Targeting TNFα-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 TNFα synthesis. We hypothesized that thalidomide would decrease the systemic and organ-specific TNFα/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). TNFα 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 TNFα 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 used to assess neuronal, glial, cardiac and intestinal damage, respectively. CA increased TNFα 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 TNFα 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 TNFα or cytokine levels in plasma, brain or extracerebral organs, or biomarkers of injury. Although CA at 3 h post resuscitation produces a robust TNFα 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 (TNFα) 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 TNFα up-regulated in cerebral ischemia. Systemic selective anti-TNFα 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 TNFα vs. other brain regions including hippocampus, where no increase in TNFα 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 TNFα localized immunohistochemically in neurons rather than microglia (6,7). Thus, unique TNFα 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 TNFα, thalidomide, an inhibitor of TNFα protein synthesis is readily capable of crossing the blood brain barrier (BBB) (10). Thalidomide has been reported to selectively decrease TNFα (11-13) and shown to be neuroprotective via destruction of TNFα mRNA in vivo (14) and in vitro (15).
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 TNFα mRNA, inhibiting TNFα production (16). In this exploratory study, we hypothesized that thalidomide would attenuate (1) systemic TNFα levels, (2) neuroinflammation as reflected by brain tissue TNFα levels, (3) extra-cerebral organ TNFα 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, Scottsdale, 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% for 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.

TNFα and cytokine measurements. Tissues were then processed for cytokine assessment for interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, interferon γ (IFNγ) and granulocyte-macrophage colony stimulating factor (GMCSF) 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 TNFα analysis. Protein levels in the supernatant were measured using the bicinchoninic acid (BCA) protein kit (Thermo Fisher Scientific, 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...
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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 sample size calculations were based on the results published by us previously. We hypothesized that thalidomide would reduce TNFα levels in the striatum by 50%. Using α=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). 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).

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

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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|         |
| pO₂, 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   |         |
| pCO₂, 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).
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).

**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,
and also demonstrated that CA resulted in an early marked TNFα response both systemically, and in other target organs. Contrasting our hypothesis, however, thalidomide in a dose reported previously to improve outcome of \textit{in vivo} models of brain ischemia did not decrease TNFα levels in the striatum, a brain region that showed the most pronounced response to ischemia in our model of experimental CA. Unfortunately, TNFα 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 TNFα 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).

| 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)   |
| IFNγ     |                    |                    |                    |                    |
| 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)   |
| TNFα     |                    |                    |                    |                    |
| 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)*  | 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)   | 0.31 (0.00-1.14)   |

Differences among groups were examined using the Kruskal-Wallis test followed by Dunn-Bonferroni test (adjusted significance, $P<0.05$ 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.
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 TNFα 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, TNFα is produced by neurons (6,7). This observation has been supported by others (24,25). It is conceivable that neuronal origin of TNFα 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

### 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  |
| CA       | 12.59 (9.97-18.80)   | 1135.50 (699.93-1777.02) | 266.41 (93.34-420.76) |
| CA+T    | 17.39 (12.43-32.68)  | 851.2 (818.11-1231.17) | 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)  | 1,421.24 (559.38-1,887.66) | 255.81 (141.62-619.25) |
| CA+T    | 59.50 (37.90-79.31)  | 1,135.49 (784.93-1,534.19) | 330.60 (229.21-863.37) |
| 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) | 6,316.17 (2,533.21-8462.35) |
| CA+T    | 129.84 (73.25-333.64) | 119.86 (86.35-169.09) | 6,756.38 (2,561.31-7775.65) |
| 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) |
| CA+T    | 133.14 (27.23-147.75) | 16.22 (13.02-16.84) | 868.66 (597.01-1336.45) |
| 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) |
| CA+T    | 10.26 (7.93-13.91)   | 31.53 (17.88-63.99) | 4,049.25 (2,830.96-7046.72) |
| IFNγ     |                      |                     |              |
| 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)    | 30.86 (12.58-105.17) |
| TNFα     |                      |                     |              |
| 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) |
| CA+T    | 1.11 (0.65-1.48)     | 3.01 (0.20-3.64)    | 67.14 (32.24-190.30) |

Differences among groups were examined using Kruskal-Wallis test followed by Dunn-Bonferroni test (adjusted significance, *P*<0.05 vs. naïve). N, naïve; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).
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 TNFα in the striatum as described by us earlier was selected as a primary target structure in our current study.

TNFα 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 TNFα, IL-6, and IL-10 were observed in the jejunum from 6 h until 7 d but not in serum in rats (27). Similarly, tissue (intestine, lung) TNFα levels, effectively ameliorating injury, thalidomide was effective to decrease both systemic and over 6 h.

produce a TNFα inhibitory effect that is 50,000 times greater effects (19). In that regard, pomalidomide has been shown to the BBB. In this light, it is possible that newly synthetized into the hippocampus, obviating the need for transport across the BBB. In an experimental model of intestinal ischemia-reperfusion injury, thalidomide was effective to decrease both systemic and tissue (intestine, lung) TNFα 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 TNFα with thalidomide treatment. After a lipopolysaccharide challenge resulting in massive cytokine response, neither plasma nor hepatic TNFα 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 synthetized 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 TNFα 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 TNFα 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 TNFα levels in plasma and extracerebral organs argues against brain penetration as the explanation for failure to effect target engagement and/or reduce secondary injury.

We explored TNFα 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 timepoints showed more robust cytokine response (6). Moreover, TNFα-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 TNFα 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 TNFα seen in that brain region vs striatum in our model.

In conclusion, this exploratory study suggests that TNFα 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 TNFα 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. KJJ performed the Lumexin assays and ELISAs. JPS and KJJ 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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