Clostridium difficile induces expansion and cytokine production by human Vγ9Vδ2 T cells

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INTRODUCTION

Clostridium difficile is a major culprit of nosocomial antibiotic-associated diarrhoea and can result in pseudomembranous colitis and life-threatening toxic megacolon. *C. difficile* mediates its pathogenesis via toxin production and recently, several dominant strains have emerged, that exhibit increased resistance to treatment, and have increased associated mortality. Isoprenoid biosynthesis is a pathway essential for cell survival, which occurs via one of two pathways: the mevalonate pathway, which is used by eukaryotes and some bacteria, and the MEP pathway, which is employed by most human pathogens. An intermediate of the MEP pathway, hydroxyl-3-methyl-but-2-enyl pyrophosphate (HMB-PP), is the most potent antigen for activating Vγ9Vδ2 T cells, and thus expansion of Vγ9Vδ2 T cells is seen upon infection with a broad range of microbial pathogens. Expression of MEP pathway genes by *C. difficile* suggests that *C. difficile* may also activate Vγ9Vδ2 T cells. We compared the ability of *C.difficile* and HMB-PP in inducing Vγ9Vδ2 T cell proliferation and cytokine production.

Method: Human PBMC were stimulated with HMB-PP or *C. difficile* supernatant

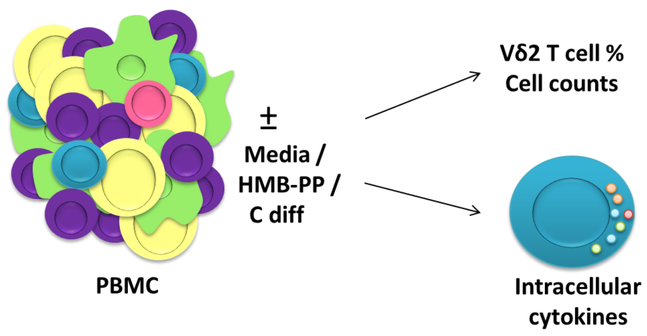

Figure 1. Supernatants and lysates of strain RT060 and hypervirulent strains RT001 and RT027 were filtered with 0.22µm and 3kDa filters. Human PBMC were cultured with HMB-PP or *C. difficile* supernatant and lysate from three *C. difficile* strains for 14 days. The cultures were then examined for Vδ2 T cell expansion and cytokine production.

Distribution of the MEP and mevalonate pathways amongst Gram-positive and Gram-negative pathogens

Gram + pathogen	MEP	Mevalo-nate	Gram - pathogen	MEP	Mevalo-nate
Bacillus anthracis	+	-	*B. abortus*	+	-
Bacillus subtilis	+	-	*Borrelia burgdorferi*	-	+
Clostridium difficile	+	-	*Chlamydia trachomatis*	+	-
Clostridium botulinum	+	-	*Chlamydia pneumonia*	+	-
Clostridium perfringens	+	-	*S. enterica*	+	-
Entercoccus faecalis	-	+	*Escherichia coli*	+	-
L. monocytogenes	+	+	*F. tularensis*	+	-
L. innocua	-	+	*Legionella pneumophila*	-	+
Listeria seeligeri	-	+	*P. aeruginosa*	+	-
Nocardia terpenica	+	-	*V. cholera*	+	-
Stahylococcus aureus	-	+	*K. pneumonia*	+	-
Streptomyces pyogenes	-	+	*Bordetella pertussis*	+	-
S. pneumonia	-	+	*Haemophilus influenza*	+	-
			Helicobacter pylori	+	-
			Shigella dysenteriae	+	-
			Neisseria gonorrhoeae	+	-
			Neisseria meningitides	+	-
			C. jejuni	+	-
			Y. enterocolitica	+	-

RESULTS

C. difficile from 3 distinct strains induced expansion of Vδ2 T cells *in vitro*

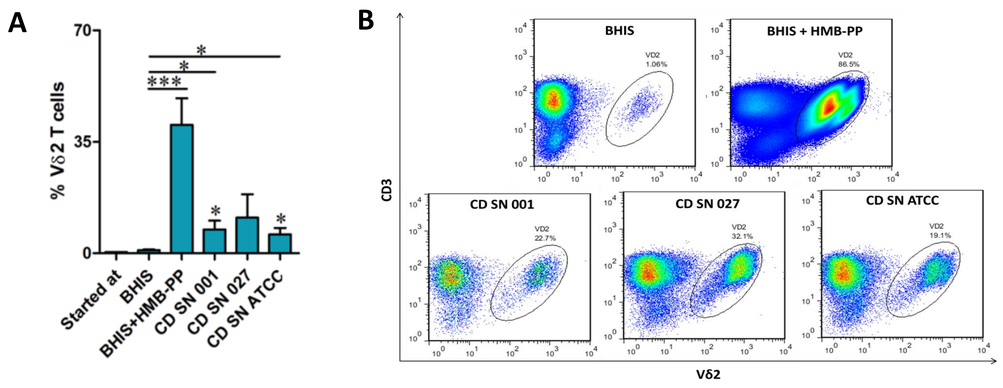

Figure 2. PBMC were stimulated with *C. difficile* supernatant (denoted CD SN) of three different *C. difficile* strains (001, 027 and ATCC) at a 9:1 ratio of RPMI to BHIS or *C. difficile* SN. The cells were expanded for 2 weeks in the presence or absence of HMB-PP and examined for CD3 and Vδ2 expression by flow cytometry. **A**, mean (±SEM) Vδ2 percentage following 2 weeks of culture (n=4-12). **B**, representative flow cytometric dot plots depicting Vδ2 percentage under the different stimuli.

C. difficile induced Vδ2 T cell expansion in a large proportion of donors tested

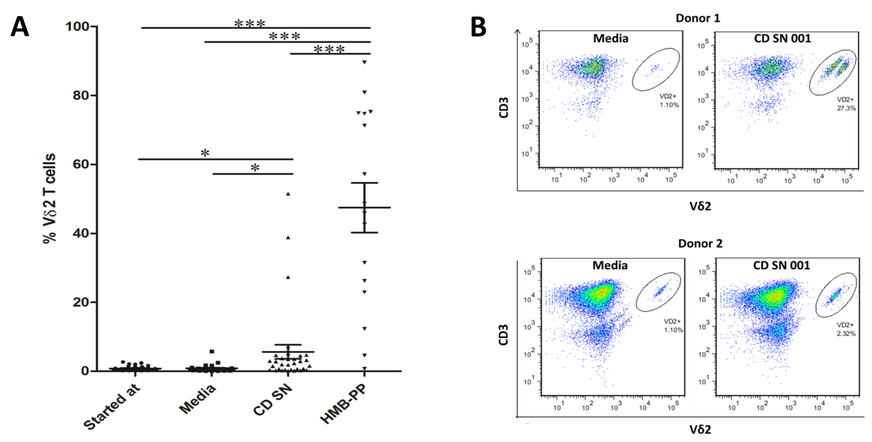

Figure 3. PBMC donors were stimulated with *C. difficile* supernatant strain 001 (denoted CD SN), BHIS media (denoted media) or HMB-PP and cultured for 2 weeks in IL-2-supplemented RPMI and examined for CD3 and Vδ2 expression by flow cytometry. **A**, scatterplot shows mean (±SEM) Vδ2 percentage following 2 weeks of culture with media (n=27), CD SN (n=32) or HMB-PP (n=16) compared to the starting percentage (n=32). **B**, representative flow cytometric dot plots depicting Vδ2 percentage following stimulation with media or *C. difficile* supernatant for two different donors.

C. difficile promotes the same cytokine profile in Vδ2 T cells as HMB-PP

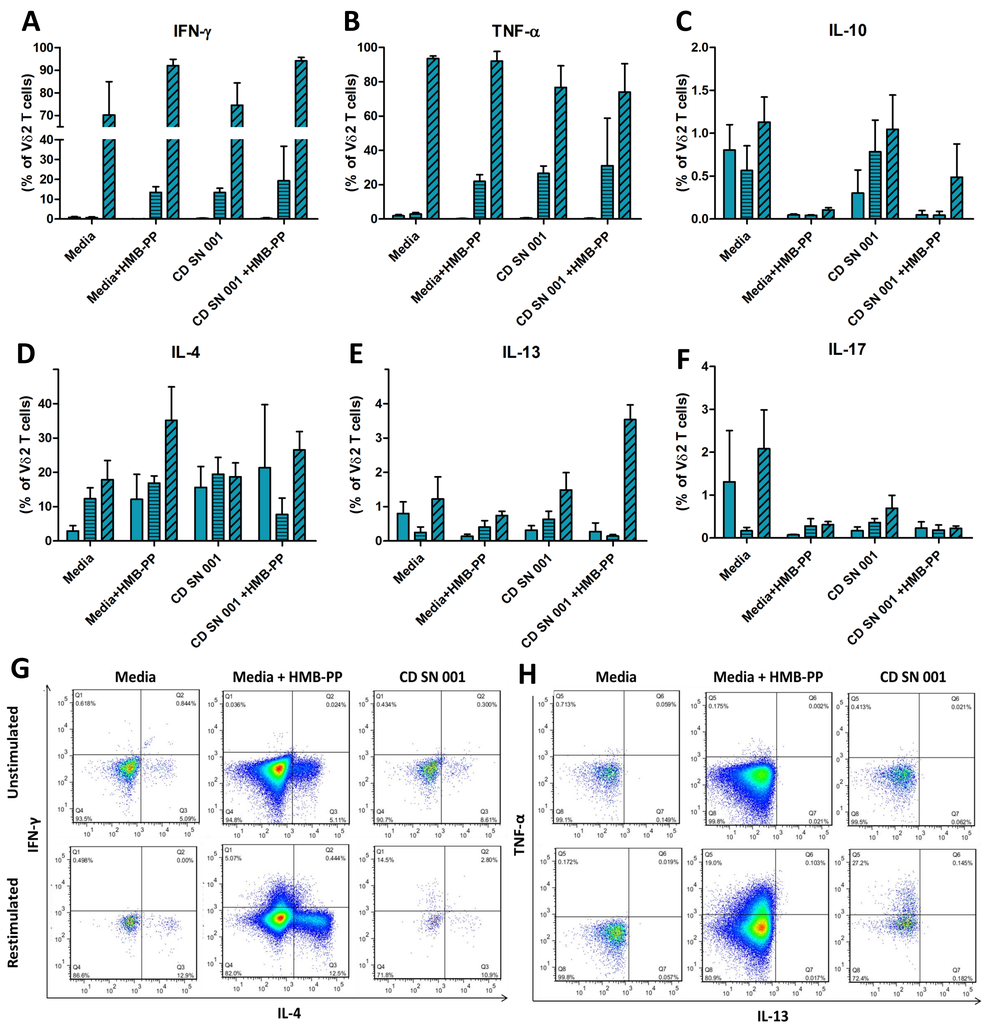

Figure 4. PBMC were expanded using *C. difficile* supernatant strain 001 (denoted CD SN 001) or BHIS media in the presence or absence of HMB-PP for 2 weeks in IL-2-supplemented RPMI. Cells were taken out of IL-2 overnight and then cultured in the presence of monensin for 4 h and left unstimulated or restimulated with the same stimulus as originally expanded with, and as a positive control, using PMA and ionomycin. The cells were then examined for CD3 and Vδ2 and intracellular expression of IFN-γ, IL-4, TNF-α, IL-10, IL-13 and IL-17 by Vδ2 T cells. **A-F**, mean (±SEM) percentage Vδ2 T cells expressing IFN-γ (**A**), TNF-α (**B**), IL-10 (**C**), IL-4 (**D**), IL-13 (**E**) and IL-17 (**F**) (n=6). **G-H**, representative flow cytometric dot plots showing IFN-γ and IL-4 (**G**) and TNF-α and IL-13 (**H**) expression by unstimulated (top panels) or restimulated (bottom panels) Vδ2 T cells.

CONCLUSIONS

We found that an unidentified factor secreted by *C. difficile* strain RT 001 was able to induce Vδ2 T cell expansion from PBMC, while the medium did not. However, the effect was not as striking as that observed with HMB-PP-stimulated PBMC. We speculated there may only be a low concentration of stimulus in the *C. difficile* supernatant. However, when a higher concentration of *C. difficile* supernatant was used, the *C. difficile* medium BHIS appeared to inhibit Vδ2 T cell expansion. Furthermore, examination of the ability of another two *C. difficile* strains RT 027 and ATCC in Vδ2 T cell activation revealed no difference in the stimulating capacities between the different strains. The *C. difficile* supernatants were devoid of toxins, as they were filtered using a 3 kDa filter, which excluded toxins and SLP. Thus, the stimulating agent in the *C. difficile* supernatant would have to be a small molecule. We further examined the role of *C. difficile* in cytokine secretion by Vδ2 T cells following *C. difficile* supernatant stimulation and compared these to HMB-PP-stimulated cells. We found that *C. difficile* induced the same cytokines as HMB-PP, the proinflammatory TH1 cytokines IFN-γ and TNF-α, while neither induced TH2 or TH17 cytokines. These results demonstrate a role for *C. difficile* in inducing an immune response through stimulation of Vγ9Vδ2 T cells. Future work will use liquid chromatography/mass spectrometry to determine whether *C. difficile* supernatant contains HMB-PP or whether it contains a distinct Vδ2 T cell-stimulating agent, such as a phosphoantigen.