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The NR4A agonist, Cytosporone B, attenuates pro-inflammatory mediators in human colorectal cancer tissue *ex vivo*

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ABSTRACT

Inflammation is a pivotal pathological factor in colorectal cancer (CRC) initiation and progression, and modulating this inflammatory state has the potential to ameliorate disease progression. *NR4A* receptors have emerged as key regulators of inflammatory pathways that are important in CRC. Here, we have examined the effect of *NR4A* agonist, *Cytosporone B* (*CsnB*), on colorectal tissue integrity and its effect on the inflammatory profile in CRC tissue *ex vivo*. Here, we demonstrate concentrations up 100 μM CsnB did not adversely affect tissue integrity as measured using transepithelial electrical resistance, histology and crypt height. Subsequently, we reveal through the use of a cytokine/chemokine array, ELISA and qRT-PCR analysis that multiple pro-inflammatory mediators were significantly increased in CRC tissue compared to control tissue, which were then attenuated with the addition of *CsnB* (such as IL-1β, IL-8 and TNFα). Lastly, stratification of the data revealed that *CsnB* especially alters the inflammatory profile of tumours derived from males who had not undergone chemoradiotherapy. Thus, this study demonstrates that *NR4A* agonist *CsnB* does not adversely affect colon tissue structure or functionality and can attenuate the pro-inflammatory state of human CRC tissue *ex vivo*.

1. Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-related morbidity and mortality worldwide, with an increase in CRC incidence in both sexes, particularly in those under the age of fifty [1]. Multiple life-style risk factors contribute to its pathogenesis including diet and smoking [1]. Additionally, inflammatory processes are known to play a pivotal role in the pathogenesis of CRC, both its initiation and progression [2]. The *nuclear orphan receptor family 4A* (*NR4A*), have emerged over the past decade as a key regulators of multiple processes such as inflammation, apoptosis, metabolism, stress response, differentiation and proliferation [3,4]. Recently molecules to manipulate these receptors have been described, and although work is in its infancy, they show promise as a therapeutic approach for treating inflammatory disease, having the capacity to concurrently repress pro-inflammatory processes and activate resolution pathways [4–6].

One of the first described natural agonists for *NR4A1* was

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Cytosporone B (*CsnB*), although recent evidence using protein structural footprinting shows it can also bind *NR4A2* [4,7]. While the biological relevance of such binding remains to be explored, such studies highlight the potential cross over of NR4A agonists. Recently the modulation of *NR4A1* using *CsnB* has been shown to be protective in inflammatory diseases of the gut [3]. Moreover, *CsnB* also has anti-fibrotic actions in mice [8]. The role of *NR4A1* in cancer remains unclear, with some studies showing it to be pro-oncogenic, while it was protective/tumour suppressing in other studies [9]. However, the role of *NR4A1* agonism on inflammatory activation in human CRC remains unexplored. In this study we have exposed CRC tissue *ex vivo* to *CsnB* and examined the impact on inflammatory outputs using array, quantitative ELISA and gene expression analysis. Here, we reveal *CsnB* attenuates production of inflammatory mediators in CRC tissue *ex vivo*, and, moreover, the attenuation was even more pronounced in males that had not undergone chemoradiotherapy. This study, to the best of our knowledge, is the first examining the effect of *CsnB* on CRC tissue *ex vivo*, and as such contributes significantly to the understanding of the efficacy of *NR4A* agonist *CsnB* on CRC.

2. Materials and methods

2.1. Patient recruitment

This study was approved by the Ethics Committee of St. Vincent's University Hospital, Dublin, and in accordance with International guidelines and Helsinki Declaration principles. Patients diagnosed with colorectal cancer and already undergoing surgical excision of tumour were recruited prospectively between September 2016 and April 2019. All patients ($n = 33$), aged between 39 and 88 years old, provided written informed consent and their demographic data including sex, age, location of tumour, and whether they had chemoradiotherapy prior to resection were noted and summarised in Supplemental Tables 1-3.

2.2. Tissue specimen preparation

Following colorectal resections, colonic human specimens (normal and tumour) were placed in 50 ml conical flasks containing RPMI 1640 GlutaMax media (Life Technologies), and transported to the laboratory (total-time 15 min). Normal colonic tissues were pinned luminal side up on the dissection board allowing for the dissection of circular and longitudinal smooth muscle from colonic mucosae. The stripped human normal colonic mucosae, and tumour specimens, were cut into 200 mg sections (Initial optimisation experiments revealed 200 mg to be the optimum weight for reliable data collection, data not shown), and added to a 12 well plate and treated as detailed in figure legends. Following treatments, media was removed and stored at −20 °C until ELISA and Human XL Cytokine/Chemokine array analysis, some tissue was placed in RNAlater and stored at −80 °C for further RNA extraction, cDNA synthesis and qRT-PCR analysis.

2.3. Ussing chamber and histological processing

Muscle stripped colonic mucosae were mounted onto the Ussing chambers (0.63 cm^2 window) and maintained in a temperature-controlled carbogenated (5% CO_2, 95% O_2) Krebs-Henseleit (KH) buffer for a period of 3 h. Each half of the chambers was filled with 5 ml kH and continuously mixed by gassing. After an equilibration period of 40–45 min, 100 μM CsnB was added to the apical compartment, the DMSO concentration did not exceed 0.1%. Baseline potential difference (PD, mV), short circuit current (Isc, $\mu A.cm^{-2}$) were measured and TEER ($\Omega.cm^2$) was calculated by Ohm's Law. Data is shown here as percentage of time 0 for each treatment to account for any variability in basal TEER ($\Omega.cm^2$) values resulting from individual tissue in different chambers over the course of the experiments. At the end of the 120 min treatment, stimulation of an Inward short-circuit current (Isc) with carbachol (CCh) (0.1–10 μM) was used as a functionality assay. At the end of the Ussing experiments, mounted tissues were then formalin-fixed (10%) and processed (Tissue-Tek® VIP, Sakura) overnight and embedded in wax. 5 μM section were cut (Leica microtome), mounted on slides, and stained with haematoxylin and eosin (H&E) stains. The slides were visualised using a light microscope and images were taken at 4x and 10x magnification with a high-resolution camera (Olympus BX43) and Image-Pro ® Plus version software (Media Cybernetics Inc., USA).

2.4. Cytokine/chemokine array

105 human cytokines, chemokines were evaluated using a Proteome Profiler Assay Human XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA) as per manufacturer's instructions. Membranes were then exposed in the same frame to X-ray film for 1–10 min. The pixel densities on the developed X-ray film selected were analysed using a transmission mode scanner and Image J® analysis software.

2.5. ELISA

Medium from treated human colonic tissue specimens (normal and tumour) was centrifuged briefly to eliminate tissue debris, and ELISA was performed for IL-8, MCP-1, and IL-1β as per manufacturer's instructions (#431504, #438804, #437004 respectively). 200 mg of tissue was used per treatment and values that varied, on occasion, were normalised to 200 mg post ELISA analysis. Tumour tissue below the weight of 100 mg was not used.

2.6. Real-time quantitative reverse transcription PCR (qRT-PCR)

RNA was extracted from *ex vivo* tissues using the GenElute Mammalian Total RNA Miniprep kit as per manufacturer's instructions (Sigma-Aldrich). cDNA synthesis and qRT-PCR was performed as previously described [10]. Primer pair sequences used are detailed in Supplemental Table 4. Relative expression/abundance levels of target gene transcripts were identified using qBase plus software (Biogazelle, Ghent University, Belgium) with adequate reference target determined using GeNorm Analysis (Supplemental Table 5).

2.7. Data presentation and statistical analysis

Results were analysed using GraphPad Prism 6 (GraphPad, San Diego, USA). All data is presented as mean ± standard error of the mean (SEM). A minimum of three individual n numbers (unless otherwise stated) were used. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test for cell line experiments (Supplemental Figure 1), and a *post-hoc* Dunnett's test for TEER experiments (Figure a,b). An unpaired Student's t-test and Mann-Whitney test were used for comparing crypt height and gene expression data (Fig. 1e) (Fig. 3c–f), while a Wilcoxon matched-pairs signed rank test was used for ELISA analysis (Fig. 3a,b,c) (Fig. 4) (Supplemental Figure 2,3). Statistical significance was considered when $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. NS=Not significant. Some p-values (p) are also included.

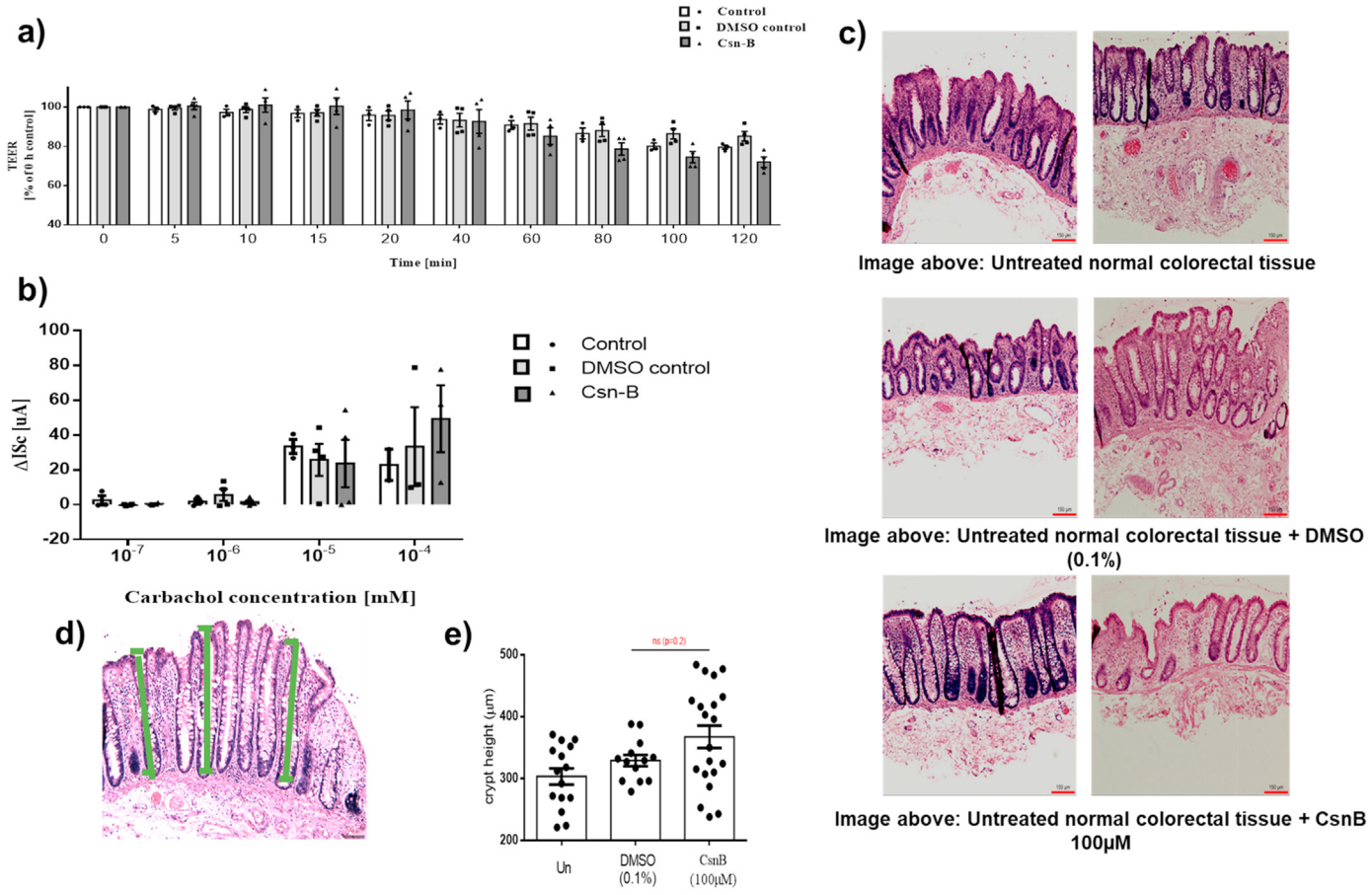

Fig. 1. The effect of Cytosporone B (CsnB) on human colorectal tissue (*ex-vivo*) integrity and functionality. a.) Non-cancerous colon tissue was mounted on an Ussing chamber. Tissue was then left untreated, treated with DMSO (0.1%) (vehicle control) or CsnB (100 μM) for 120 min. During the experiment baseline potential difference and short circuit current were measured. At the end of the experiment TEER was calculated by Ohm's law. Data are expressed as percentage of TEER at time 0hr ± SEM. b) At the end of the TEER experiment Carbachol was added at time 0 (10^{-7}mM), and then every 5 min thereafter the concentration was increased with the addition to the 10^{-6}mM, 10^{-5}mM, and 10^{-4}mM. Data are expressed as raw values representing Inward Short circuit current (ISc) in μA ±SEM. c.) Tissue was fixed and sectioned for histological analysis. Microscopic images of H&E stained slides at 4x magnification were taken using Olympus BX43 microscope (scale bar 150 μm). d) This is an example of how the crypt height was then calculated (green line) in μm. Olympus BX43 microscope was used and Image-Pro Premier 9.2 (Media Cybernetics) was utilised to calculate the crypt height. e) Bar graph representing crypt height calculated from 4 individual crypts from 3 separate sections in each treatment group. Data was expressed as μm ±SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. CsnB does not adversely affect human colorectal tissue integrity and functionality ex vivo

Preliminary studies using *in vitro* cell lines Caco-2 and THP-1 examined the effect of *CsnB* at various concentrations on cell viability. Supplemental Fig. 1a-h demonstrates positive control Etoposide significantly reduced metabolic activity of both cell lines, as measure using MTT and resazurin assays, and increases caspase 3/7 activity, both indirect and direct measurements of viability. While examination of the assays as a whole, indicate that *CsnB* at all concentrations up to 100 μM does not adversely affect viability (Supplemental Fig. 1a-h). Subsequently, we exposed non-cancerous colonic tissue to 100 μM CsnB, as selected from *in vitro* experiments, and examined gut integrity using established methods [11]. No changes in transepithelial electrical resistance (TEER) were observed between groups (Untreated control, control with DMSO (0.1%) (vehicle control for *CsnB*), and tissue exposed to 100 μM CsnB) over 120 min, indicating that 100 μM CsnB does not adversely alter colonic tissue integrity (Fig. 1a).

To confirm functionality of colonic tissue, an Isc was evoked by carbachol at varying concentrations after the 120 min Ussing experiment. A concentration response relationship was observed with increasing concentrations of carbachol in all groups, indicating that *CsnB* did not adversely affect tissue functionality (Fig. 1b). Lastly, histological analysis of this tissue showed no overt changes in architecture/histology, or crypt height, across all treatments (Fig. 1c,d,e).

3.2. CsnB attenuates pro-inflammatory mediators from colorectal tumours ex vivo

Having established *CsnB* did not adversely affect colonic tissue integrity or functionality we examined the affects it had on inflammatory output from CRC tissue. A cytokine/chemokine array was then performed using media collected at 8 h from vehicle-treated control colon tissue, untreated tumour tissue, tumour tissue +100 μM CsnB and tumour tissue +20 μM CsnB for 8 h. For analysis, media from four experiments was pooled per treatment to increase reliability of the data output. Supplemental Table 6 illustrates the raw pixel values of the 105 inflammatory mediators measured. 35 targets were increased by two-fold in tumour tissue compared with 'normal' control tissue and subsequently decreased

greater than 2-fold again with the addition of *CsnB* (either 20 μM or 100 μM or both). TNFα was included for further analysis as it is a well established *NR4A* target, albeit it is increased only 1.92 fold from tumour compared to normal tissue, while decreased 2.05 with the addition of *CsnB* (20 μM). These 36 targets are highlighted in yellow on Supplemental Table 6. Fig. 2a shows the raw array image following initial ImageJ analysis with positive control (internal array control), negative control (internal array control) and some relevant targets, IL-8, MCP-1 and IL-1β. Fig. 2b shows a heatmap displaying the 36 targets described above. Circles beside each target identify those that have been previously shown in the literature to be altered/regulated by *NR4A1* (Blue circle), *NR4A2* (Red circle) and *NR4A3* (Orange circle) (Fig. 2c), [12–23]. Fig. 2d illustrates the fold over normal untreated control tissue values for these 16 targets, taken from Supplemental Table 6.

The results of the cytokine/chemokine array identified targets of interest which were then analysed and validated using individual ELISAs and gene expression. Fig. 3a–c shows ELISA data for IL-8, MCP-1 and IL-1β. IL-8 was included as it is a well established *NR4A* target, a pivotal cytokine in CRC, albeit it was not identified from the array as it displayed high/saturated pixel density in all treatments. However, ELISA analysis revealed that both IL-1β and IL-8 were significantly secreted from tumour tissue compared to control tissue, while no significant changes were observed in MCP-1 (Fig. 3a–c). Although IL-8 and IL-1β secretion was maintained significant in tumour tissue treated with *CsnB* compared to untreated control a trend toward a reduced concentration of secreted protein was observed, and more pronounced in IL-1β. Next we examined the gene expression profile of targets IL-8, MCP-1 and also TNFα. MCP-1 gene expression confirms the ELISA data showing no significant changes in any treatments, while both IL-8 and TNFα displayed significant increases in gene expression in tumour tissue compared to untreated control tissue, and this significance was attenuated with increasing concentrations of *CsnB* (Fig. 3d–f). Gene expression was not performed for IL-1β given it is processed primarily post-transcriptionally [24]. Thus, taken together, data here supports that CsnB can attenuate inflammatory mediators in CRC tissue *ex vivo*.

3.3. Stratification of data reveals CsnB may affect CRC dependent on sex and whether the tumour was exposed to chemoradiotherapy

We next stratified the ELISA data based on sex, location, chemoradiotherapy treatment yes/no, sex with chemoradiotherapy treatment yes/no for IL-8, MCP-1 and IL-1β. Stratifying the ELISA data allowed for statistical analysis given the fact that comparable samples sizes were present in most of the stratifications. Fig. 4 shows the stratification data of interest discussed here, while other stratification data is shown in Supplemental Fig. 2 and 3. Of note, *CsnB* affected IL-8 and IL-1β levels from tumours that were derived from males compared to females, colon versus rectum and those that did not receive chemoradiotherapy versus had chemoradiotherapy, separately (Fig. 4a,b,c). When combining some of the stratifications, a reduction in IL-8 and IL-1β secretion following treatment with *CsnB ex vivo* was observed in males who had not undergone chemoradiotherapy versus those that had, albeit not

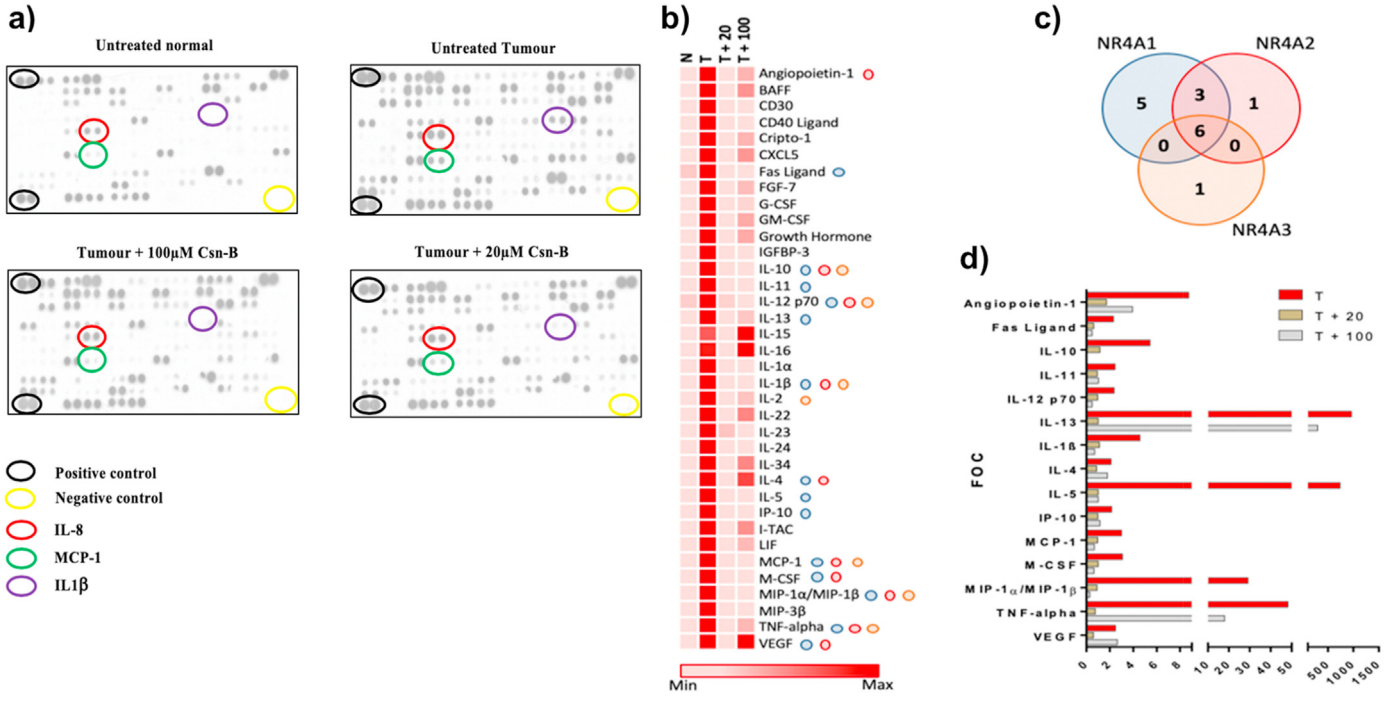

Fig. 2. The effect of Cytosporone (CsnB) on cytokines/chemokines secretion from human colorectal cancer tissue *ex vivo*. Non-cancerous colon and tumour tissue (T) with/without 20 μM and 100 μM Cytosporone B (CsnB) (n = 4) were incubated for 8hrs in a tissue culture incubator. Media was removed from 4 separate experiments, pooled and a cytokine/chemokine array was performed for 105 inflammatory targets and internal positive/negative controls. a.) Dot plot images of the array are displayed post Image J software analysis. Each dot (in duplicate) represents one target. This image shows the membranes (Untreated normal, Untreated Tumour, Tumour + 100 μM CsnB, Tumour + 20 μM CsnB) highlighting the areas of the positive control, negative control, and key targets of interest, IL-8, MCP-1, and IL1β. 3b) Following pixel density analysis using image J analysis (See supplemental table 6), displayed here is a heat map representing 35 targets identified in the array to be greater than 2-fold increase in tumour (T) compared to untreated normal control tissue (N), which were subsequently decreased greater than 2-fold with the addition of either 20 μM or 100 μM CsnB (T+20 and T+100 respectively) (TNFα is also included in this analysis and the rationale for this is explained in the manuscript). Coloured circles beside targets highlight those identified from the literature as *NR4A* targets (Blue = NR4A1, Red = NR4A2 and Orange = NR4A3). 3c) A Venn diagram represents these targets and how many were identified in the literature to have been regulated by all three, two or one *NR4A* family members. 3d) Raw pixel density of targets in part c are shown as fold over untreated normal control tissue in treatments T, T+20 (μM CsnB) and T+100 (μM CsnB). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

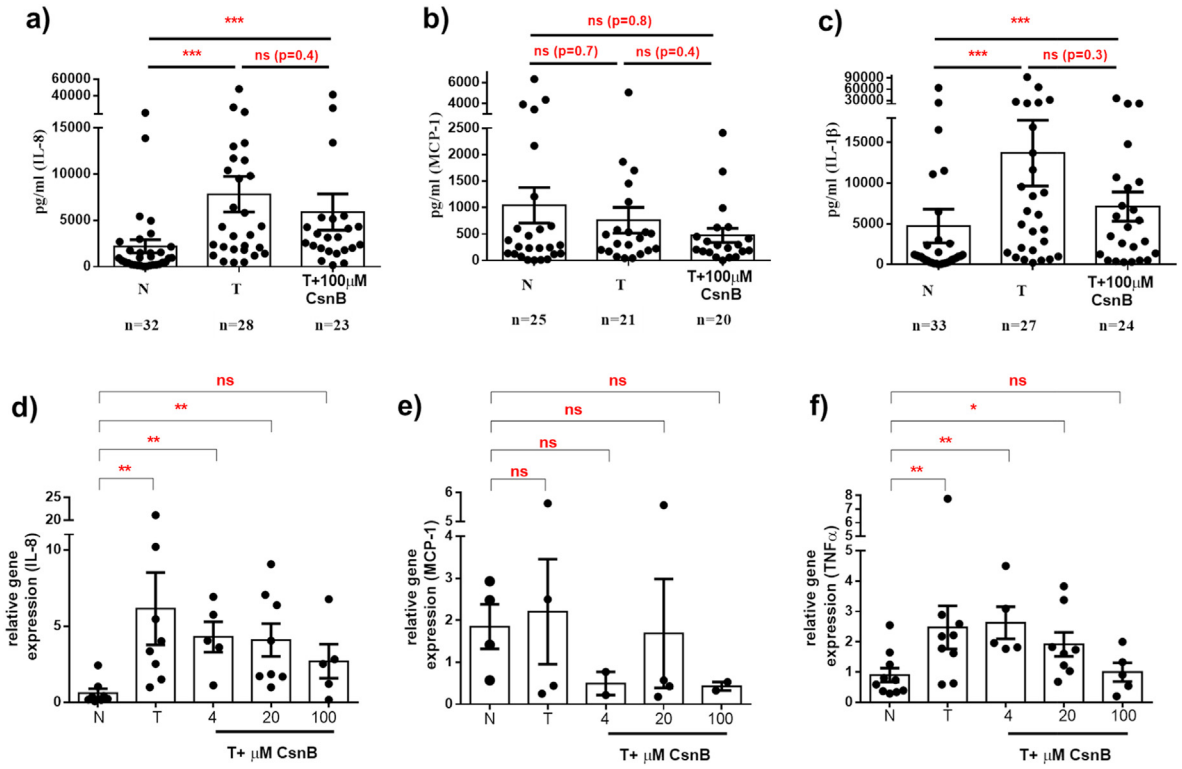

Fig. 3. The effect of Cytosporone B (CsnB) on IL-8, MCP-1, IL-1β and TNFα levels from colorectal cancer tissue *ex vivo*. a-f) Tumour tissues (T) were treated with/without CsnB (100 μM, a-c) (4, 20 and 100 μM, d-f) alongside untreated non-cancerous control tissue (N) for 8hrs, followed by ELISA of the media for IL-8, MCP-1, and IL-1β (a–c) and RNA isolation followed by qRT-PCR for IL-8, MCP-1, TNFα and housekeeping genes H3F3A and YWHAZ (d–f). Data were expressed as pg/ml ±SEM (a) and raw CNRQ values ± SEM (b) (sample numbers are written under each treatment on the graph itself).

significantly decreased when compared to tumour alone (Fig. 4d and e). Regarding MCP-1 stratification, colon derived tumours versus rectum displayed enhanced MCP-1 secretion, which trended toward an attenuation with by *CsnB*, albeit not significantly (Fig. 4f). Thus, following stratification, *CsnB* addition displays a trend toward a more pronounced reduction in IL-8 and IL-1β secretion from CRC tissue in males that have not undergone chemoradiotherapy.

4. Discussion

Multiple studies have suggested anti-inflammatory effects of the NR4A agonist CsnB in various models of inflammatory disease [3,8,25]. This study, to our knowledge, is the first to investigate the effects of CsnB on human CRC tissue *ex-vivo*, examining both toxicity and inflammatory outcome. The range of concentrations CsnB used did not adversely affect cell or tissue viability and functionality as measured using the experimental approaches utilised herein. While some studies support our *in vitro* viability findings [26,27], it is important to note others have shown CsnB at concentrations of 100 μM to be toxic to *in vitro* cells, albeit we can conclude CsnB associated toxicity appears to be cell-type specific [5,7,28]. Regarding colon tissue, CsnB up to concentrations of 100 μM does not adversely affect tissue integrity/functionality as measured using the Ussing chamber and subsequent treatment with carbachol. Moreover, CsnB did not overtly alter human colonic crypt height, which is supported by previous work using Nurr77+/+, Nurr77−/− mice models treated with CsnB and displaying no change in crypt height [3].

We then examined what affect CsnB would have on the inflammatory output from CRC tissue. Array analysis revealed 35 targets which were 2-fold higher in CRC tissue compared to normal, which were then decreased 2 fold with the addition of CsnB (with either 20 μM or 100 μM). Given that CsnB is a confirmed NR4A1 agonist, and not much data exists for CsnB, we performed an extensive literature for the 36 targets to examine which had been shown to be NR4A targets previously, which resulted in 16 targets [12–23]. MCP-1, TNFα, IL-1β (identified by the array), and the well established NR4A target IL-8, were focused on in this current study as they are known to not only be NR4A1 targets but also play a substantial role in CRC in terms of stimulating tumour growth, metastasis and chemoresistance [2,29]. Gene expression analysis and/or ELISA reveals that the addition of CsnB resulted in a trend towards or in some cases a significant reduction in IL-8, IL-1β and/or TNFα. Additionally, stratifying the ELISA results revealed that CsnB seemed to have a more pronounced effect at attenuating IL-8 and IL-1β from males who had no chemoradiotherapy prior to resection. The sex-specific effects of CsnB have not been reported to our knowledge, while sex differences in NR4A1 KO mice have [30]. Several other studies revealed sex-specific differences in colorectal cancer incidence, tumour location, stage at diagnosis, and mortality rates [31]. The sex-specific impact on CRC is complex, from initiation right through to treatment, and although the data collected here highlights a potential sex-specific effect of CsnB in CRC, it does not allow for simple conclusions to be drawn at this point, but highlights further investigation is warranted.

Taken together, the results from this study show that CsnB can modulate multiple pro-inflammatory mediators in colorectal cancer tissue *ex vivo* with no evident cell cytotoxicity and is tolerated in human colorectal tissue as evidenced by experimental approaches utilised herein. These data, to the best of our knowledge, are the first to describe an anti-inflammatory effect of CsnB on human CRC tissue *ex vivo*. Bearing that in mind, further research is warranted to

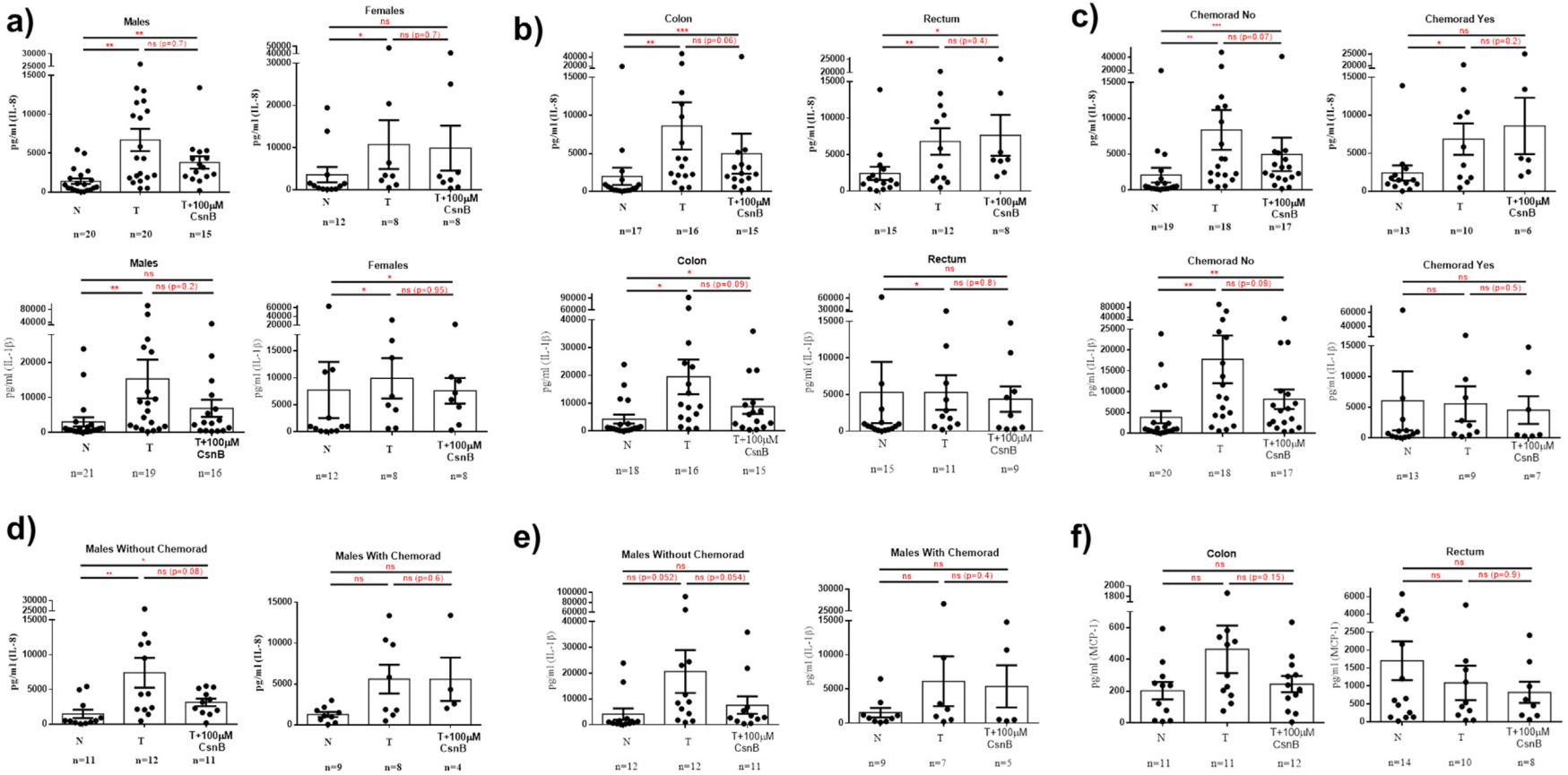

Fig. 4. The effect of Cytosporone B (CsnB) on IL-8, IL-1β and MCP-1 secreted protein from patients following stratification based on sex, location, having had chemoradiotherapy or combinations thereof. ELISA data from Fig. 3, Tumour tissues (T), T + with CsnB (100 μM) alongside untreated non-cancerous control tissue (N) for 8hrs is shown based on a.) sex, b.f.) location, c.) having had chemoradiotherapy (Chemorad Yes) or not (Chemorad No), and sex + Chemorad Yes/Chemorad No. Data were expressed as pg/ml ±SEM (sample numbers are written under each treatment on the graph itself).

support this data and to better understand the anti-inflammatory mechanism of action of CsnB on human CRC tissue.

Declaration of competing interest

The authors of the article below declare no conflicts of interest associated with this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.03.110.

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