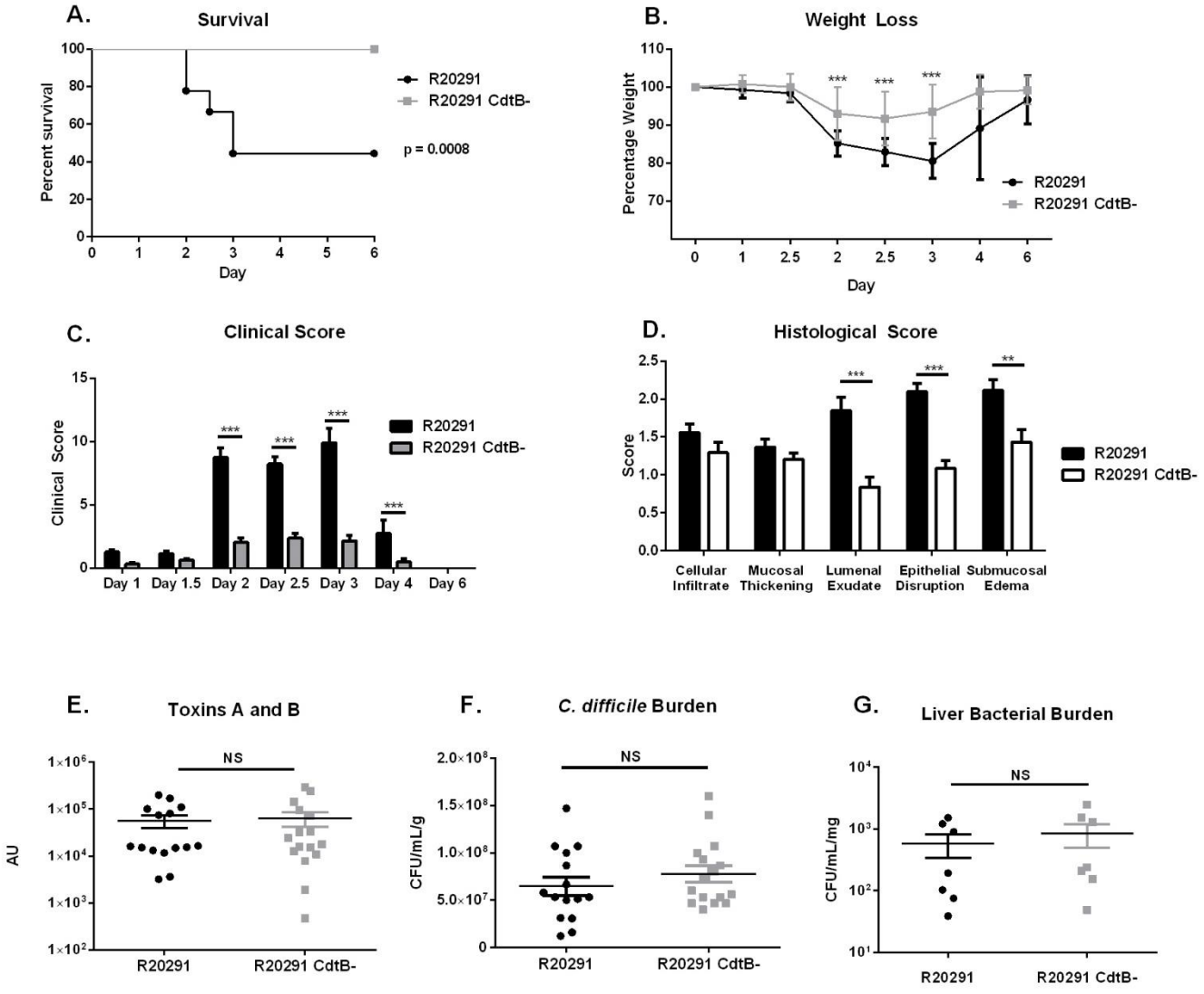
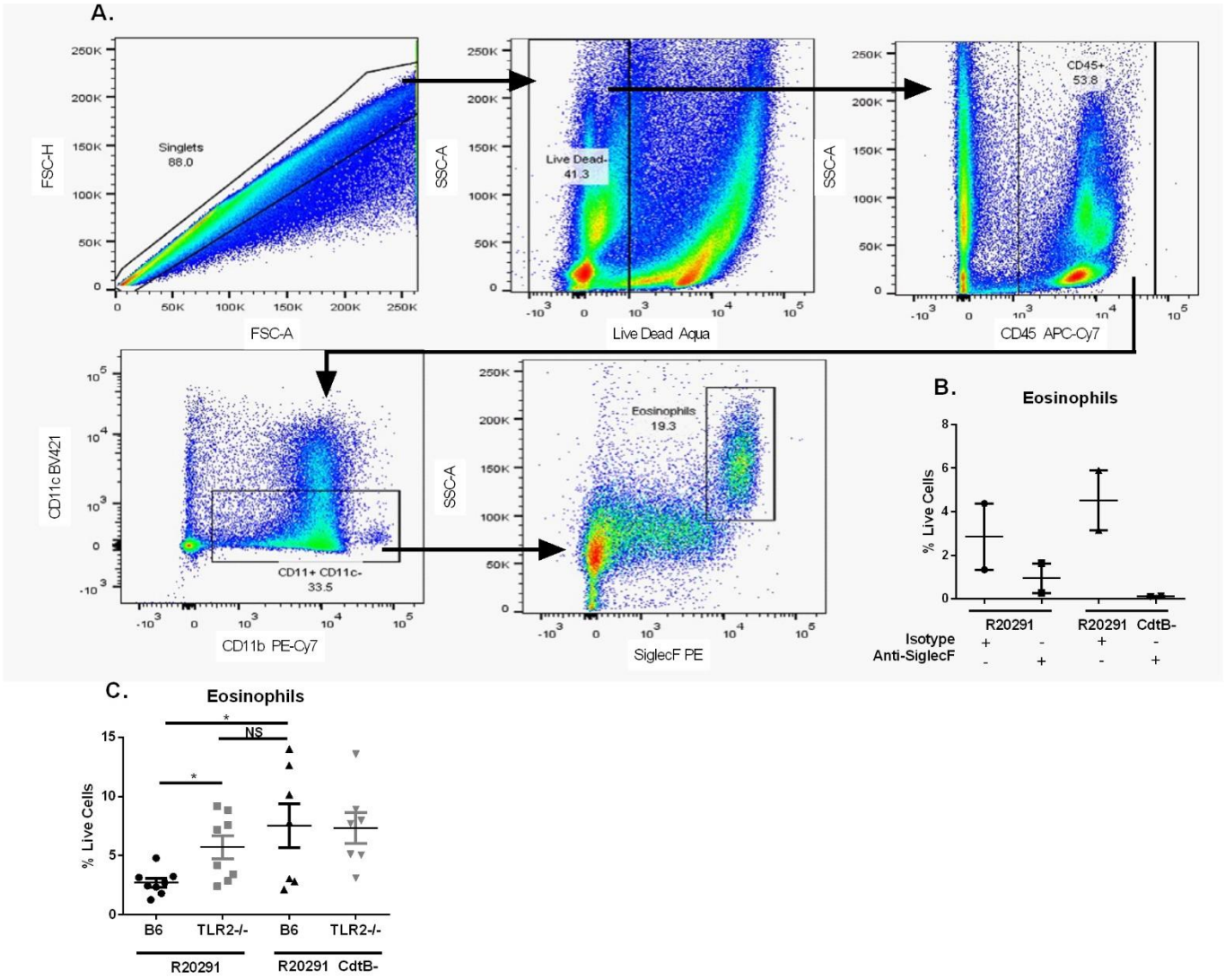
Supplementary Figure 1: *C. difficile* burden, Toxins A and B, translocation of commensals and histology scoring.



(A-C) 8 week old C57BL/6J mice underwent an antibiotic regimen prior to infection with 10^7 CFU of vegetative *C. difficile* strain R20291 or the isogenic mutant lacking the binding domain of CDT (R20291 CdtB-) (data shown combined from three independent experiments, n=24). **(D)** Mice were sacrificed on day 2 of infection and cecal sections were fixed in Bouin's solution for 18 hours before undergoing paraffin embedding, sectioning and hematoxylin & eosin staining.

Samples were scored blinded based on 5 parameters (submucosal edema, inflammatory infiltrate, epithelial disruption, luminal exudate and mucosal thickening). **(E)** Mice were sacrificed on day 3 and Toxins A and B in the cecal contents were assessed via ELISA. **(F)** Cecal *C. difficile* burden was enumerated anaerobically on Brain-Heart Infusion agar. **(G)** Total liver bacterial burden was determined by plating liver homogenate on non-selective BHI and incubating aerobically overnight. Data shown combined from two independent experiments (n=13 in **D,** n=15 in **E-F**, and n=7 in **G**). * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Kaplan-Meier Analysis (**A**) or Mann-Whitney test (**B-G**). NS = not significant. Error bars shown represent S.D. **(B)** or S.E.M. **(C-G)**.

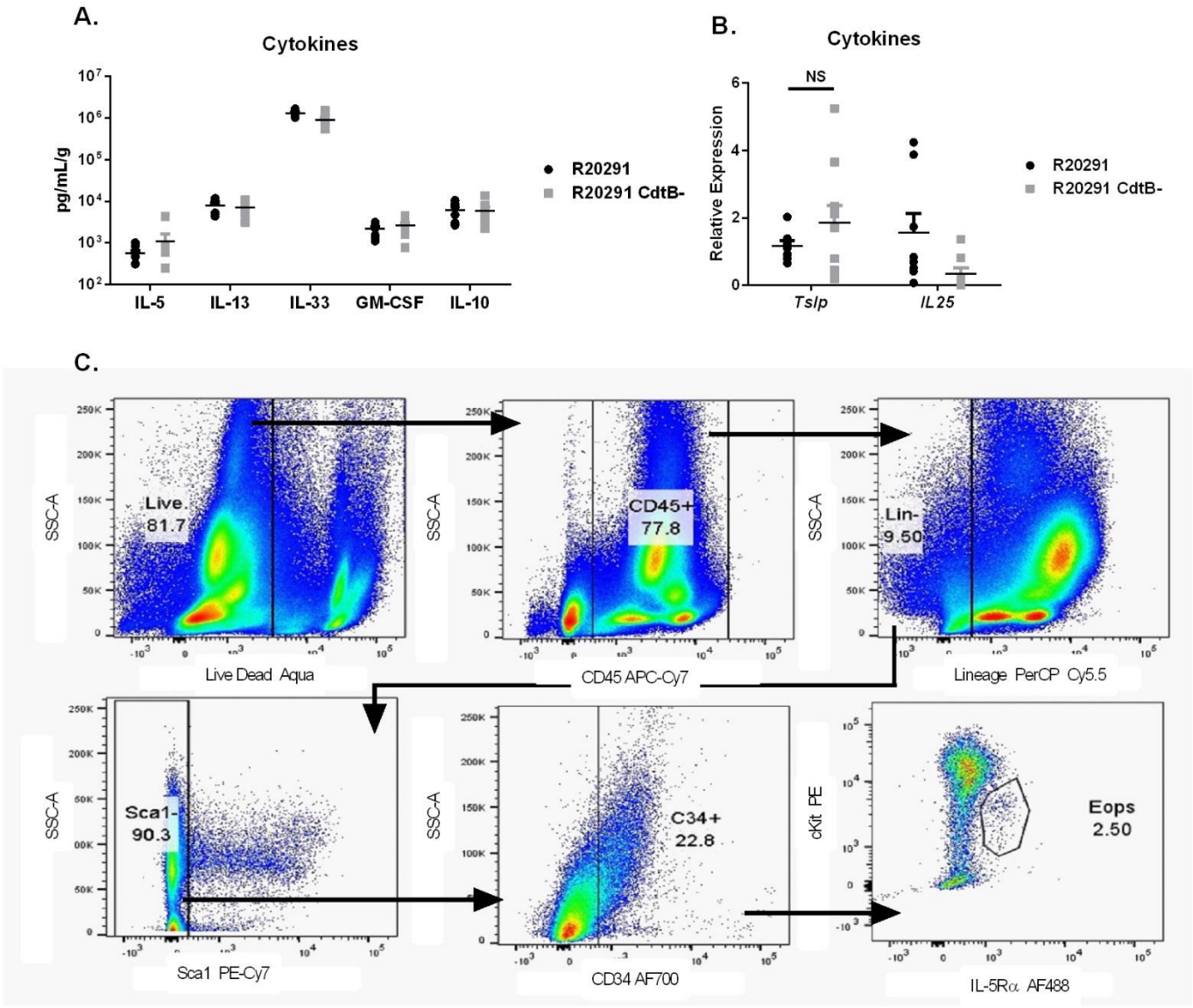
Supplementary Figure 2: Eosinophil gating strategy and depletion with anti-SiglecF.



(A) Mice were sacrificed on day 3 of infection and colonic eosinophils were measured by flow cytometry following tissue processing and staining. The gating strategy identified singlets, Live dead negative cells, CD45+ cells, CD11b+ CD11c- and SiglecF+ side scatter high populations as eosinophils. Data shown are representative of 2 independent experiments. **(B)** Eosinophil depletion was quantified on day 3 of infection in mice treated with anti-SiglecF or an isotype control antibody (two IP injections of 40 ug each on the day before and the day following infection) infected with R20291 or R20291 CdtB- (n=2). **(C)** TLR2$^{-/-}$ mice or C57BL/6J mice were infected

with wild-type R20291 or the isogenic CDT mutant R20291 CdtB- and were sacrificed on day 3 of infection. Colonic eosinophils ($CD45^+$ $CD11b^+$ $SiglecF^+$ SSC^{hi}) were measured by flow cytometry following tissue processing and staining (data shown as a percent of live cells, combined from two independent experiments, n=8). * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Welch's unequal variance *t*-test (**C**). NS = not significant. Error bars shown represent S.E.M.

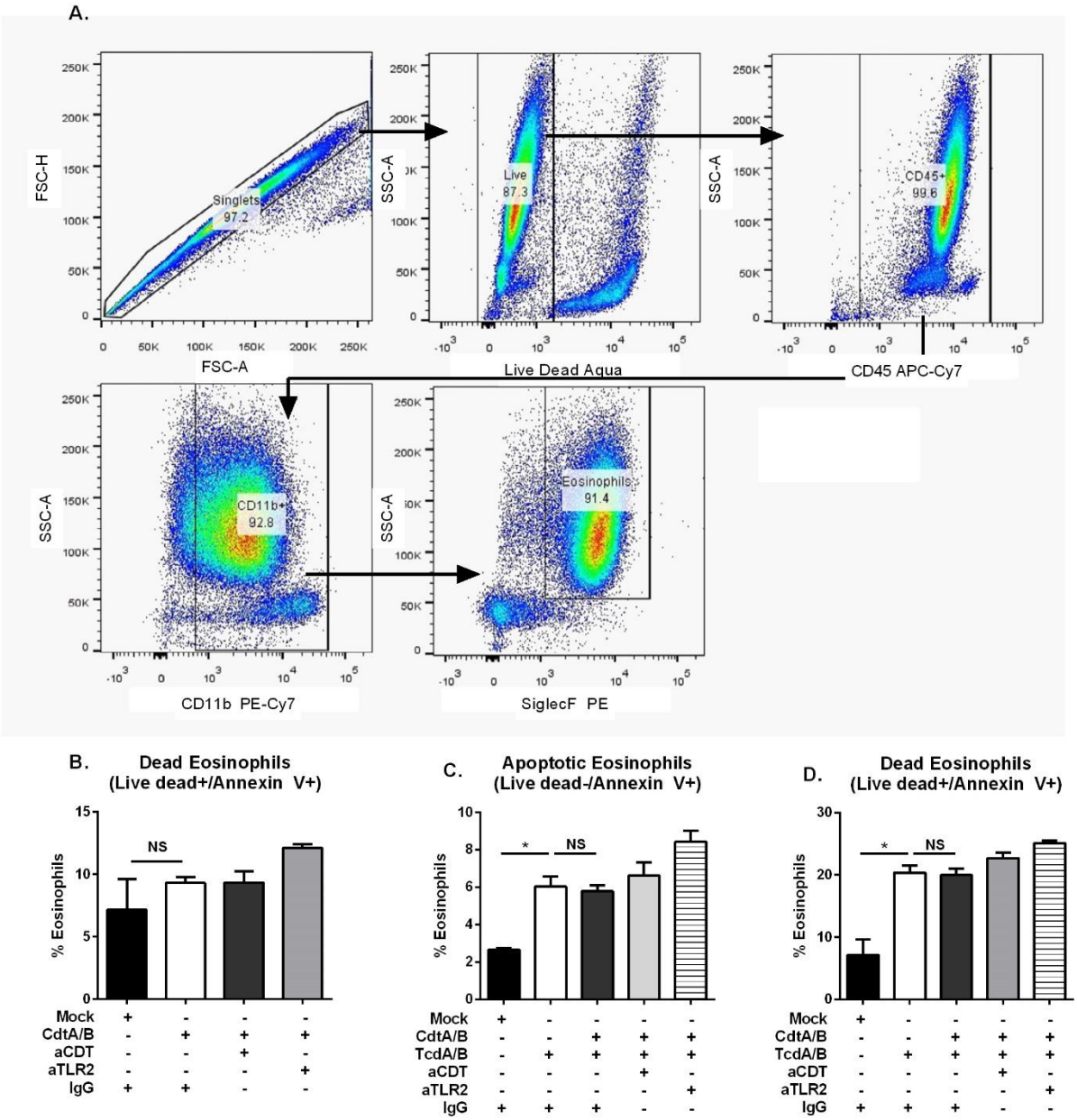
Supplementary Figure 3: Assessment of Th2 Cytokines and measurement of eosinophil progenitors.



(A) Mice were sacrificed on day 3 of infection and cecal cytokines assessed in total cecal lysate by Luminex (IL-5, IL-13) or ELISA (IL-33, GM-CSF, IL-10). Values are shown normalized to total protein concentration, and are combined from two independent experiments (n=7). **(B)** Gene expression in total cecal RNA from mice on day 3 post-infection was quantified by qRT-PCR and shown normalized to GAPDH as a housekeeping gene, data shown combined from two

independent experiments, (n=7). **(C)** Eosinophil progenitors (Eops) were identified as Lin- CD34+ Sca-1- IL-5Rα+ cKit^{in} cells by flow cytometry (data shown are representative of 2 independent experiments, n=6). Lineage gate consisted of TCRβ, CD3ε, CD49b, B220, GR1, CD11b and CD11c on the PerCP Cy5.5 channel. Data shown are representative of two independent experiments. P values determined by Mann-Whitney test (**A, B**). NS = not significant. Error bars shown represent S.E.M.

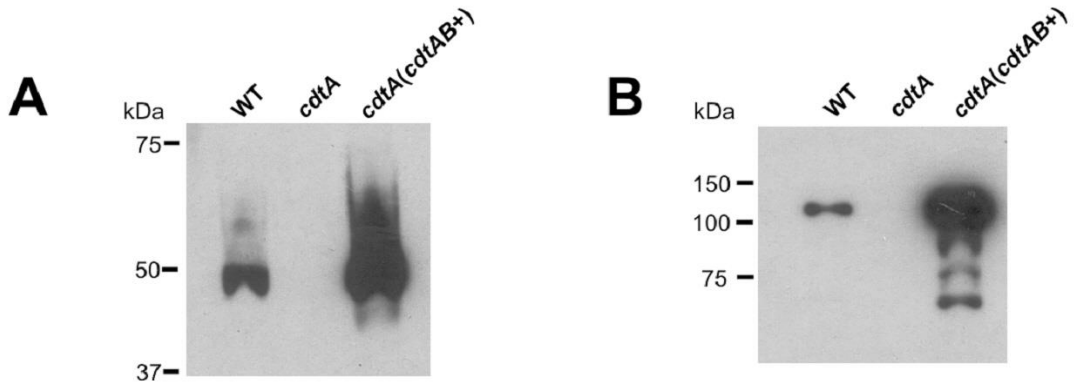
Supplementary Figure 4: Generation of bone marrow-derived eosinophils.



(A) Total bone marrow was harvested from C57BL/6 knockout mice and frozen in Fetal Bovine Serum with 10% DMSO until use. Thawed cells were grown in the presence of 100 ng/mL FLT3L and 100 ng/mL SCF for 4 days. The media was then removed and fresh media, supplemented with 10 ng/mL IL-5 was added. The cells were grown an additional 6 days, with fresh media added

every other day. Purity of the culture was assessed by flow cytometry on day 10. Eosinophils were identified as Live, $CD45^{+}$, $CD11b^{+}$, $SiglecF^{+}$ SSC^{hi} cells. Data shown are representative of two independent experiments. **(B)** BM Eos were incubated for 8 hours with 200 ng/mL CDTa and 200 ng/mL CDTb in the presence or absence of anti-TLR2 neutralizing antibody (aTLR2) or anti-CDT neutralizing nanobody (aCDT). Eosinophils were stained with Live dead or Annexin V and cell death was assessed by flow cytometry, data shown are representative of 3 independent experiments assayed in duplicate. **(C-D)** BM Eos were incubated for 8 hours with 200 ng/mL CDTa and 200 ng/mL CDTb and 2ng/mL Toxin A and 2 ng/mL Toxin B. Eosinophils were stained with Live dead or Annexin V and cell death was assessed by flow cytometry, data shown are representative of 3 independent experiments assayed in duplicate * = p value < 0.05 by Mann-Whitney test. NS = not significant. Error bars shown represent S.E.M.

Supplementary Figure 5: Complementation of CDT in strain M7404.



Western blot of concentrated supernatant from *C. difficile* strains M7404 (WT), M7404 CdtA- (cdtA), and M7404 CdtAComp (cdtA(cdtAB+)). Blots were probed with CDTa-specific antibody (**A**) or an antibody recognizing *Clostridium perfringens* Ib which cross-reacts with CDTb **(B**).

Supplementary Table 1: Antibodies used for Flow Cytometry.

Fluorochrome	Antibody	Source	Clone
Brilliant Violet 421	CD11c	BioLegend	N418
AlexaFluor 488	CD125/IL-5Rα	BD Biosciences	T21
PE	SiglecF	BD Pharmingen	E50-2440
PeCy7	CD11B	BioLegend	M1/70
APC-CY7	CD45	BioLegend	30-F11
AlexaFluor 647	CD193/CCR3	BD Biosciences	83103
PerCP-Cy5.5	Gr1	BioLegend	RB6-8C5
AlexaFluor 488	Annexin V	Life Technologies	
PE	c-Kit/CD117	BioLegend	2B8
AlexaFluor 700	CD34	BD Biosciences	RAM34
PeCy7	Sca-1	BioLegend	D7
PerCP-Cy5.5	TCRβ	BioLegend	H57-597
PerCP-Cy5.5	CD3ε	BioLegend	17A2
PerCP-Cy5.5	CD49b	BioLegend	DX5
PerCP-Cy5.5	B220	BioLegend	RA3-6B2
PerCP-Cy5.5	CD11c	BioLegend	N418
PerCP-Cy5.5	CD11b	BioLegend	M1/70
FITC	LY6C	BD Biosciences	AL-21
PE-CY7	LY6G	BD Biosciences	1A8