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The role of IL-1 β and TNF- α signaling in the genesis of cancer treatment related symptoms (CTRS); a study using cytokine receptor-deficient mice

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Abstract

Cytotoxic chemotherapeutic agents often induce a cluster of cancer treatment related symptoms (CTRS). The purpose of this study was to develop a mouse model of CTRS to examine the role of IL-1 β and TNF- α signaling in the genesis of these symptoms. CTRS (change in wheel running activity, food intake, and body weight from baseline) were examined in wild type (WT) mice or mice lacking the TNF- α p55 (type 1) receptor (TNFR1 $^{-/-}$) and/or IL-1 β type 1 receptor (IL-1R1 $^{-/-}$) injected with four doses of cyclophosphamide/Adriamycin/5-fluorouracil (CAF) at 20-day intervals. Inflammatory cytokines in blood and tissues were measured using multiplex immunoassays and quantitative RT-PCR. ANOVA was used to examine differences between genotype and/or treatment group. Kaplan-Meier analysis was used to estimate survival rate. CAF rapidly increased IL-1 β and TNF- α signaling in WT mice. CAF induced acute CTRS immediately following drug injection which returned to baseline prior to the next CAF dose. Persistent CTRS were evident 3 weeks after the 4th CAF dose. Acute but not persistent CTRS were associated with increased levels of IL-7, IL-9, KC, MCP-1, GCSF, and IP-10. This CAF induced inflammatory response was blunted in IL-1R1 deficient mice and absent in IL-1R1/TNFR1-deficient mice. IL-1R1 $^{-/-}$ mice showed an identical pattern of CTRS to their WT counterparts. The assessment of CTRS in IL-1R1/TNF-R1-deficient mice was precluded by severe toxicity. Our data suggest that

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Competing interests

The authors declare that they have no competing interests

Authors' contributions

LJW conceived of and designed the study. LJW, LBS, CA, and TJW performed the experiments. MCL and LJW performed the statistical tests. LJW, LBS, and KW drafted the manuscript and figures. All authors read and approved the final manuscript.

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an important function of the IL-1 β and TNF- α driven inflammatory cascade is to promote recovery following exposure to cytotoxic agents.

Keywords

Sickness behavior; IL-1 β ; TNF- α ; cancer related fatigue

Background

Cancer patients undergoing treatment with cytotoxic chemotherapy often experience a cluster of symptoms including fatigue, loss of appetite (anorexia), sleep disturbance, pain, and cognitive dysfunction, which can have profound negative effects on physical functioning and quality of life during and after treatment [1,2]. Cancer treatment related symptoms (CTRS) typically occur in the days following chemotherapy infusion [3–5]. In approximately large proportion of cancer survivors, CTRS, most notably fatigue, can persist for months or years after cytotoxic chemotherapy has ended [6]. The etiological mechanisms underlying CTRS are unclear. It has long been suspected, however, that CTRS are homologous to sickness behavior, a physiological response to activation of the innate immune system triggered by increased production and synergistic action of the pro-inflammatory cytokines interleukin 1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) [7–9].

IL-1 β and TNF- α are produced by macrophages, monocytes and other cells of the innate immune system in response harmful stimuli such as cytotoxic agents. Cytotoxic chemotherapy kills tumor cells but also healthy cells in the hematopoietic and gastrointestinal systems. The intracellular components liberated from these dying cells act as “*Danger Signals*” which prime resident tissue macrophages via activation of their surface Toll-Like-Receptors (TLRs) [10]. In addition, priming of these cells via TLR activation also occurs following bacterial translocation (passage of bacteria or bacterial products i.e. endotoxin, across the intestinal mucosal barrier) related to chemotherapy induced gastrointestinal damage [11,12]. In addition to these indirect effects, cytotoxic chemotherapy directly increase the synthesis and release of IL-1 β from macrophages via activation of the mitogen activated protein 3 kinase (MAP3K) ZAK and formation of the NLRP3 inflammasome [13,14]. Once released IL-1 β and TNF- α bind to specific IL-1 (i.e. IL-1R1-receptor) or TNF (TNFR1-receptor) receptors on target cells which in turn produce additional inflammatory cytokines and chemokines including IL-6, IL-8, and IL-10. The resulting systemic inflammatory response mobilizes the immune system to fight infection, initiates tissue repair mechanisms, and reorganizes homeostatic mechanisms to favor survival [15].

Although IL-1 β and TNF- α generally act locally in an autocrine and paracrine manner, a pro-inflammatory stimulus of sufficient magnitude can cause a transient increase in the levels of these cytokines in peripheral blood along with increased levels of several other inflammatory cytokines and chemokines [22]. One such stimulus is bacterial lipopolysaccharide (LPS), which is often used as an inflammatory stimulus in rodent and human models of sickness behavior. Following peripheral administration of LPS, IL-1 β and

TNF- α levels peak in peripheral blood within minutes to hours and this rise occurs at the onset of sickness behavior [16,17]. Fatigue, anorexia, weight loss and other signs of sickness behavior, which can persist for several days after immune challenge, are mediated by the action of IL-1 β and TNF- α on specific populations of inflammatory-responsive neurons in the hypothalamus and cerebellum [18–22]. While the short term production of IL-1 β and TNF- α promotes survival, prolonged production of these cytokines, is no longer beneficial, and is associated with a variety of illness states including depression [23], muscle wasting [24], bone loss [25], and anemia [26], all of which can occur in cancer patients towards the end of treatment after exposure to repeated cycles of cytotoxic chemotherapy.

Pre-clinical studies have shown that cytotoxic chemotherapy increases blood levels of IL-1 β and TNF- α [13,27,28] although this increase was often observed after multiple daily doses of chemotherapy or following administration of high drug doses. Several other studies have shown that cytotoxic chemotherapy induces fatigue, anorexia, cognitive dysfunction, and other signs of cytokine induced sickness behavior when injected into rodents [29–32]. Yet few of these studies have demonstrated a relationship between chemotherapy induced inflammation and CTRS, or determined the specific role of IL-1 β and TNF- α in the genesis of these symptoms. The purpose of the present study was to test the hypothesis that CTRS are caused by cytotoxic chemotherapy mediated induction of IL-1 β and TNF- α using a clinically relevant adjuvant breast cancer chemotherapy treatment mouse model. Tumor bearing animals were not incorporated into our experiments because our previous work showed no effect of tumor burden on sickness behavior-like symptoms in mice administered etoposide [32]. In addition we wanted to focus attention on CTRS in the context of adjuvant chemotherapy for breast cancer which is administered post-surgery when tumor burden is negligible.

Methods

Mice

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University. Female mice lacking the interleukin-1 beta type 1 receptor (IL1R1: B6.129S7-Il1r1tm1Imx), and both IL1R1 and tumor necrosis factor alpha p55 (type 1) receptor (TNFR1: B6.129S-Tnfrsf1atm1Imx Il1r1tm1Imx/J), and genetically matched wild type (WT) control mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice for CTRS assessment were housed singly in a cage fitted with a running wheel (diameter 30 cm) so that voluntary wheel running activity (VWRA) was monitored throughout the experiment (Minimitter, Sun River OR). All mice had ad lib access to food and drinking water supplemented with antibiotics (1.3mg/ml sulfamethoxazole and 0.3mg/ml trimethoprim) to prevent infection related to neutropenia, secondary to chemotherapy-induced bone marrow suppression. Mice were monitored daily for signs of morbidity due to the experimental procedure and were removed from the study if they lost 20% of their body weight, or had a moribund appearance (ruffled dull fur, hunched posture, abdominal distention). Mice displaying these signs for > 2 days were removed from the study and sacrificed according to protocols established at OHSU Department of Comparative Medicine.

Cytotoxic chemotherapy administration in mice

Mice were administered CAF, a combination of cyclophosphamide (Cytosan), doxorubicin (Adriamycin), and 5-fluorouracil (5-FU) at concentrations of 167mg/kg, 4mg/kg, and 167mg/kg respectively. This drug regimen was chosen to reflect a clinically relevant adjuvant treatment regimen in breast cancer patients. Mice were first injected intraperitoneally (ip) with Cytosan + Adriamycin in a volume of 1 mL normal saline (NS). One hour later mice were injected IP with 1mL NS containing 5-FU. NS-treated mice were injected with the same volume of NS without drug. All injections began at 3pm and finished at 5pm. In Experiments 2, 3, and 4, CAF-treated mice were administered 4 doses of CAF at approximately 10–20 day intervals to mimic the dosing schema in the clinical setting.

Experiment 1: Assessment of IL-1 β and TNF- α expression in blood and tissues of CAF- and NS-treated mice

For the assessment of cytokines in peripheral blood, female mice were separated into two groups. Mice in Group 1 (n = 50) were injected with CAF while mice in Group 2 (n = 50) were injected with NS. Blood was collected from 9–10 mice from each group at 1-, 3-, 6-, 14- and 24-hours post injection. Serum was prepared from all blood samples and levels of IL-1 β and TNF- α were measured as described below. For the measurement of tissue levels of IL-1 β and TNF- α mRNA, a separate cohort of female mice, housed under identical conditions as above were separated into two groups of 12 mice; mice in Group 1 were injected with CAF while mice in Group 2 were injected with NS. At 3-, 6-, 14-, and 24-hours post-injection 3 mice from each Group were terminally sedated and perfused with ice cold-NS containing 10 units per mL of heparin sulfate. Each mouse was perfused for 5 minutes after which time liver, and spleen, were removed from each animal and stored in RNA later (Qiagen) for 24 hrs then frozen at –80°C. Hypothalamus and cerebellum was removed from mice at the 24-hour time point only. Relative IL-1 β and TNF- α mRNA levels in each tissue type were quantified and normalized to GAPDH gene expression using two-step qRT-PCR as described below.

Experiment 2: Assessment of patterns of CTRS in mice administered 4 doses of CAF—CTRS as evidenced by changes VWRA, food intake, and body weight from baseline were monitored daily. Female mice were housed singly in pathogen-free rooms (12-hour light-dark cycle) in shoebox cages modified to include an activity wheel (diameter 30 cm) (Minimitter, Sun River, OR). All mice had ad lib access to drinking water supplemented with antibiotics (1.3mg/ml sulfamethoxazole and 0.3mg/ml trimethoprim). VWRA was monitored for each mouse using the VitalView Data Acquisition System (Respironics, Sun River, OR). To measure daily food intake, approximately 10 g (2–3 pellets) of Rodent Diet 5001 (PMI Nutrition International, Brentwood, MO) were placed in the bottom of the cage each day. The following day, uneaten food was weighed and the amount of food eaten during the previous 24 hours was calculated. Mice were weighed daily between 3–5 pm. After a two week acclimation period, each mouse was allowed to establish a 10-day baseline VWRA, after which they were separated into two groups. Mice in Group 1 (n = 15) were injected with CAF (as described above) while mice in Group 2 (n=16) were injected with NS. After injection mice were returned to their home cages. Mice received subsequent doses of either CAF or NS every 19 days. VWRA, food intake, and body weight were assessed throughout 4 doses of CAF or NS up until 19-days after the last dose.

Experiment 3: Measurement of serum inflammatory cytokine markers of CTRS

—Female mice were housed singly as described in Experiment 2. Mice were allowed to establish a 10-day baseline VWRA level after which time they were separated into two groups of 20 mice. Mice in Group 1 were injected with CAF whereas mice in Group 2 were injected with NS. Mice were returned to their home cages and daily VWRA was monitored. Twenty-four hours post CAF-injection, when CAF-treated mice were considered acutely symptomatic, 6–8 mice from each group were terminally sedated and peripheral blood collected by cardiac puncture. The remaining mice remained in their home cages and daily VWRA monitored until there was no significant difference between VWRA between CAF and NS-treated mice. At this time point, when CAF treated mice were considered asymptomatic, 6–7 mice from each Group were terminally sedated and peripheral blood was collected by cardiac puncture. The remaining mice in Group 1 (n=6) and Group 2 (n=6) were administered 3 more doses of CAF at 19 day intervals. Twenty days after the last dose of CAF or NS, when CAF-treated mice were considered persistently symptomatic, the remaining mice in each Group were terminally sedated and peripheral blood was collected by cardiac puncture. Serum was prepared from all blood samples and levels of levels of eotaxin, G-CSF, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, KC (CXCL1), IL-9, IL-10, IL-12(p40), IL12(p70), IL-13, IL-15, IL-17, IP-10 (CXCL10), LIF, LIX (CXCL5), MCP-1 (CCL2), M-CSF, MIG (CXCL9), MIP-1 α (CCL3), MIP-1 β (CCL4), MIP-2 (CXCL2), RANTES (CCL5), TNF- α , and VEGF were measured as described below.

Experiment 4: Examining the specific role of IL-1 β and TNF- α signaling in

CTRS—Mice were housed as described in Experiment 2. Prior to establishing a 10-day baseline VWRA level, mice underwent body composition assessment by dual-energy x-ray absorptiometry (DEXA) densitometry using a Lunar PIXImus II (software version 1.42.006.010; Lunar Corp, Madison, Wisconsin). After scanning, mice from each genotype were separated into 2 groups of 18 mice. Nine of the 18 mice in each group were injected with CAF (as described above) while the remainder were injected with the same volume of NS without drug. After injection, mice were returned to their home cages and daily VWRA, food intake, and body weight were monitored throughout 3 additional CAF or NS-injections. Mice were monitored daily for signs of morbidity due to the experimental procedure and removed from the study if they met the criteria described above. Three weeks after the 4th injection of CAF or NS, all surviving mice underwent body composition scanning. The following day mice were terminally sedated and peripheral blood collected by cardiac puncture. Red blood cell (RBC) count and hemoglobin (Hgb) level were measured using the Vet ABC-Diff Hematology Analyzer (Heska Corp.).

Serum Analysis of Inflammatory Cytokines and Chemokines

Blood collected by cardiac puncture was allowed to clot for 1 hour, centrifuged at 8,000 rpm in a microcentrifuge and the serum removed, aliquoted and immediately stored at -87°C prior to analysis. Serum cytokines and chemokines were measured in duplicate using a bead-based immunofluorescence assay according to the manufacturer's instructions. In Experiment 1 only serum levels of IL-1 β and TNF- α were measured whereas a larger panel of inflammatory cytokines and chemokines was measured in Experiment 3 using a pre-

mixed bead assay (Millipore Inc. # MPXMCYTO70KPMX32). Data were collected and analyzed using the Luminex-100 system Version IS (Luminex, Austin, TX, USA). A four or five-parameter regression formula was used to calculate the sample concentrations from the standard curves. To minimize lot-to-lot and user-to-user variation in analyte levels sera collected from each experiment were analyzed by the same technician using kits with the same lot number.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Analysis

Relative IL-1 β and TNF- α mRNA levels in liver, spleen, hypothalamus, and cerebellum were quantified and normalized to GAPDH gene expression using two-step qRT-PCR. In the first step, purified RNA was quantified and first-strand cDNA synthesis was performed on ~3 μ g RNA in a 30 μ l reaction using an Omniscript RT kit (Qiagen, Valencia, CA, USA). In the second step, IL-1 β , TNF- α , and GAPDH transcription in each sample was quantified in separate reactions on an Opticon2 DNA Engine (MJResearch, Waltham, MA, USA) using target-specific primers and a target-specific fluorescence resonance energy transfer (FRET) probe (Biosource, Camarillo, CA, USA). Primer and FRET probe sequences were as follows: IL-1 β 5'-CTGTCGGACCCATATGAGC-3' (forward), 5'-GCTCATGGAGAATATCACTTGTG-3' (reverse), 5'-FAM-AAGCTCTCCACCTCAATGGACAGA-BHQ1-3' (FRET probe); TNF- α 5'-AGACCCTCACACTCAGATCATG-3' (forward), 5'-TCAGCCACTCCAGCTGCT-3' (reverse), 5'-FAM-CCACGTCGTAGCAAACCACCA-BHQ1-3' (FRET probe); GAPDH 5'-CCTCAACTACATGGTCTACATGTTC-3' (forward), 5'-GCTCCTGGAAGATGGTGATG-3' (reverse), 5'-FAM-CATTCTCGGCCTTGACTGTGCCGT-BHQ1-3' (FRET probe). Each qRT-PCR target was measured with 5 replicate wells for each sample (n=5). PCR reaction efficiency (i.e. the linearity of the CT versus log relationship) was monitored by performing qRT-PCRs on serial 10-fold dilutions of cDNAs derived from each tissue type.

Analysis

One way analysis of variance (ANOVA) was used to determine significant differences between treatment type (CAF or NS) at the 95% confidence interval where $p < 0.05$. Cytokine CT values for each sample and primer pair combination and primer efficiency were used to determine fold expression values for IL-1 β and TNF- α in CAF treated relative to control mice using the Relative Expression Software Tool (REST-2009, <http://www.gene-quantification.de/rest-2009.html>). The examination of statistical significance ($p < 0.05$) with this tool was done by a Pair Wise Fixed Reallocation Randomization Test as previously described [33]. Daily VWRA was calculated from the number of wheel turns in a 24-hour period (12-hour (LD) cycle). VWRA base10 was calculated as the average daily VWRA for the 10-day period prior to the start of treatment. The distribution of some of the inflammatory analytes measured did not follow a normal distribution and therefore transformations were applied. The types of transformations used for each serum analyte are presented in Table S1 (Supplemental Data). The decision to transform a serum analyte variable was based solely on whether or not the distribution was consistent with the assumptions of the analysis [34,35]. We inspected the distribution for each serum analyte variable separately, and chose the transformation that brought the distribution closest to

normal. We also performed a sensitivity analysis by using all of the variables in their original metric, and there was no case where the follow-up univariate F-test went from non-significant to significant as a result of the transformation. A 3 X 2 between-subjects multivariate analysis of variance (MANOVA) was used to determine whether an interaction existed between CTRS state (acute, non, and persistent) and treatment (CAF or Control) on serum inflammatory markers. Follow-up univariate F tests were performed on the dependent variables in the presence of a significant multivariate interaction. Repeated measures ANOVA were used to examine patterns of change in VWRA, food intake, or body weight between genotype and/or treatment group. Kaplan-Meier analysis was used to estimate survival rate. Log-rank testing was used to evaluate the equality of survival curves.

Results

Cytotoxic chemotherapy causes systemic increases in IL-1 β and TNF- α

LPS administration in rodents causes a rapid and systemic increase in IL-1 β and TNF- α expression that coincides with the onset of sickness behavior. We hypothesized that cytotoxic chemotherapy triggers a similar systemic response when injected into mice. To test this hypothesis we injected mice with CAF or NS and at 1-, and/or, 3-, 6-, 14-, and 24-h post-injection collected peripheral blood, liver, spleen, hypothalamus and cerebellum (24-h time point only) from each mouse. Figure 1 A. shows fold increase in serum IL-1 β and TNF- α levels in CAF- treated mice relative to NS-treated control mice at each time point. CAF treatment had no observed effect on blood levels of IL-1 β and TNF- α at 1-hour post injection ($F(1,18)=.000$, $p=.993$ and $F(1,18)=.468$, $p=0.503$ respectively). Three hours post-injection, however, blood levels of IL-1 β were 4–5 fold higher than in controls ($F(1,18)=29.822$, $p<0.0001$) and TNF- α levels were 3-fold higher than controls ($F(1,18)=60.868$, $p<0.0001$). This relative increase, however, was short-lived, as six-hours post injection serum levels of IL-1 β and TNF- α were significantly lower than in control mice ($F(1,18)=14.900$, $p=0.001$ and $F(1,18)=17.069$, $p=0.001$ respectively). At 14- to 24-hours post-injection serum levels of IL-1 β increased again in CAF treated mice relative to controls ($F(1,18)=.6334$, $p=0.022$ and $F(1,18)=11.36$, $p=0.004$ respectively). A similar increase in serum TNF- α in CAF-treated mice was also evident at 14- and 24-hours post-injection ($F(1,18)=5.468$, $p=0.032$ and $F(1,18)=2.586$, $p=.126$ respectively). This highly variable serum response to CAF was not evident when examining IL-1 β and TNF- α gene expression in liver and spleen. Relative to control mice, IL-1 β mRNA levels in liver (Fig. 1 B) and spleen (Fig. 1C) of CAF-treated mice were consistently 2–4 fold higher in CAF-treated mice relative to controls at each time point (see box and whisker plots in Fig. 1B and C for median fold increase, standard error, 95% confidence intervals and p-values). A similar pattern of TNF- α expression was observed, albeit to a lower level (Fig. 1 B and C.). Twenty-four hours after CAF or NS injection levels of IL-1 β mRNA were significantly increased in the cerebellum (1.6-fold, $p=0.041$) and non-significantly increased in the hypothalamus (1.4-fold, $p=0.092$) (Fig. 1D). There was also a significant increase in TNF- α expression in the cerebellum (3.0-fold, $p=0.011$) and a strong trend towards significance in the hypothalamus (1.7-fold, $p=0.057$) (Fig. 1D). Taken together these data demonstrate that like LPS, cytotoxic chemotherapy causes a rapid and systemic increase in IL-1 β and TNF- α levels.

Cytotoxic chemotherapy induces acute and persistent CTRS: relationship to serum inflammatory cytokines and chemokines

Prior rodent studies have shown that cytotoxic chemotherapy, like LPS, induces fatigue and other sickness behaviors when injected into rodents. Few of these studies, however, have demonstrated a relationship between these symptoms and chemotherapy induced IL-1 β and TNF- α production. We hypothesized that if CAF-induced IL-1 β and TNF- α expression is the trigger for CTRS, then blood levels of IL-1 β , TNF- α and associated inflammatory cytokines and chemokines would be elevated in symptomatic but not asymptomatic CAF-treated mice. To test this hypothesis we first examined daily VWRA, body weight, and food intake in mice injected with CAF or NS every 19-days, to identify the time during a clinically relevant treatment scenario when CAF treated mice were symptomatic and asymptomatic. Figure 2A. shows plots of average daily VWRA, body weight, and food intake of mice in each group. Figure 2B. shows the average VWRA, body weight, and food intake during baseline (B), and each of the four cycles (C1 through C4). Repeated measure ANOVA revealed no significant difference between groups in daily VWRA ($F(9,261)=1.497$, $p=.149$), food intake ($F(9,261)=.705$, $p=.704$), or body weight ($F(9,261)=.612$, $p=.787$) during the 10-day baseline period (Fig 2A. and B). In the 24-hours post injection, however, CAF-treated mice displayed a significant decline in VWRA ($F(1,29)=95.043$, $p<0.0001$), food intake ($F(1,29)=37.783$, $p<0.0001$) and body weight ($F(1,29)=8.006$, $p=0.008$) compared to control mice (Fig. 2A.). While food intake and body weight returned to baseline levels 3- and 4- days post CAF injection respectively, VWRA in CAF-treated mice was suppressed for 9-days post-injection (Fig. 2A.). Following the 4th CAF dose, however, the decline in VWRA was prolonged. VWRA levels did not recover to baseline but remained significantly lower in CAF-treated mice versus control mice for 19-days post-injection ($F(1,29)=14.241$, $p=0.001$). Repeated measure ANOVA revealed a significant time by group interaction in average VWRA ($F(4,116)=13.216$, $p<0.0001$) across the 4 treatment cycles (Fig. 2B). CAF treatment had no effect on the onset of dark phase VWRA or time to peak dark phase VWRA (data not shown). Weight loss in CAF treated mice was similar to the pattern observed with VWRA (Fig. 2B.). Following the first CAF injection, body weight decreased significantly and remained reduced relative to control mice for 4-days post-injection before returning to baseline. The same pattern of acute weight loss occurred following the 2nd and 3rd CAF doses. Body weight returned to a level similar to control mice prior to the next treatment. However, after the 4th dose, body weight remained significantly lower in CAF treated mice relative to control mice for 19-days post injection ($F(1,29)=4.348$, $p=0.046$). Repeated measure ANOVA revealed a significant time by group interaction in average body weight over the 4 treatment cycles ($F(4,116)=14.381$, $p<0.0001$). The effect of CAF treatment on food intake was less pronounced than for VWRA and body weight. Food intake was suppressed for 3 days after the first CAF injection but rebounded rapidly, eventually surpassing intake in control mice for a 2-day period (Fig. 2A). A similar pattern of food intake was evident following the 2nd and 3rd CAF doses. The effect of repeated CAF administration on food intake became evident after the 4th CAF dose. Food intake was suppressed in CAF treated mice for over a week after the 4th dose but did return to baseline by the end of the study ($F(1,29)=.842$, $p=.366$). Repeated measure ANOVA revealed a significant time by group interaction in average food intake across the 4 treatment cycles ($F(4,116)=16.938$, $p<0.0001$). Taken together these data

demonstrate a distinct temporal pattern of CTRS in CAF treated mice; acute symptoms occurring immediately following chemotherapy injection, and persistent symptoms evident following the 4th and final dose of CAF.

To determine the relationship between CTRS and chemotherapy induced inflammation we set up a similar experiment in which VWRA was monitored in mice administered 4 doses of CAF (n = 20) or NS (n = 20) at 19-day intervals. Six-to 7 mice in each group were sacrificed and peripheral blood collected at 3 time points during treatment; 24-hours after the 1st injection when VWRA was significantly decreased relative to control mice (Acute CTRS), 9 days after the 1st injection when VWRA in CAF-treated mice had returned to baseline and was no different to the control group (No CTRS), and 20-days after the 4th injection when VWRA in CAF treated mice was persistently decreased relative to controls (Persistent CTRS). Serum levels of IL-1 β , TNF- α and several other inflammatory cytokines and chemokines were measured at each time point. Table 1 shows serum levels of each analyte for CAF-treated and NS-treated mice at each time point. The distribution of some of the inflammatory analytes measured did not follow a normal distribution and therefore transformations were applied. The types of transformations used for each serum analyte are presented in Table S1 (Supplemental Data). Based on the results of a 3 X 2 MANOVA using Wilk's Criterion, we observed differences in the pattern of expression of several inflammatory cytokines and chemokines between CAF-treated and control mice in the Acute CTRS, No CTRS, and Persistent CTRS groups ($F(64,4)=22.00$, $p=.004$). The univariate F tests for the interaction for each inflammatory analyte are presented in Table 2. Several inflammatory analytes had p values below 0.05 for the interaction of symptom and treatment group (CAF or Control) including IL-7 ($F(2,33)=4.86$, $p=0.014$), IL-9 ($F(2,33)=4.51$, $p=0.019$), G-CSF ($F(2,33)=5.21$, $p=0.011$), IP-10 ($F(2,33)=7.19$, $p=0.003$), KC ($F(2,33)=12.12$, $p<0.0001$), and MCP-1 ($F(2,33)=15.38$, $p<0.0001$). Relative to control mice, serum levels of IL-7, IL-9, G-CSF, KC, IP-10 and MCP-1 were increased in CAF-treated mice with acute CTRS whereas there was no difference in the levels of these or any of the inflammatory analytes measured in CAF-treated mice with persistent CTRS. Although serum IL-1 β , TNF- α and IL-6 were elevated in acutely symptomatic CAF-treated relative to control mice (see Table 1) there was no significant interaction between levels of these cytokines, CTRS and treatment group ($p=0.136$, $p=0.094$, and $p=0.094$ respectively, Table 2). Taken together our data support a role for CAF-induced inflammation in the etiology of CTRS.

Blockade of IL-1 β and TNF- α signaling impairs the peripheral inflammatory response to CAF and decreases survival

To determine the specific role of IL-1 β and TNF- α signaling in the CAF induced inflammation and CTRS we determined whether CAF induced inflammation and CTRS were reduced in mice lacking functional IL-1 receptors (IL-1R1 $^{-/-}$) or in mice lacking both IL-1 and TNF receptors (IL-1R1/TNFR1 $^{-/-}$). Serum levels of GCSF, IL-6, KC, and MCP-1 were measured in WT, IL-1R1-deficient- and IL-1R1/TNFR1-deficient mice sacrificed 24-hours after a single injection of CAF or NS. In WT mice, CAF caused a significant increase in serum levels of GCSF ($F(1,13)=49.105$, $p<0.0001$), IL-6 ($F(1,13)=6.841$, $p=0.023$), KC ($F(1,13)=30.165$, $p<0.0001$), and MCP-1 ($F(1,13)=18.336$, $p=0.001$) (Fig. 3A). Although

the CAF-induced cytokine response was blunted in IL-1R1-deficient mice, CAF treatment still significantly increased serum levels of GCSF ($F(1,8)=10.019$, $p=0.016$), KC ($F(1,8)=20.454$, $p=0.003$), and MCP-1 ($F(1,8)=36.784$, $p=0.001$) but not IL-6 ($F(1,8)=1.927$, $p=.208$) (Fig. 3A). In contrast, CAF failed to induce IL-6 ($F(1,13)=.584$, $p=.473$), KC ($F(1,13)=2.597$, $p=.133$), or MCP-1 ($F(1,13)=.975$, $p=.343$) in IL-1R1/TNFR1-deficient mice (Fig. 3A).

In the next experiment we sought to determine whether the blunted CAF-induced inflammatory response observed in IL-1R1 and IL-1R1/TNFR1 deficient mice decreased CTRS and/or survival following exposure to multiple doses of CAF. Daily VWRA, food intake, and body weight were assessed in WT, IL-1R1-deficient, and IL-1R1/TNFR1-deficient mice injected with 4 doses of CAF or NS at 19-day intervals. In addition, the effects of CAF treatment on lean body mass (LBM), fat mass (FM), bone mass (BM), hemoglobin (Hgb) level and red blood cell count (RBC) were also assessed in these experiments because prolonged production of IL-1 β and TNF- α induces fat and skeletal muscle wasting, bone loss, and anemia and these symptoms can also occur in cancer patients undergoing multiple rounds of chemotherapy.

Survival curves shown in Figure 3B revealed no significant difference in survival between CAF treated *IL-1R1*^{-/-} and WT mice (Fig. 2A, $\chi^2=1.39$, $df=1$, $p=0.197$). However, CAF treatment significantly reduced survival of CAF-treated IL-1R1/TNFR1-deficient mice relative to CAF-treated WT mice (Fig. 3B, $\chi^2=9.27$, $df=1$, $p=0.003$). None of the NS-treated (WT or IL-1R1/TNFR1 deficient) and only one mouse in the WT-CAF treatment group died during the study. Although an extensive necropsy and histological examination was not performed, CAF-treated IL-1R1/TNFR1-deficient mice showed enlarged abdomens and reduced fecal evacuation, ascites, erosion of the mesenchymal connective tissue, and innervations of the gastrointestinal tract (data not shown), indicative of pronounced GI toxicity [21]

Because of the reduced survival in CAF-treated IL-1R1/TNFR1-deficient mice, only changes in CTRS in IL-1R1-deficient mice and their WT counterparts are shown. Figure 4A shows plots of average VWRA, body weight, and food intake during baseline and each of the 4 treatment for mice that survived 4 doses of CAF or NS. At baseline IL-1R1-deficient mice ran significantly less than WT mice ($11,425 \pm 1995$ vs. 8961 ± 2845 , $F(1,38)=9.703$, $p=0.004$), whereas body weight and food intake did not differ between the two groups ($F(1,38)=2.569$, $p=.117$ and $F(1,38)=.512$, $p=.479$ respectively). A significant time x treatment interaction was observed in VWRA ($F(4, 116)=49.8$, $p<0.0001$), body weight ($F(1,29)=71.9$, $p<0.0001$), and food intake ($F(4,116)=32.45$, $p<0.0001$). Nonetheless, we did not observe a significant time x treatment x genotype effect on VWRA ($F(4,116)=0.957$, $p=0.434$), body weight ($F(4,116)=.298$, $p=0.879$), or food intake ($F(4,116)=.207$, $p=.934$). There was no difference in the onset of dark phase wheel running between WT and *IL-1R1*^{-/-} – CAF-treated mice; mice in all groups initiated wheel running at the beginning of the dark phase but CAF treated mice of both genotypes ran significantly less during each hour of the dark phase (data not shown).

Sham treated mice of both genotypes gained BMC, FM, and LBM during the course of the study (Fig. 4B). In contrast to sham-treated mice, CAF-treated WT mice lost FM ($F(1,16)=8.998$, $p=.008$), LBM ($F(1,16)=8.870$, $p=0.009$), and gained less BMC ($F(1,16)=14.646$, $p=0.001$). CAF-treated IL-1R1-deficient mice also lost FM ($F(1,13)=5.094$, $p=.042$), LBM ($F(1,13)=10.040$, $p=0.007$), and gained less BMC ($F(1,13)=5.664$, $p=0.034$) than their sham-treated counterparts. There was, however, no observed effect of genotype on the change in FM ($F(3,29)=.130$, $p=0.721$), LBM ($F(3,29)=.065$, $p=0.800$), or BMC ($F(3,29)=.179$, $p=0.675$).

Compared to sham, CAF-treatment led to a statistically significant decrease in RBC count in WT and IL-1R1-deficient mice ($F(1,16)=6.276$, $p=.023$ and $F(1,13)=6.186$, $p=.027$ respectively), but there was no effect of treatment x genotype on RBC count ($F(3,29)=.018$, $p=.894$). CAF treatment also led to a significant decrease in HgB level in WT mice ($F(1,16)=5.316$, $p=.035$) but not in IL-1R1-deficient mice ($F(1,13)=2.067$, $p=.174$) although again therein CAF-treated *IL-1R1*^{-/-} and WT mice relative to controls, these differences were not significant (Fig 4B). Moreover there was no effect of treatment x genotype on HgB level ($F(3,29)=.259$, $p=.614$).

Discussion

Although there have been studies examining the behavioral effects of cytotoxic chemotherapy in mice to our knowledge the present study is the first to examine the relationship between daily patterns of CTRS and inflammatory signaling in mice administered multiple doses of a multi-drug regimen over a clinically relevant time frame (i.e. 2–3 weeks). Using this clinically relevant approach we observed a distinct pattern of CTRS that was remarkably similar to that of cancer patients undergoing treatment; acute CTRS, occurring in the days following chemotherapy infusion and persistent CTRS that became evident after several doses of chemotherapy [1,3–5]. Whereas acute CTRS that occurred immediately following CAF treatment were associated with elevated levels of inflammatory cytokines and chemokines persistent CTRS were not. This finding does not necessarily preclude a role for chemotherapy induced inflammatory processes in the induction of persistent CTRS but it suggests that some factors which drive acute and persistent symptoms are distinct. For instance, acute fatigue, anorexia, and weight loss may be related to systemic dehydration and intestinal permeability since, cytotoxic agents alter gastrointestinal (GI) function by decreasing absorption from the small intestine and increasing GI permeability [36,37]. These symptoms were found to cluster together in colorectal cancer patients undergoing cytotoxic chemotherapy [38]. CAF-induced inflammation may contribute to altered GI function since IL-1 β and TNF- α are key regulators of intestinal permeability through their ability to modulate the function of tight junctions [39].

Based on recent findings by Weymann et al., the acute fatigue which occurred during the 24-hours post-CAF injection is likely the result of reduced orexin signaling [18]. Orexin, a neurotransmitter which regulates arousal and wakefulness is secreted by a small population of hypothalamic neurons [40]. These neurons are highly sensitive to pro-inflammatory signals and the observed increase in hypothalamic IL-1 β and TNF- α gene expression

observed in the present study coincides with the reduction in orexin neuron activity [18,19]. Weymann also demonstrated prolonged reduction in orexin neuron activity in mice administered 4 doses of CAF using the same dosing schema described in the present study. This finding suggests that long term suppression of hypothalamic orexin signaling may contribute to persistent fatigue observed in CAF treated mice.

Loss of lean-, fat- and bone-mass contribute to persistent weight loss observed after 4 doses of CAF. Whether the observed changes in body composition are caused by a related change in dietary intake, and/or reduced physical activity secondary to increased fatigue, or due to the catabolic effects of inflammatory cytokines (i.e. TNF- α) remain unclear. Loss of skeletal muscle mass and strength have been implicated in cancer related fatigue and may explain why resistance training decreases perceived fatigue in cancer survivors while increasing muscle mass and strength [41]. Reduced HgB and RBC count also likely contribute to the reduced locomotor activity observed in persistently fatigued mice. Anemia is common in cancer patients undergoing repeated rounds of cytotoxic chemotherapy and not surprisingly has a profound impact on level of perceived fatigue, energy level and physical functioning [42]. Several clinical trials have demonstrated the efficacy of erythropoietin in increasing hemoglobin levels and decreasing fatigue in cancer patients receiving myelosuppressive chemotherapy [43].

Despite growing evidence that treatment related increases in IL-1 β signaling may underlie many CTRS and are central to coordinating the immune response to harmful stimuli, the pattern of CTRS and survival in CAF-treated IL-1R1 deficient mice was not noticeably different from their WT counterparts. In IL-1R1 deficient mice, TNF- α compensates for the lack of IL-1 β signaling to induce sickness behavior [9]. This finding may explain why we did not see any difference in CTRS or survival between IL-1R1 deficient and WT mice. Although the GCSF, KC, and MCP-1 response was blunted in IL-1R deficient mice compared to WT mice, this response may have been sufficient to promote sickness behavior and allow recovery following CAF treatment. In this case TNF- α may have been able to compensate for the lack of IL-1 β signaling since blockade of both IL-1 β and TNF- α signaling completely abolished the CAF induced KC, IL-6, and MCP-1 response, suggesting that together IL-1 β and TNF- α mediate the effect of CAF on these inflammatory proteins. The cytokines and chemokines induced by CAF play important roles in the immune system response to infection and tissue damage. Specifically, IL-7, IL-9, and GCSF play roles in the proliferation and differentiation of T and B lymphocytes and neutrophils and other granulocytes [44]. MCP-1 and KC are chemokines which regulate migration and infiltration of monocytes, macrophages, and neutrophils into areas of damaged and inflamed tissue [45,46]. Moreover, studies in KC-deficient mice have demonstrated an important role for KC in mediating the infiltration of neutrophils into inflamed intestinal tissue and restoring the integrity of the gastric mucosa following injury [47]. Given the widespread hematologic and gastrointestinal toxicity induced by cytotoxic chemotherapy failure to mount this peripheral inflammatory response may explain the reduced survival observed in CAF-treated IL-1R1/TNFR-1 deficient mice.

Preventing the transmission of inflammatory signals from the periphery to the brain, and/or reducing the neuronal effects of brain inflammation, while leaving the peripheral

inflammatory response intact may be a more promising approach to ameliorating CTRS than systemic blockade of IL-1 β and TNF- α signaling. The work by Weymann et al., support this approach as does the recent study by Mahoney et al., who examined 5-fluorouracil induced sickness behavior in mice lacking MCP-1 [18,30]. In addition to its peripheral effects, MCP-1 mediates infiltration of activated monocytes into the hippocampus, basal ganglia, and motor cortex in mouse models of sickness behavior [48]. Mahoney et al (2013) reported that mice lacking MCP-1 recovered significantly faster following 5 daily doses of 5-FU (60mg/kg) administration than their WT counterparts [30]. Although brain infiltration of white blood cells was not examined, this work demonstrates that altering chemokine expression can lessen symptoms of fatigue and improve recovery. These recent pre-clinical studies provide new avenues of investigation aimed at reducing CTRS in cancer patients.

Study Limitations

Although the model used to guide the present study focused on adjuvant chemotherapy related inflammation and CTRS, the contribution of tumor-derived inflammation in the genesis of these symptoms is important to evaluate. Tumor cells are capable of producing large quantities of cytokines including IL-8, IL-1 β , TNF- α , and IL-6, either directly or through their association with activated macrophages, monocytes, and other cell types derived from the host [49]. These tumor derived cytokines likely explain the significant symptom burden in those with advanced cancer. Another limitation of this study is that in using cytokine receptor deficient mice to evaluate the role of IL-1 β and TNF- α in the genesis of CTRS we were unable to demonstrate a time sensitive effect of IL-1 β and TNF- α on CTRS and survival following exposure to cytotoxic chemotherapy. Transiently blocking the activity of these cytokines in the periphery or blocking their activity in the brain rather than the brain and periphery may help elucidate their role in the genesis of CTRS. Finally, all experiments were conducted with female mice and CAF was administered without regard to estrus cycle. What influence estrus cycle has on CTRS is not known. Future experiments are needed to determine whether there are gender differences in CAF-induced inflammation and CTRS.

Conclusions

Although our data further support a relationship between cytotoxic chemotherapy, pro-inflammatory signaling and CTRS, it suggests that one purpose of the chemotherapy induced acute inflammatory response, at least in the periphery, is the recovery from exposure to cytotoxic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

An important function of the chemotherapy induced acute pro-inflammatory cytokine response is to promote systemic recovery from exposure to cytotoxic agents.

Figure 1. CAF-induced IL-1 β and TNF- α production in mice

A) Fold increase in serum levels of IL-1 β and TNF- α in CAF-treated relative to NS-treated mice sacrificed at 1, 3, 6, 14 and 24 hours post-injection. Each bar represents the mean $\pm$ SEM of each value. Fold-increase in IL-1 β and TNF- α mRNA levels in B) livers, and C) spleens of CAF-treated mice relative to control mice sacrificed at 3, 6, 14 and 24 hours post-injection. (D) Fold-increase in IL-1 β and TNF- α mRNA levels in cerebellum (Cer) and hypothalamus (Hyp) in CAF-treated mice relative to controls at 24-hours post-injection. Box and whisker plots represent the data with boxes ranging from the 25th to the 75th percentile of the observed values, the solid line representing the median value and the whiskers spanning the minimum and maximum values. The asterisks indicate a statistically significant difference in serum protein level (pg/mL) or tissue specific fold-increases in mRNA expression levels between groups (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

Figure 2. CAF-induced changes in CTRs in mice

A.) Daily VWRA, body weight, and food intake during baseline (B), and 4 cycles (C1–C4) of CAF (n= 17, filled square) or NS (n=17, open diamond). Each data point represents the mean of each value. Error bars are not included in the figure to allow discrimination of data trends. (B.) Average VWRA, body weight, and food intake during baseline (B) and each of the 4 treatment cycles (C1 to C4). Each data point represents the mean $\pm$ SEM of each value. Asterisks indicate a statistically significant difference in levels of VWRA, body weight and food intake between groups ($p < 0.05$).

Figure 3. Blockade of IL-1 β and TNF- α signaling impairs the peripheral inflammatory response to CAF and decreases survival

A) Fold increase in serum levels of GCSF, IL-6, KC and MCP-1 in CAF-treated relative to NS-treated WT, IL-1R1- and IL-1R1/TNFR1-deficient female mice sacrificed 24- hours post-injection. The number of mice in the NS and CAF treated groups for each genotype are indicated in parentheses respectively. Each bar represents the mean $\pm$ SEM of each value. (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$). B) Survival curves of CAF-treated or NS-treated wild type (WT), IL-1R1 deficient (IL-1R1^{-/-}) and IL-1R1/TNFR1-deficient (IL-1R1/TNFR1^{-/-}) mice during 4 treatment cycles.

Figure 4. Patterns of CTRS in IL-1R1-deficient mice

A.) Average VWRA, body weight, and food intake during baseline (B) and each of the 4 treatment cycles (C₁ to C₄). Each data point represents the mean $\pm$ SEM of each value. Time x treatment x genotype effect was evaluated using repeated measure ANOVA. **B.)** Changes in fat mass (FM), lean mass (LM), and bone mineral content (BMC) derived from pre-and post-treatment DXA scans and C) hemoglobin (HGB) and red blood cell (RBC) counts prior to sacrifice. Two-way ANOVA was used to detect significant treatment x genotype interactions. Asterisks indicate a statistically significant difference in levels of VWRA, body weight and food intake between groups (*p < 0.05, **p<0.001, ***p<0.0001).

Table 1

Serum levels of inflammatory cytokines in CAF- and NS-treated mice.

Serum inflammatory cytokine levels in NS treated mice (Control) and CAF-treated mice (CAF) in the Acute CTRS, No CTRS, and Persistent CTRS groups. The number of animals per group is indicated in parentheses. Mean concentration of each analyte in pg/mL $\pm$ standard deviation (SD) is shown. The threshold for detection was 3.2 pg/mL.

Analytes	A. Acute CTRS			B. No CTRS			C. Persistent CTRS		
	Control (6)	CAF (8)	Mean $\pm$ SD	Control (6)	CAF (7)	Mean $\pm$ SD	Control (6)	CAF (6)	Mean $\pm$ SD
	Mean $\pm$ SD	Mean $\pm$ SD		Mean $\pm$ SD	Mean $\pm$ SD		Mean $\pm$ SD	Mean $\pm$ SD	
TNF- α	3.5 $\pm$ 0.5	6.5 $\pm$ 3.2	4.0 $\pm$ 1.2	4.0 $\pm$ 1.2	3.3 $\pm$ 0.4	3.3 $\pm$ 0.4	4.0 $\pm$ 0.8	6.3 $\pm$ 3.8	6.3 $\pm$ 3.8
IL-1 α	739 $\pm$ 707	1078 $\pm$ 712	599 $\pm$ 303	599 $\pm$ 303	482 $\pm$ 156	482 $\pm$ 156	291 $\pm$ 292	153 $\pm$ 97	153 $\pm$ 97
IL-1 β	10.0 $\pm$ 4.9	28.7 $\pm$ 19.5	18.6 $\pm$ 14.1	18.6 $\pm$ 14.1	12.3 $\pm$ 7.7	12.3 $\pm$ 7.7	6.6 $\pm$ 4.8	6.8 $\pm$ 5.6	6.8 $\pm$ 5.6
IL-2	4.7 $\pm$ 2.1	4.9 $\pm$ 1.8	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0	3.2 $\pm$ 0	3.4 $\pm$ 0.6	3.6 $\pm$ 0.9	3.6 $\pm$ 0.9
IL-3	5.8 $\pm$ 3.1	7.4 $\pm$ 3.3	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0	3.2 $\pm$ 0	3.2 $\pm$ 0.0	3.6 $\pm$ 0.7	3.6 $\pm$ 0.7
IL-4	3.8 $\pm$ 1.4	4.1 $\pm$ 1.6	3.2 $\pm$ 0	3.2 $\pm$ 0	3.2 $\pm$ 0	3.2 $\pm$ 0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0
IL-5	14.9 $\pm$ 8.3	45.2 $\pm$ 26.7	9.2 $\pm$ 3.5	9.2 $\pm$ 3.5	27.7 $\pm$ 15.2	27.7 $\pm$ 15.2	14.6 $\pm$ 8.0	22.0 $\pm$ 12.6	22.0 $\pm$ 12.6
IL-6	16.1 $\pm$ 10.4	52.1 $\pm$ 14.4	4.2 $\pm$ 1.6	4.2 $\pm$ 1.6	8.2 $\pm$ 5.9	8.2 $\pm$ 5.9	5.8 $\pm$ 2.9	10.7 $\pm$ 2.7	10.7 $\pm$ 2.7
IL-7	3.1 $\pm$ 0.2	7.3 $\pm$ 4.6	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	4.1 $\pm$ 1.2	3.4 $\pm$ 0.4	3.4 $\pm$ 0.4
IL-9	19.5 $\pm$ 11.9	55.6 $\pm$ 33.4	32.4 $\pm$ 18.0	32.4 $\pm$ 18.0	16.5 $\pm$ 2.7	16.5 $\pm$ 2.7	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0
IL-10	7.2 $\pm$ 4.1	13.5 $\pm$ 8.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0
IL-12(p40)	27.1 $\pm$ 6.6	54.3 $\pm$ 38.8	10.6 $\pm$ 7.3	10.6 $\pm$ 7.3	3.6 $\pm$ 0.7	3.6 $\pm$ 0.7	6.4 $\pm$ 3.2	5.5 $\pm$ 2.2	5.5 $\pm$ 2.2
IL-12(p70)	77.0 $\pm$ 60.1	85.8 $\pm$ 73.1	11.3 $\pm$ 11.7	11.3 $\pm$ 11.7	10.4 $\pm$ 9.5	10.4 $\pm$ 9.5	12.5 $\pm$ 7.5	15.6 $\pm$ 7.7	15.6 $\pm$ 7.7
IL-13	47.8 $\pm$ 18.6	39.8 $\pm$ 18.6	49.6 $\pm$ 26.1	49.6 $\pm$ 26.1	35.7 $\pm$ 9.0	35.7 $\pm$ 9.0	29.5 $\pm$ 22.8	33.1 $\pm$ 12.2	33.1 $\pm$ 12.2
IL-15	23.4 $\pm$ 18.5	26.3 $\pm$ 17.2	9.8 $\pm$ 7.8	9.8 $\pm$ 7.8	10.8 $\pm$ 6.6	10.8 $\pm$ 6.6	17.6 $\pm$ 9.1	23.2 $\pm$ 16.2	23.2 $\pm$ 16.2
IL-17	8.3 $\pm$ 4.2	10.8 $\pm$ 5.5	4.8 $\pm$ 2.0	4.8 $\pm$ 2.0	8.5 $\pm$ 6.4	8.5 $\pm$ 6.4	3.3 $\pm$ 0.3	9.3 $\pm$ 3.2	9.3 $\pm$ 3.2
IFN- γ	18.4 $\pm$ 9.9	25.3 $\pm$ 11.9	6.9 $\pm$ 3.1	6.9 $\pm$ 3.1	6.4 $\pm$ 4.1	6.4 $\pm$ 4.1	3.6 $\pm$ 0.3	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0
G-CSF	390 $\pm$ 177	1362 $\pm$ 744	346 $\pm$ 133	346 $\pm$ 133	424 $\pm$ 130	424 $\pm$ 130	294 $\pm$ 75	301 $\pm$ 107	301 $\pm$ 107
GM-CSF	6.4 $\pm$ 4.1	8.2 $\pm$ 7.4	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	6.9 $\pm$ 3.0	5.3 $\pm$ 1.0	5.3 $\pm$ 1.0
M-CSF	30.8 $\pm$ 25.9	41.5 $\pm$ 25.1	4.0 $\pm$ 1.3	4.0 $\pm$ 1.3	3.3 $\pm$ 0.3	3.3 $\pm$ 0.3	9.5 $\pm$ 4.6	9.3 $\pm$ 2.7	9.3 $\pm$ 2.7
IP-10	98.7 $\pm$ 25.8	209.9 $\pm$ 44.8	350.7 $\pm$ 41.5	350.7 $\pm$ 41.5	355.7 $\pm$ 42.6	355.7 $\pm$ 42.6	142 $\pm$ 73.9	132 $\pm$ 21	132 $\pm$ 21
KC	46.2 $\pm$ 14.0	151.2 $\pm$ 66.5	69.8 $\pm$ 16.6	69.8 $\pm$ 16.6	47.8 $\pm$ 16.9	47.8 $\pm$ 16.9	33.8 $\pm$ 21.5	34.2 $\pm$ 9.9	34.2 $\pm$ 9.9

	A. Acute CTRS		B. No CTRS		C. Persistent CTRS	
	Control (6)	CAF (8)	Control (6)	CAF (7)	Control (6)	CAF (6)
Analytes	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
LIF	3.2 $\pm$ 0	5.5 $\pm$ 3.3	3.2 $\pm$ 0	3.2 $\pm$ 0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0
LIX	8.3 $\pm$ 1.5	7.7 $\pm$ 3.1	4.2 $\pm$ 3.6	3.6 $\pm$ 1.5	4.9 $\pm$ 2.0	4.0 $\pm$ 2.6
MCP-1	30.3 $\pm$ 17.4	166.8 $\pm$ 49.4	4.9 $\pm$ 2.6	4.5 $\pm$ 1.6	40.7 $\pm$ 28.8	27.5 $\pm$ 11.9
MIG	145 $\pm$ 58	173 $\pm$ 74	184 $\pm$ 72	225 $\pm$ 134	366 $\pm$ 487	86 $\pm$ 31
MIP-1 α	54.7 $\pm$ 13.0	60.3 $\pm$ 10.5	50.5 $\pm$ 33.4	41.6 $\pm$ 21.0	3.3 $\pm$ 0.3	12.5 $\pm$ 4.2
MIP-1 β	33.4 $\pm$ 15.4	84.6 $\pm$ 51.7	56.5 $\pm$ 22.0	66.8 $\pm$ 31.4	32.7 $\pm$ 24.7	32.0 $\pm$ 9.0
MIP-2	41 $\pm$ 58.6	52.8 $\pm$ 57.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0
Rantes	84.8 $\pm$ 47.5	100.8 $\pm$ 54.9	61.5 $\pm$ 33.3	69.2 $\pm$ 20.3	49.3 $\pm$ 29.8	42.9 $\pm$ 29.6
VEGF	4.6 $\pm$ 1.8	6.5 $\pm$ 3.1	3.3 $\pm$ 0.1	3.6 $\pm$ 0.5	3.2 $\pm$ 0.1	3.6 $\pm$ 0.8
Eotaxin	1307 $\pm$ 387	1044 $\pm$ 254	1505 $\pm$ 207	1751 $\pm$ 225	625 $\pm$ 361	616 $\pm$ 409

Table 2

Serum inflammatory cytokine markers of CTRS.

Inflammatory markers that had p values below 0.05 for the interaction of symptom and treatment group (CAF or Control) including A) IL-7 (F(2,33)=4.86, p=0.014), B) IL-9 (F(2,33)=4.51, p=0.019), C) G-CSF (F(2,33)=5.21, p=0.011), D) IP-10 (F(2,33)=7.19, p=0.003), E) KC (F(2,33)=12.12, p<0.0001), and F) MCP-1 (F(2,33)=15.38, p<0.0001).

Analytes	Between SS	Error SS	Between MS	Error MS	F	p-value
IL-1 β	2.70	20.96	1.35	0.64	2.12	0.136
TNF- α	23.53	152.57	11.77	4.62	2.54	0.094
IL-1 α	2.42	26.05	1.21	0.79	1.53	0.231
IL-6	1.51	9.92	0.76	0.30	2.51	0.096
IL-2	0.12	49.40	0.06	1.50	0.04	0.960
IL-3	4.35	126.86	2.17	3.84	0.57	0.574
IL-4	0.21	27.67	0.10	0.84	0.12	0.884
IL-5	0.94	13.84	0.47	0.42	1.12	0.339
IL-7	45.19	153.46	22.60	4.65	4.86	0.014
IL-9	3.87	14.18	1.94	0.43	4.51	0.019
IL-10	80.78	1364.35	40.39	41.34	0.98	0.387
IL-12(p40)	1.73	19.69	0.86	0.60	1.45	0.250
IL-12(p70)	0.11	43.49	0.06	1.32	0.04	0.959
IL-13	487.81	12997.16	243.90	393.85	0.62	0.544
IL-15	0.04	19.45	0.02	0.59	0.04	0.964
IL-17	19.67	614.46	9.83	18.62	0.53	0.595
IFN- γ	147.32	1768.05	73.66	53.58	1.37	0.267
G-CSF	3.10	9.81	1.55	0.30	5.21	0.011
GM-CSF	79.28	839.52	39.64	25.44	1.56	0.226
M-CSF	0.80	10.94	0.40	0.33	1.21	0.311
IP-10	28919.92	66375.07	14459.96	2011.37	7.19	0.003
KC	4.09	5.57	2.05	0.17	12.12	0.000
LIF	11.27	77.12	5.64	2.34	2.41	0.105
LIX	19.64	7485.06	9.82	226.82	0.04	0.958

Analytes	Between SS	Error SS	Between MS	Error MS	F	p-value
MCP-1	8.94	9.59	4.47	0.29	15.38	0.000
MIG	0.00	0.02	0.00	0.00	3.14	0.057
MIP-1 α	576.70	9911.98	288.35	300.36	0.96	0.393
MIP-1 β	15.14	189.21	7.57	5.73	1.32	0.281
MIP-2	0.59	41.04	0.29	1.24	0.24	0.791
Rantes	0.16	9.97	0.08	0.30	0.26	0.775
VEGF	1.03	28.07	0.51	0.85	0.60	0.552
Eotaxin	431531.1	3216004.5	215765.6	97454.7	2.21	0.125