

Targeting TNF α -mediated cytotoxicity using thalidomide after experimental cardiac arrest in rats: An exploratory study

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Abstract. Cardiac arrest (CA) results in a central and systemic cytokine and inflammatory response. Thalidomide has been reported to be neuroprotective by selectively decreasing TNF α synthesis. We hypothesized that thalidomide would decrease the systemic and organ-specific TNF α /cytokine response and biomarkers of injury in rats subjected to 10 min CA. Naïves, CA treated with vehicle (CA) and CA treated with thalidomide (50 mg/kg; CA+T) were studied (n=6 per group). TNF α and key cytokines were assessed at 3 h after resuscitation in the cortex, hippocampus, striatum, cerebellum, plasma, heart and lung. Neuron specific enolase (NSE), S100b, cardiac troponin T (cTnT) and intestinal fatty acid binding protein (IFABP) were used to assess neuronal, glial, cardiac and intestinal damage, respectively. CA increased TNF α and multiple pro-inflammatory cytokines in plasma and selected tissues with no differences between the CA and CA+T groups in any region. NSE, S100b, cTnT and IFABP were increased after CA or CA+T vs. in the naïve group (all P<0.05) without significant differences between the CA and CA+T groups. In conclusion, CA resulted in a TNF α and cytokine response, with increased biomarkers of organ injury. Notably, thalidomide at a dose reported to improve the outcome in *in vivo* models of brain ischemia did not decrease TNF α or cytokine levels in plasma, brain or extracerebral organs, or biomarkers of injury. Although CA at 3 h post resuscitation produces a robust TNF α response, it cannot be ruled out that an alternative dosing regimen or assessment at other time-points might yield different results.

The marked systemic and regional cytokine response to CA remains a potential therapeutic target.

Introduction

Overall mortality and morbidity after cardiac arrest (CA) remain high, despite improvements in resuscitation and critical care. Neurocognitive disabilities are frequently observed in survivors from CA. Histological damage including neuronal cell loss was characterized in multiple experimental global ischemia-reperfusion insults (1). Several selectively vulnerable regions have been identified, namely hippocampus, cerebellar Purkinje neurons, lamina IV cortical layer and striatum. While early ischemic brain injury is the result of energy failure, neuro-inflammation could contribute, or even represent a major cause of delayed neuronal death. Tumor necrosis factor alpha (TNF α) is a pivotal cytokine that can induce neuronal apoptosis and/or necroptosis, and increase neuroinflammation (2-4).

CA also triggers a sepsis-like inflammatory response with expression of TNF α up-regulated in cerebral ischemia. Systemic selective anti-TNF α therapies improved early recovery from CA, in both small and large animal models (4,5). Our prior studies in multiple models of CA in rats, identified a unique early cytokine response specifically in striatum, showing a dramatic >100-fold increase of TNF α vs. other brain regions including hippocampus, where no increase in TNF α was seen (6-8).

The striatum is a region with selective vulnerable neuronal death in our model (9). Surprising is the fact our prior studies showed TNF α localized immunohistochemically in neurons rather than microglia (6,7). Thus, unique TNF α production in striatal neurons may mediate the region-specific neuronal loss in striatum after CA. Thus, region-specific neuronal therapies may be required to best target neuronal death after CA.

Among several readily translatable potential strategies for CA to target TNF α , thalidomide, an inhibitor of TNF α protein synthesis is readily capable of crossing the blood brain barrier (BBB) (10). Thalidomide has been reported to selectively decrease TNF α (11-13) and shown to be neuroprotective via destruction of TNF α mRNA *in vivo* (14) and *in vitro* (15).

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Specifically, thalidomide and its derivatives, including both lenalidomide and pomalidomide, termed immunomodulatory imide drugs (IMiDs), are a class of drugs that target the 3'-untranslated region (3'-UTR) of TNF α mRNA, inhibiting TNF α production (16). In this exploratory study, we hypothesized that thalidomide would attenuate (1) systemic TNF α levels, (2) neuroinflammation as reflected by brain tissue TNF α levels, (3) extra-cerebral organ TNF α levels, and (4) markers of organ injury after prolonged CA in rats. We have also assessed (5) complex cytokine response to CA to elucidate the potential downstream effect of thalidomide on other cytokines. Naïve rats and rats treated with vehicle served as controls.

Materials and methods

Institutional approval. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Protocol no. 13021161; 'Neuroinflammation after prolonged cardiac arrest'). We used our previously established model of ventricular fibrillation (VF) CA (17).

Preparation phase. In brief, adult male Sprague-Dawley rats (350–400 g) were obtained from a licensed vendor (Hilltop Lab Animals, Scottdale, PA) and housed under 12/12 h light/dark in a holding facility for at least two days prior to the experiment. Water was provided ad libitum until the experiment. Standard chow was removed 12 h prior to experiment. On the day of the experiment, rats were anesthetized with 4% isoflurane (Baxter) in pure oxygen in a plexiglass jar, intubated with a 14-gauge cannula (Becton Dickinson), and mechanically ventilated (Harvard Ventilator 683, Harvard Rodent Apparatus) with tidal volume 8 ml/kg, PEEP 3 cm H₂O and respiratory rate 30–40/min to maintain normocapnia. Anesthesia was maintained with 2% isoflurane (FiO₂ of 0.5).

CA and resuscitation phase. Three groups (n=6 per group) were studied: i) Naïve rats; ii) rats subjected to VFCA without thalidomide (CA); and iii) rats subjected to VFCA with thalidomide (CA+T). Naïve rats were deeply anesthetized with isoflurane 4% over 4 min, midline laparotomy and sternotomy were performed, and rats were perfused transcardially with 250 ml of ice-cold heparinized normal saline.

In rats subjected to VFCA, arterial (PE50) and venous (PE90) femoral catheters were inserted via cut-downs for blood pressure monitoring and drug administration. For VFCA, 5F pacing catheter was introduced via the jugular vein to the conjunction of right atrium and right ventricle.

Electrocardiogram (ECG) and mean arterial pressure (MAP) were continuously monitored and recorded (Polygraph, Grass Instruments). Rectal temperature was controlled at 37.0±0.5°C with a temperature controlled operating table, overhead heating lamp and a fan. After surgery, the FiO₂ was reduced to 0.3 and isoflurane was gradually weaned to 0% over 10 min in rats scheduled for CA.

No-flow was then induced by a 2-minute impulse of 12 V/50 Hz alternating current and ensured by ECG readings and reduction in MAP <10 mmHg. The pacing catheter was then removed and jugular vein ligated. After 10 min of

VFCA, manual chest compressions were started at a rate ~360/min along with mechanical ventilation with 100% oxygen. Epinephrine (Abbott) 0.01 mg/kg was given with start of compressions. Additional epinephrine 0.005 mg/kg was given at 1 min resuscitation time (RT). Sodium bicarbonate (Abbott) 1 mEq/kg was also given at start of resuscitation. At 2 min after the start of resuscitation (2 min RT), defibrillation was attempted with biphasic 10 J impulse (Zoll M series defibrillator; Zoll). If unsuccessful, subsequent shocks were delivered every 30 seconds, with maximum 5 attempts over 4 min resuscitation effort. Return of spontaneous circulation (ROSC) was defined as sustained supraventricular rhythm with MAP >50 mmHg. In rats subjected to thalidomide treatment (CA+T group), 50 mg/kg thalidomide (Enzo Life Sciences; cat. no. BML-T115-0100) dissolved in dimethyl sulfoxide (DMSO) was administered i.p. at 5 min after initiation of resuscitation. This dose was selected based on prior studies reporting benefits (18–21). Control rats (CA group) received an identical volume of DMSO at the corresponding timepoint.

Postoperative care. After ROSC, rats were mechanically ventilated with FiO₂ 1.0, Vt 8 ml/kg, PEEP 5 cmH₂O and respiratory rate adjusted to maintain normocapnia. Epinephrine infusion was titrated to maintain MAP >65 mmHg. Controlled normothermia (36.5–37.5°C) was maintained for 3 h. At 3 h RT, serum samples were obtained, rats were deeply anesthetized with isoflurane 4%, and perfused transcardially with 250 ml of ice-cold heparinized normal saline. Rats were then decapitated, hearts and brains removed and dissected into four regions of interest: cortex, hippocampus, striatum, and cerebellum. Plasma, heart, and lung samples were also obtained. Individual tissue samples were then snap-frozen in liquid nitrogen and stored in -70°C freezer until further processing.

TNF α and cytokine measurements. Tissues were then processed for cytokine assessment for interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12, interferon γ (IFN γ) and granulocyte-macrophage colony stimulating factor (GM-CSF) using Luminex-200 multiplex analyzer (Luminex) using a rat-specific kit (Millipore). All values were corrected for protein concentration. The tissue was homogenized in phosphate-buffered saline (PBS) by using Dounce homogenizer for 20 strokes. The homogenate was then sonicated for 10 seconds for three times with an interval of 20 seconds, followed by centrifugation at 16,000 x g for 30 min. The supernatant was used for TNF α analysis. Protein levels in the supernatant was measured using the bicinchoninic acid (BCA) protein kit (Thermo Fisher Scientific, Inc.). TNF α concentrations that were read as 'out of range below' were nominally assigned a value at one hundredth of the lowest level on the calibration scale.

Biomarkers of organ-specific injury. Biomarkers of neuronal injury (neuron-specific enolase, NSE) and glial injury (S100b) were assessed using kits (MyBioSource kits catalogue nos. MBS262217 and MBS849461, respectively; MyBioSource, Inc.) according to manufacturer's instructions. Myocardial injury was assessed using rat-specific cardiac troponin T (cTnT) ELISA kit (MyBioSource catalogue no. MBS730382). Intestinal injury was assessed using rat specific ELISA kit for

Table I. Physiologic and biochemical profile after CA.

Variable	BL	RT5	RT30	RT60	RT120	RT180	P-value
HR, bpm							
CA	324±11	272±86	324±40	364±27	374±15	390±20	0.88
CA+T	344±25	302±22	340±32	384±43	378±46	390±31	
MAP, mmHg							
CA	86±4	146±18	75±8	70±5	80±12	85±16	0.21
CA+T	91±12	133±22	72±4	66±7	79±18	81±12	
pHa							
CA	7.39±0.04	7.31±0.13	7.18±0.04	7.43±0.03	7.48±0.02	7.45±0.01	<0.0001
CA+T	7.40±0.02	7.31±0.09	7.18±0.04	7.35±0.08	7.38±0.04	7.39±0.03	
paO ₂ , mmHg							
CA	189±31	343±50	354±44	366±31	369±68	370±45	0.75
CA+T	227±15	363±73	413±102	324±90	397±79	379±75	
paCO ₂ , mmHg							
CA	37±6	33±10	42±6	34±4	29±5	33±3	0.10
CA+T	38±5	34±7	39±4	35±7	39±6	40±6	
BE, mEq/l							
CA	-2.1±1.4	-9.5±3.8	-11.9±2.2	-1.4±2.5	-1.8±2.7	-0.8±1.4	0.36
CA+T	-1.1±2.4	-8.9±2.0	-13.0±0.7	-5.3±3.8	-1.7±4.8	-0.7±4.8	
Lactate, mmol/l							
CA	1.4±0.8	9.8±1.6	9.4±2.6	4.5±1.8	3.1±0.9	2.5±0.7	0.82
CA+T	1.4±0.5	10.3±1.0	8.4±2.7	3.9±0.7	2.8±0.7	3.1±1.0	
Hct, %							
CA	43±2	46±3	50±3	48±3	45±4	47±4	0.33
CA+T	43±3	45±2	47±5	45±4	47±1	48±3	
Glucose, g/dl							
CA	166±31	105±42	121±28	142±41	145±24	161±46	0.76
CA+T	178±35	86±14	94±22	125±34	190±26	199±60	

Differences among groups (P-value) were examined using Generalized Estimating Equations models for treatment (RT5-RT180 in CA vs. CA+T). BL, baseline; RT, resuscitation time (min); HR, heart rate; MAP, mean arterial pressure; BE, base excess; Hct, hematocrit; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).

intestinal fatty acid binding protein (IFABP; MyBioSource catalogue no. MBS024910).

Statistical analysis. The analyses were performed using IBM SPSS Statistics 26.0 software (IBM Corp.). The samples size calculations were based off the results published by us previously. We hypothesized that thalidomide would reduce TNFα levels in the striatum by 50%. Using alpha=0.05 and power=80%, number of rats per group needed was five. Anticipating ~20% mortality in our model, we randomized 6 rats per group.

Data were tested for normality using the Kolmogorov-Smirnov test. Hemodynamic and biochemical data are presented as mean ± standard deviation (SD). Differences between groups (P-value) were tested using Generalized Estimating Equations models for treatment (RT5-RT180 in CA vs. CA+T). Cytokine levels are presented as median and the first and the third quartile. Differences in plasma cytokines were assessed using one-way analysis of

variance (ANOVA) with post hoc Tukey's test. Differences in extracerebral organs cytokines at three hours after CA were assessed using Kruskal-Wallis test followed by Dunn's test with post hoc Bonferroni test. The differences in biomarkers of organ-specific damage between groups were evaluated with a one-way ANOVA with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Survival. One rat in each group died. One rat in the CA group died from hemodynamic collapse at RT 10 min, whereas one rat in the CA+T group did not achieve ROSC. Data from both rats were excluded from further analyses.

Biochemical and hemodynamic profiles. As anticipated in our model, CA induced profound metabolic acidosis with increased lactate that illustrated the severity of the insult. The metabolic derangements progressively resolved by the 180 min RT. There

Table II. Biomarkers of brain and extracerebral organ injury after CA.

Variable	Naïve	CA	CA+T
NSE, ng/ml	0.01±0.02	1.19±0.16 ^a	1.13±0.22 ^a
S100b, pg/ml	22±1	196±26 ^a	177±37 ^a
cTnT, pg/ml	0.64±0.38	1.20±0.06 ^a	1.17±0.30 ^a
IFABP, pg/ml	239±73	368±39 ^a	393±70 ^a

^aP<0.05 vs. naïve; no significant differences for CA vs. CA+T. Differences among groups were examined using one-way analysis of variance with post hoc Tukey's test. NSE, neuron specific enolase; S100b, S100 calcium binding protein beta; cTnT, cardiac troponin-T; IFABP, intestinal fatty acid binding protein; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).

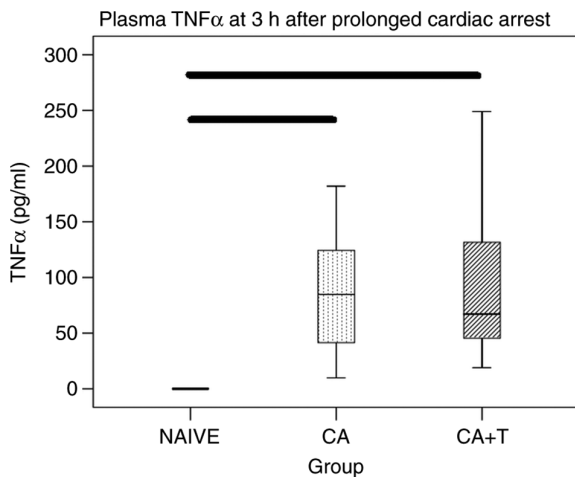


Figure 1. TNFα levels in plasma at 3 h after prolonged ventricular fibrillation CA in rats. Boxes represent the 25-75% interquartile range, the line across the box represents the median, and the whiskers are min-max values. The thick horizontal line signifies P<0.05 between groups (one-way ANOVA with post hoc Tukey's test). CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).

were no major differences in hemodynamic or biochemical profiles between CA and CA+T groups except pHa (Table I).

Plasma TNFα. CA resulted in an anticipated marked increase in plasma TNFα (both naïve vs. CA and naïve vs. CA+T P<0.05). However, no significant differences were found in plasma TNFα between CA and CA+T treatment groups (Fig. 1).

TNFα in individual brain regions. In general, brain cytokines in naïve rats were low or undetectable. Although the TNFα levels were increased after CA in most surveilled brain regions, no statistically significant difference was found between any groups for CA, CA+T and naïve in the cerebellum (P=0.236), cortex (P=0.297), or in the hippocampus (P=0.067). As anticipated from our prior studies, TNFα was significantly increased after CA in the striatum vs. naïve (P<0.05) but was not found to be significantly different between CA and CA+T (P=1.0), failing to support our hypothesis (Fig. 2).

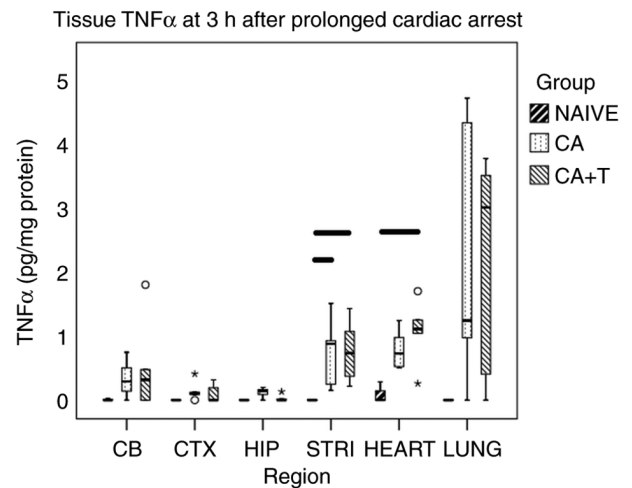


Figure 2. TNFα levels in selected brain regions and extracerebral organs at 3 h after prolonged ventricular fibrillation CA in rats. Boxes represent the 25-75% interquartile range, the line across the box represents the median, and the whiskers are min-max values. The thick horizontal line signifies P<0.05 between groups (Kruskal-Wallis test followed by Dunn-Bonferroni post-hoc test). The round marker and the asterisk represent outliers ('out' and 'far out' values). CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group); CB, cerebellum; CTX, cortex; HIP, hippocampus; STRI, striatum.

TNFα in extracerebral organs. TNFα levels were numerically increased after CA or CA+T vs. naïve in lung tissue but this trend did not reach statistical significance (P=0.077). There were no differences in lung TNFα between CA and CA+T. In contrast, the myocardial tissue levels of TNFα were significantly increased after CA+T vs. naïve (P<0.05) but were not found to be significantly different between CA and CA+T (Fig. 2).

Biomarkers of organ-specific injury. Rats subjected to CA had significantly increased biomarkers of end-organ injury over naïves across all the organs sampled: NSE (P<0.05), S100b (P<0.05), cardiac troponin T (P<0.05), and IFABP (P<0.05), without significant differences between CA and CA+T groups (Table II).

Cytokine response in individual brain regions. Rats subjected to CA showed a significant increase of IL-6 in the hippocampus and TNFα in the striatum over naïve rats, with no differences between CA and CA+T groups (Table III).

Cytokine response in plasma and extracerebral organs. CA resulted in statistically significant increases of selected cytokines in plasma, namely IL-1β, IL-6, IL-10 and IL-12. None of these cytokines were affected by thalidomide administration. Increases of selected cytokines in heart (IL-1α, IL-1β, IL-6, TNFα) and lung (IL-1α, IL-1β, IL-6, IFNγ) were also not affected by thalidomide (Table IV).

Discussion

We previously reported that this model of 10 min of VFCA results in extensive neuronal death and dramatic increases in cytokines. In the current study, we confirmed our findings of early marked increases in TNFα levels in the striatum,

Table III. Cytokine profile in individual brain regions at 3 h after CA.

Variable	CTX, pg/mg protein	HIP, pg/mg protein	STRI, pg/mg protein	CEREB, pg/mg protein
IL-1a				
N	0.00 (0.00-1.97)	0.08 (0.00-1.25)	0.00 (0.00-0.00)	0.00 (0.00-0.65)
CA	1.43 (0.00-3.11)	2.56 (1.12-4.47)	2.91 (0.00-4.44)	0.47 (0.00-2.17)
CA+T	0.00 (0.00-1.47)	2.04 (0.10-4.35)	3.40 (1.47-4.72)	1.38 (0.00-1.76)
IL-1b				
N	2.90 (2.27-4.42)	6.45 (3.40-7.96)	6.90 (2.23-10.62)	7.87 (7.10-9.02)
CA	8.02 (4.85-11.77)	7.93 (7.25-14.23)	7.12 (6.38-14.44)	9.72 (4.84-14.44)
CA+T	6.81 (3.58-9.76)	8.29 (6.15-10.0)	10.42 (7.28-11.76)	12.73 (9.78-13.51)
IL-2				
N	6.12 (1.41-50.59)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	4.98 (4.08-5.47)
CA	0.00 (0.00-14.04)	4.55 (0.00-9.10)	0.00 (0.00-26.60)	5.75 (0.00-52.85)
CA+T	0.00 (0.00-22.33)	0.00 (0.00-2.82)	24.28 (2.83-40.66)	4.08 (0.00-10.12)
IL-4				
N	0.02 (0.01-0.02)	0.02 (0.01-0.02)	0.03 (0.01-0.04)	0.02 (0.02-0.02)
CA	0.02 (0.02-0.02)	0.02 (0.01-0.02)	0.02 (0.02-0.04)	0.02 (0.01-0.02)
CA+T	0.02 (0.02-0.03)	0.02 (0.01-0.02)	0.02 (0.02-0.04)	0.02 (0.02-0.02)
IL-6				
N	0.00 (0.00-0.00)	0.00 (0.00-0.34)	1.01 (0.00-2.05)	0.41 (0.06-0.90)
CA	2.88 (1.05-10.92)	1.52 (0.25-3.05)	1.88 (0.63-3.47)	2.29 (0.92-6.82)
CA+T	2.20 (0.61-3.28)	1.34 (0.81-2.32)	2.66 (1.91-4.40)	3.96 (2.65-4.41)
IL-10				
N	5.72 (1.06-9.15)	1.75 (0.00-4.14)	17.48 (4.27-28.86)	1.55 (0.00-4.12)
CA	9.41 (5.96-10.83)	1.90 (1.19-4.99)	13.52 (11.28-24.75)	4.56 (0.00-15.32)
CA+T	13.76 (7.75-18.65)	4.83 (2.35-6.94)	18.51 (8.88-23.71)	5.45 (1.49-7.99)
IL-12				
N	0.00 (0.00-2.45)	1.13 (0.00-2.48)	0.00 (0.00-0.00)	1.33 (0.08-2.91)
CA	1.56 (0.62-3.06)	1.50 (0.76-3.55)	0.00 (0.00-0.57)	0.00 (0.00-1.49)
CA+T	2.31 (0.90-4.05)	1.04 (0.39-2.4)	1.37 (0.58-3.19)	0.97 (0.00-2.35)
IFN γ				
N	0.48 (0.10-1.12)	0.33 (0.10-0.48)	0.33 (0.18-1.32)	0.42 (0.37-0.50)
CA	0.38 (0.00-0.57)	0.26 (0.11-0.37)	0.31 (0.03-0.78)	0.24 (0.09-0.54)
CA+T	0.40 (0.00-0.65)	0.27 (0.12-0.49)	0.54 (0.18-1.32)	0.50 (0.25-0.64)
TNF α				
N	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.16)
CA	0.10 (0.04-0.27)	0.14 (0.04-0.18)	0.88 (0.20-1.22) ^a	1.29 (0.07-0.63)
CA+T	0.00 (0.00-0.25)	0.00 (0.00-0.08)	0.74 (0.29-1.25) ^a	0.31 (0.00-1.14)

Differences among groups were examined using the Kruskal-Wallis test followed by Dunn-Bonferroni test (adjusted significance, ^aP<0.05 vs. naïve). N, naïve; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group); CTX, cortex; HIP, hippocampus; STRI, striatum; CEREB, cerebellum.

and also demonstrated that CA resulted in an early marked TNF α response both systemically, and in other target organs. Contrasting our hypothesis, however, thalidomide in a dose reported previously to improve outcome of *in vivo* models of brain ischemia did not decrease TNF α levels in the striatum, a brain region that showed the most pronounced response to ischemia in our model of experimental CA. Unfortunately, TNF α levels in plasma, other brain regions or extracerebral organs were also not attenuated by thalidomide. Selected

pro- and anti-inflammatory cytokines increased in brain, plasma, heart or lung after CA were not affected by thalidomide. The reason for the failure of this approach to attenuate the increase in striatal and/or other levels of TNF α in our model and/or exhibit neuroprotective effects is unclear.

We used a dose previously reported to be effective in an experimental incomplete brain ischemia in mice. However, only pre-treatment with that dose was effective; post-treatment was not (20). To evaluate the potential of this approach

Table IV. Cytokine profile in extracerebral organs and plasma at 3 h after CA.

Variable	Heart, pg/mg protein	Lung, pg/mg protein	Plasma, pg/ml
IL-1a			
N	0.00 (0.00-0.00)	46.49 (44.14-52.17) (43.95-46.49)	0.00 (0.00-0.00)
CA	12.59 (9.97-18.80) ^a	1135.50 (699.93-1777.02)	266.41 (93.34-420.76)
CA+T	17.39 (12.43-32.68) ^a	851.2 (818.11-1231.17) ^a	216.04 (105.97-405.33)
IL-1b			
N	4.82 (3.23-6.12)	45.21 (43.90-47.95)	2.68 (0.07-11.99)
CA	54.57 (34.17-75.14) ^a	1,421.24 (559.38-1,887.66) ^a	255.81 (141.62-619.25)
CA+T	59.50 (37.90-79.31) ^a	1,135.49 (784.93-1,534.19) ^a	330.60 (229.21-863.37) ^a
IL-2			
N	118.71 (86.42-142.46)	0.00 (0.00-0.00)	179.61 (80.27-1495.17)
CA	84.71 (65.65-120.83)	13.38 (1.95-18.84)	1033.3 (661.27-1,903.21)
CA+T	148.08 (49.9-194.49)	24.15 (0.00-64.48)	1,053.10 (672.88-2,526.03)
IL-4			
N	0.28 (0.12-0.25)	0.02 (0.02-0.04)	0.17 (0.14-1.02)
CA	0.07 (0.04-0.25)	0.03 (0.02-0.04)	0.38 (0.32-0.87)
CA+T	0.19 (0.02-0.25)	0.04 (0.03-0.05)	0.41 (0.26-0.76)
IL-6			
N	0.00 (0.00-1.85)	0.00 (0.00-0.00)	2.31 (0.00-7.29)
CA	61.26 (26.02-117.38)	131.05 (20.75-165.54) ^a	6,316.17 (2,533.21-8462.35) ^a
CA+T	129.84 (73.25-333.64) ^a	119.86 (86.35-169.09) ^a	6,756.38 (2,561.31-7775.65) ^a
IL-10			
N	145.01 (96.93-194.58)	10.74 (1.91-16.33)	0.00 (0.00-0.00)
CA	75.55 (48.46-157.16)	17.02 (4.06-24.36)	925.86 (453.14-1390.16) ^a
CA+T	133.14 (27.23-147.75)	16.22 (13.02-16.84)	868.66 (597.01-1336.45) ^a
IL-12			
N	8.24 (6.54-12.78)	21.69 (18.22-30.02)	1,479.62 (1,142.40-1704.93)
CA	9.45 (6.81-15.84)	32.29 (27.04-67.66)	4,754.44 (2,533.69-9116.59) ^a
CA+T	10.26 (7.93-13.91)	31.53 (17.88-63.99)	4,049.25 (2,830.96-7046.72) ^a
IFN γ			
N	3.04 (2.26-3.55)	0.37 (0.25-0.48)	1.89 (0.35-7.90)
CA	1.35 (0.90-2.05)	0.63 (0.50-2.40)	17.39 (7.74-190.14)
CA+T	3.01 (0.55-3.35)	0.78 (0.70-1.50) ^a	30.86 (12.58-105.17)
TNF α			
N	0.00 (0.00-0.21)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
CA	0.73 (0.52-1.05)	1.24 (0.49-4.50)	84.74 (25.68-153.13) ^a
CA+T	1.11 (0.65-1.48) ^a	3.01 (0.20-3.64)	67.14 (32.24-190.30) ^a

Differences among groups were examined using Kruskal-Wallis test followed by Dunn-Bonferroni test (adjusted significance, ^aP<0.05 vs. naïve). N, naïve; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).

in a clinically relevant translational CA model, we used a post-treatment paradigm.

Early brain cytokine response to global brain ischemia has been documented by us (6,7) and others (22). Microglia are deemed to be a major source of brain TNF α in the later phases after the insult or in neuro-inflammatory diseases in which thalidomide was shown to be effective, e.g., Alzheimer's disease (23). However, we previously reported that in the early phase post resuscitation, TNF α is produced by neurons (6,7). This observation has been supported by others (24,25). It is

conceivable that neuronal origin of TNF α in this early phase of reperfusion could contribute to the lack of a definitive effect of thalidomide that primarily targets glia cells in brain (16). Also, thalidomide selectively targets microglia-mediated neuroinflammatory response rather than astrocytes (26). We reported that the cytokine production at the early stages of post-CA syndrome is not mediated by microglia but rather neurons and astrocytes (7).

Most studies focused on neuronal loss in hippocampus, a selectively vulnerable region with extensive neuronal

degeneration after cerebral ischemia. In recent studies focused on the neuro-inflammatory response to CA, however, we noted dramatic regional dependence of the cytokine response in brain after CA (6,8). These studies suggest the potential need for a paradigm shift in the approach to the development of neuroprotective therapies in CA—namely, region specific therapies tailored to individual brain regions. Dopaminergic neurons in the striatum may represent an alternative, selectively vulnerable region in the prolonged CA setting. The early surge of TNF α in the striatum as described by us earlier was selected as a primary target structure in our current study.

TNF α has also been documented to be increased after ischemia-reperfusion also in plasma and extracerebral organs.

In rats subjected to a shorter, 6 min VFCA, increased levels of TNF α , IL-6, and IL-10 were observed in the jejunum from 6 h until 7 d but not in serum in rats (27). Similarly, Wender *et al* (28) did not observe increased serum TNF α levels past 24 h in a rat CA model. In contrast, Yang *et al* (29) reported that serum IL-6 and TNF α levels were both increased at 6 h after 6 min VFCA in rats.

In a porcine model of VFCA, systemic TNF α was detected early (15 min after reperfusion), and peak plasma levels coincided with myocardial depression (30) and with poor survival (31). Zhu *et al* (32) recently reported early increases of both serum TNF α and IL-6 levels that continued to rise over 6 h.

In an experimental model of intestinal ischemia-reperfusion injury, thalidomide was effective to decrease both systemic and tissue (intestine, lung) TNF α levels, effectively ameliorating biomarkers of injury, edema and resulting histologic damage. However, pre-treatment with a large dose (400 mg/kg p.o.) was used (33). Not all studies reported decrease of systemic TNF α with thalidomide treatment. After a lipopolysaccharide challenge resulting in massive cytokine response, neither plasma nor hepatic TNF α levels were decreased with thalidomide (34,35).

Our study has several limitations. Although thalidomide has been shown to cross the BBB, most studies documenting the salutary effects of thalidomide were performed in models with markedly disrupted BBB, (e.g., traumatic brain injury, stroke (20) or lipopolysaccharide-induced chronic neuroinflammation). Mohammed *et al* (36) reported a salutary effect of thalidomide was elicited by direct injection of the drug into the hippocampus, obviating the need for transport across the BBB. In this light, it is possible that newly synthesized derivatives of thalidomide, e.g., 3,6'-dithiothalidomide or pomalidomide, that penetrate BBB more easily, could have been more effective in ameliorating neuronal injury (37-39).

We also did not perform a dose response in our model. Instead, we selected for our study the highest (and the only effective) dose used in other studies (18,19,21). Importantly the same dose given to naïve rats did not elicit notable adverse effects (19). In that regard, pomalidomide has been shown to produce a TNF α inhibitory effect that is 50,000 times greater than thalidomide, and thus might have more potential in a CA scenario, where a rapid inhibitor effect is needed in the setting of an intact BBB (16). Also, the time-course of TNF α response in traumatic brain injury and models of chronic neuroinflammation seems to be delayed (40), providing a more favorable scenario for thalidomide or its derivatives to exert the effects

on the injured brain even with oral administration (16). However, the lack of effect of thalidomide on increases in TNF α levels in plasma and extracerebral organs argue against brain penetration as the explanation for failure to effect target engagement and/or reduce secondary injury.

We explored TNF α levels only at a singular timepoint (3 h RT). We chose this timepoint based on results from our prior study using VFCA model in which we have explored both early (3 and 6 h RT) and late periods (14 days). Earlier time-points showed more robust cytokine response (6). Moreover, TNF α —as the major target for thalidomide effects—peaks early, perhaps within the first hour after reperfusion (22). We have also tested a single, high dose of thalidomide shown previously to be beneficial and safe. However, we cannot rule out that alternative dosing regimen or assessments at other time-points might yield different results. Biomarkers of end-organ injury were also assessed at an early timepoint. Despite this limitation, we were able to document significant increases in CA groups over naïve controls.

We studied healthy young male rats only. More pronounced systemic cytokine response was observed in aged rats subjected to asphyxial CA (41). A significant difference in plasma cytokine response to CA between sexes have been observed by others (30).

Finally, we observed a trend toward reduced TNF α levels in the hippocampus in rats treated with thalidomide ($P=0.067$). We cannot rule out a possible effect in that brain region that would need to be explored with a larger sample size to appropriately test that hypothesis given the modest increase in TNF α seen in that brain region vs striatum in our model.

In conclusion, this exploratory study suggests that TNF α is increased early after CA systemically, in the brain and in extracerebral organs. Thalidomide, used early after reperfusion at high-dose previously showed to confer benefits, failed to decrease TNF α levels or other increased cytokines assessed at this timepoint in our experimental CA model. Biomarkers of end-organ injury were markedly increased after CA without any effect of thalidomide. Early systemic and organ-specific cytokine response after CA remains a valuable therapeutic target for future interventions in both acute and longitudinal studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AAP, PMK and TD designed the study, analyzed and interpreted the data, and wrote the manuscript. JPS performed the experiments. KJF performed the Luminex assays and ELISAs. JPS and KJF confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (protocol no. 13021161; 'Neuroinflammation after prolonged cardiac arrest'), in compliance with ARRIVE guidelines and the AVMA euthanasia guidelines 2020.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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