Systemic treatment with human amnion epithelial cells after experimental traumatic brain injury

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Abstract
Background: Systemic administration of human amnion epithelial cells (hAECs) was recently shown to reduce neuropathology and improve functional recovery following ischemic stroke in both mice and marmosets. Given the significant neuropathological overlap between ischemic stroke and traumatic brain injury (TBI), we hypothesized that a similar hAEC treatment regime would also improve TBI outcomes. Methods: Male mice (12 weeks old, n=40) were given a sham injury or moderate severity TBI by controlled cortical impact. At 60 minutes post-injury, mice were given a single tail vein injection of either saline (vehicle) or 1x10^6 hAECs suspended in saline. At 24 h post-injury, mice were assessed for locomotion and anxiety using an open field, and sensorimotor ability using a rotarod. At 48 h post-injury, brains were collected for analysis of immune cells via flow cytometry, or histological evaluation of lesion volume and hAEC penetration. To assess the impact of TBI and hAECs on lymphoid organs, spleen and thymus weights were determined. Results: Treatment with hAECs did not prevent TBI-induced sensorimotor deficits at 24 h post-injury. hAECs were detected in the injured brain parenchyma; however, lesion volume was not altered by hAEC treatment. Robust increases in several leukocyte populations in the ipsilateral hemisphere of TBI mice were found when compared to sham mice at 48 h post-injury; however, hAEC treatment did not alter brain immune cell numbers. Both TBI and hAEC treatment were found to increase spleen weight. Conclusions: Taken together, these findings indicate that — unlike in ischemic stroke — treatment with hAEC was unable to prevent immune cell infiltration and sensorimotor deficits in the acute stages following controlled cortical impact in mice. Although further investigations are required, our data suggests that the lack of hAEC-induced neuroprotection in the current study may be explained by the differential splenic contributions to neuropathology between these brain injury models.

Background
Traumatic brain injury (TBI) is a leading causing of death and disability worldwide [1, 2]. Although numerous treatments have proved promising in rodent models of experimental TBI, all pharmacological agents investigated to date have failed to improve outcomes in clinical trials [3–6]. The diverse nature of clinical TBI is likely to be a major contributor to such failures, with individual
differences in clinical variables including injury biomechanics, location and severity of injury, among others, which together result in significant heterogeneity in TBI pathophysiology [3, 4, 7, 8]. As such, therapies capable of modifying multiple rather than single pathophysiological mechanisms may be most effective for improving clinical TBI outcomes.

Cell-based therapies have particular appeal in regenerative medicine due to their ability to act on multiple biological mechanisms. Several pre-clinical studies have shown that local or systemic application of pluripotent stem cells harvested from a variety of tissues can have neuroprotective and neurorestorative actions, including in the central nervous system in the context of injury or disease [9–12]. Perinatal stem cells and stem cell-like cells, such as amnion epithelial cells that line the amniotic membrane, have particular appeal in neuroregenerative medicine [13, 14]. Human amnion epithelial cells (hAECs) have pluripotent potential, and can exert several protective and restorative functions, including immunomodulatory and anti-apoptotic actions [15, 16]. Their use is also ethically and practically advantageous over other stem cells as they can be harvested in large quantities from a tissue that is typically discarded after birth. Further, hAECs are also known to have relatively low tumorigenicity and immunogenicity [16].

Intravenous (IV) administration of hAECs has recently shown great potential for improving outcomes following ischemic brain injury [17]; an injury with considerable pathophysiological overlap with TBI [8, 18, 19]. Evans and colleagues found that when administered at acute or delayed stages following experimental ischemic stroke, hAECs migrated to the injured brain as well as the spleen, resulting in reduced infarct size and improved functional recovery in mice [17]. Notably, hAEC treatment had a profound effect on immune cell infiltration into the injured brain, with reductions in the number of neutrophils, T cells and macrophages detected in the first 24–72 h post-injury [17]. Similar immunomodulatory effects of systemically delivered hAECs has also been observed in experimental autoimmune encephalomyelitis [20]. In the context of TBI, a preliminary study have found that intracerebroventricular (ICV) injections of hAECs reduced axonal degeneration after penetrating ballistic-like brain injury in rats [21]. Given the promise of the aforementioned studies, here we aimed to determine whether the systemic administration of a single dose of hAECs, as used in the recent
experimental stroke study [17], could mitigate immune cell infiltration, reduce lesion volume and improve neurobehavioral outcomes in the acute stages following a moderate severity TBI in adult mice.

Methods
Animals
A total of 40 C57Bl/6 male mice were obtained from the Australian Animal Resource Centre (ARC, Western Australia) for use in this study. Mice were 12 weeks of age at the time of injury, were group housed under a 12-hour (h) light/dark cycle, and were given access to food and water ad libitum for the duration of the experiment. All procedures were approved by the Animal Ethics Committee at La Trobe University (AEC #18-032), were within the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (the Australian National Health and Medical Research Council), and were in compliance with the ARRIVE guidelines on reporting of animal experiments [22].

Experimental Design
Animals received either sham or TBI procedures, and were randomly allocated to either vehicle or hAEC treatment groups. All mice underwent behavioral testing at 24 h post-injury, followed by tissue collection at 48 h for either histological or flow cytometric analysis as detailed in Table 1.

|                  | Sham vehicle | TBI vehicle | Sham hAEC | TBI hAEC |
|------------------|--------------|-------------|-----------|---------|
| Behavior         | 8            | 12          | 8         | 12      |
| Histology        | 3            | 4           | 2         | 5       |
| Flow Cytometry   | 5            | 8           | 6         | 7       |

Numbers indicate group n’s. Abbreviations: hAEC, human amnion epithelial cells; TBI, traumatic brain injury.

Controlled Cortical Impact (cci)
The CCI model was used to induce an experimental TBI of moderate severity in mice [23]. In brief, animals were anesthetized with 1.5% isoflurane then secured within a stereotaxic frame. Mice were administered 0.05 mg/kg subcutaneous (s.c.) buprenorphine for analgesia prior to surgery, and isotonic sterile saline (0.9% NaCl s.c.) post-surgery to aid in rehydration. The skull was exposed by midline incision, and a 4 mm craniotomy was performed on the left parietal bone, midway between Bregma and Lambda, to expose the intact dura. For animals randomly assigned to receive a TBI, the
impact was delivered via a Leica Impact One device, using a 3 mm convex tip, 4.5 m/s velocity, 1.5 mm depth for 100 ms duration. Following impact, bleeding and swelling were observed then the scalp was sutured closed. Sham animals underwent identical surgical preparation with the exception of the actual impact. Following surgery, mice were individually housed under a heat lamp until a righting reflex was observed and normal activity was resumed, then returned to group housing. Body weights were monitored daily post-surgery.

Haec Isolation And Injection
hAECs were isolated from term placenta donated by healthy volunteers who underwent elective cesarean section delivery as previously described [15] and with approval from Monash Health Human Research Ethics (approval no. 12223B). All volunteers provided informed consent and information were collected in accordance to National Health and Medical Research Council guidelines on Human Research Ethics. Cells were thawed and washed in 2% human serum albumin (HSA) in saline. Cell viability was assessed by trypan blue exclusion and isolates with a minimum of 80% viability were used, with cells were suspended in 2% HSA at 5 × 10^6 cells/mL. Sixty mins after TBI, mice were randomly assigned to receive either 2% HSA (vehicle) or 1 × 10^6 hAECs administered via tail vein injection.

Behavioral Testing
Mice underwent behavioral testing in the open field and rotarod at 24 h post-injury, by an experimenter blinded to group assignment.

Open Field
An open field was used to assess locomotion/activity at 24 h post-sham/TBI, similar, to that as described previously [24, 25]. Briefly, this task consisted of a 50 × 50 cm square arena, with 25 cm-high walls to prevent escape. Mice were released into the center of the field and allowed to explore for 5 min before they were returned to their home cage. Total distance traveled was determined. As a measure of anxiety, the arena was divided into a central inner zone (30 × 30 cm) and an outer zone, and the time spent in the inner zone was determined.

Rotarod
An accelerating rotarod was used to assess sensorimotor ability similar to that to that described
previously [26]. Briefly, testing apparatus consisted of 3 cm diameter rotating rod, with 5.7 cm lanes and a 16 cm fall height (47650 Rotarod, Ugo Basile®, Italy). Two days prior to injury, mice firstly completed rotarod training at a constant speed of 16 rpm for 5 min, with mice manually returned to the rod upon falling. On the day prior to injury, mice completed 3 baseline trials. Each trial consisted of the mouse being placed on the rotating barrel, with the speed gradually increased from 5 to 50 rpm over a period of 5 min. The average duration of time the mouse was able to stay on the rotating barrel was recorded for each trial period (maximum time of 5 min). This procedure was repeated at 24 h post-injury. Latency to fall data for each mouse was expressed as a ratio of mean post-injury to mean baseline values.

Tissue Perfusion / Collection
For flow cytometry, mice were euthanized at 48 h post-injury by carbon dioxide asphyxiation, intracardially perfused with phosphate buffered saline (PBS) and then decapitated for brain collection. Brain hemispheres were separated after removal of the cerebellum and olfactory bulbs. Spleen and thymus weights were determined.

For histology, mice were euthanized at 48 h post-injury by carbon dioxide asphyxiation, intracardially perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were collected and post-fixed in 4% PFA for 24 h, cryoprotected in 30% sucrose for 48 h, snap frozen in isopentane and stored at -80 °C until sectioned.

Flow Cytometry
Mice were euthanized at 48 h post-injury by carbon dioxide asphyxiation, intracardially perfused with PBS and then decapitated for brain collection. Brain hemispheres were separated after removal of the cerebellum and olfactory bulbs. The left hemisphere was mechanically dissociated in digestion buffer containing collagenase type XI (125 U/mL), hyaluronidase (60 U/mL), and collagenase type I-S (450 U/mL) in Ca²⁺/Mg²⁺-containing PBS, and incubated at 37 °C for 45 min with gentle agitation (100 rpm). The suspension was then passed through a 70 μm nylon cell strainer (Falcon, BR Biosciences) to yield a single-cell suspension. Cells were washed with PBS (350 g, 5 min at 4 °C), the pellet was resuspended in 3 mL of 30% Percoll (GE Healthcare), underlaid with of 2 mL of 70% Percoll,
and centrifuged at 1,400 g at room temperature for 20 min without the use of a brake. Mononuclear cells at the interphase of the two Percoll density gradients were collected and washed with PBS. Cells were stained with the antibodies listed in Table 2. Cells were firstly incubated with LIVE/DEAD™ Fixable Aqua Dead Cell Stain (1:1000 dilution, Invitrogen) for 15 min at 4 °C. Cells were then washed with PBS containing 1% bovine serum albumin (BSA; 350 g, 5 min at 4 °C). An antibody cocktail containing all antibodies except FoxP3-PE-Cy5.5 was then prepared and cells were stained for 25 min at 4 °C. Cells were washed with PBS containing 1% BSA (350 g, 5 min at 4 °C) and incubated with FIX & PERM Cell Fixation & Cell Permeabilization Kit (Invitrogen) for 20 min at 4°C. After incubation, the samples were washed with Permeabilization Wash (Invitrogen, 350 g, 5 min at 4 °C). For intracellular staining of FoxP3, a marker of regulatory T cells, cells were stained with FoxP3 antibody for 15 min at room temperature. The samples were washed with Permeabilization Wash (350 g, 5 min at 4 °C) and resuspended in 1% formalin in PBS containing 1% BSA. Stained cells were quantified on a Cytoflex LX flow cytometer (Beckman Coulter). The total number of brain-infiltrating immune cells and resident microglia were analyzed using FlowJo software (Version 10, Treestar). All data are derived from gating of the live cell population.

Gating strategy

Forward and side scatters were used to identify single cells. Dead cells were excluded with the LIVE/DEAD™ stain. Live cells were gated for CD45<sup>+</sup> high and then divided into myeloid cells (CD45<sup>+</sup> highCD11b<sup>+</sup>), and subdivided into monocytes (CD45<sup>+</sup> highCD11b<sup>+</sup>Ly6C<sup>+</sup>), macrophages (CD45<sup>+</sup> highCD11b<sup>+</sup>F4/80<sup>+</sup>), neutrophils (CD45<sup>+</sup> highCD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup>), and lymphoid cells, which included T cells (CD45<sup>+</sup> highCD3<sup>+</sup>CD4<sup>+</sup>), helper T cells (CD45<sup>+</sup> highCD3<sup>+</sup>) and regulatory T cells (CD45<sup>+</sup> highCD3<sup>+</sup> CD4<sup>+</sup>FoxP3<sup>+</sup>). All cells are presented as the number of cells per hemisphere.
Table 2
Antibodies used for flow cytometry analysis.

| Antibody          | Dilution | Target cells          | Host/isotype | Supplier     |
|-------------------|----------|-----------------------|--------------|--------------|
| CD3-APC           | 1:500    | T cells               | Hamster IgG  | BioLegend    |
| CD4-BV605         |          | Helper T cells        | Rat IgG2a, κ | BioLegend    |
| CD11b-BV421       |          | Myeloid cells         | Rat IgG2b, κ | BioLegend    |
| CD45-A700         |          | Leukocytes            | Rat IgG2b, κ | BioLegend    |
| F4/80-APC-Cy7     |          | Microglia/Macrophages | Rat IgG2a, κ | BioLegend    |
| Ly6C-FITC         |          | Monocytes             | Rat IgG2c, κ | BioLegend    |
| Ly6G-PE-Cy7       | 1:1000   | Neutrophils           | Rat IgG2a, κ | BioLegend    |
| FoxP3-PE-Cy5.5    | 1:500    | Regulatory T cells    | Rat IgG2a, κ | eBioscience  |

Brain Sections Following Pfa Perfusion:

In a subset of mice (n = 2–5 per group), brain coronal sections spanning the entire injury site (Bregma − 0.9 to -3.5 mm) were collected to determine the extent of tissue damage by histology, as well as immunofluorescence to confirm the presence of hAECs in the injured brain (representative n = 1 per group). Sections were thaw-mounted onto Superfrost™ plus slides (Thermo Scientific, USA) and stored at -80 °C until analysis.

Histology/lesion Volume Measurements

Six equidistant sections per brain (10 µm thickness, 180 µm apart) were stained with 0.25% cresyl violet acetate (15 min) followed by differentiation in descending ethanol concentrations prior to clearance in xylene and coverslip adherence with DPX mountant (Sigma). Brightfield images were captured using a Leica Aperio AT Turbo slide scanner in the Monash Histology Platform, then exported to FIJI (https://imagej.net/Fiji) for analysis using the unbiased Cavalieri method with grid point counting, whereby volume = number of points counted x area represented by each point x distance between sections (taking into account the sampling frequency and section thickness) [27, 28]. Volume measurements were made of the dorsal cortex and hippocampus, both ipsilateral and contralateral to the injury site as previously described in depth [29].

Immunohistochemistry

PFA-fixed coronal brain sections (10 µm) were thaw-mounted onto Superfrost™ plus slides (Thermo Scientific, USA) and immunofluorescently labeled with HLA-G to identify the presence of hAECs in the brain following TBI. Sections were fixed in 4% PFA for 5 min, washed in 0.01 M PBS (2 × 5 min), and then blocked with a Mouse on Mouse (M.O.M.™) Ig blocking reagent (Vector Laboratories, USA) for 1 h. Following wash with 0.01 M PBS (2 × 2 min), sections were incubated in M.O.M.™ diluent (Vector
Laboratories, USA) for 5 min, prior to HLA-G antibody incubation for 30 min (1:500; Ab52455; Abcam, UK). Sections were then washed with 0.01 M PBS (2 × 2 min) and incubated with M.O.M Biotinylated Anti-Mouse IgG reagent (Vector Laboratories, USA) for 10 min. Following washes with 0.01 M PBS (2 × 2 min), fluorescein-Avidin DCS (Vector Laboratories, USA) was applied for 5 min. Sections were then washed with 0.01 M PBS (2 × 5 min), cover-slipped with Vectashield DAPI® (Vector Laboratories, USA), and examined with an Olympus fluorescence microscope. Positive HLA-G stains were confirmed upon co-localization with DAPI⁺ nuclei.

Statistical Analysis
All outcomes were analyzed using GraphPad Prism version 8.02 for macOS (GraphPad Software, CA, USA). All data was analyzed with two-way analysis of variance (ANOVA) and are presented as mean + standard error of the mean (SEM), with Sidak’s multiple comparisons conducted where appropriate. Statistical significance was set at p < 0.05.

Results
Behavioral outcomes
Sensorimotor function was assessed using the rotarod in mice at baseline and again 24 h post-injury. Relative latency to fall (post-injury latency relative to baseline latency; Fig. 1A) was analyzed by two-way ANOVA, revealing a main effect of injury (F(1, 36) = 9.46, p < 0.01), with no effect of treatment, nor an injury x treatment interaction. An open field was used to observe locomotor and anxiety-like behaviors of mice at 24 h post-sham/TBI, with no significant effects of injury nor treatment found for either total distance traveled (Fig. 1B) or time spent in the center zone (Fig. 1C).

Total body, spleen and thymus weights
Total body weight, spleen and thymus weights were recorded, and relative tissue to total body weights calculated, to investigate potential effects of CCI and hAEC treatment. There were main effects of injury (F(1, 35) = 7.467, p < 0.01) and treatment (F(1, 35) = 7.176, p < 0.05) on relative spleen weight at 48 h (Fig. 2A). These main effects were also found for raw spleen weight (data not shown). Note that the spleen weight value for one mouse from the sham hAEC group was found to be a statistical outlier with a conservative outlier test (ROUT, Q = 0.1%) and was subsequently removed from the spleen weight analysis. There were no significant effects found for total body weight at 48 h.
post-injury, however a non-significant trend \( (p = 0.053) \) was found suggestive of reduced body weight in hAEC treated animals (data not shown). There were no significant effects found for relative thymus weight (Fig. 2B), nor raw thymus weight (data not shown).

**Detection of hAECs in injured brain parenchyma**

An HLA-G antibody was used to detect the presence of hAECs in the brain parenchyma at 24 h post-sham/TBI. Immunostaining was detected in mice with TBI only, with reactivity restricted to areas within and around the site of the cortical lesion.

**Lesion volume**

Volumetric analysis of the healthy tissue volume of the ipsilateral cortex at 48 h post-injury (Fig. 4A-C) revealed a significant main effect of injury \( (F_{(1, 10)} = 66.76, p < 0.0001) \) with no significant effects of treatment nor interaction found.

**Immune cell infiltration**

Flow cytometry was used to analyze immune cell population in the brain at 48 h post-injury. Two-way ANOVA revealed a main effect of injury for total CD45+ leukocytes \( (F_{(1, 22)} = 10.79, p < 0.01; \) Fig. 4A, C), myeloid cells \( (F_{(1, 22)} = 11.76, p < 0.01; \) Fig. 4B, D), neutrophils \( (F_{(1, 22)} = 8.98, p < 0.01; \) Fig. 4E) and total T cells \( (F_{(1, 22)} = 4.42, p < 0.05; \) Fig. 4F). There were non-significant trends of a main effect of injury on helper T cells \( (F_{(1, 22)} = 4.02, p = 0.06; \) Fig. 4G) and regulatory T cells \( (F_{(1, 22)} = 2.46, p = 0.13; \) Fig. 4H). There was a main effect of injury on expression of Ly6C Lo monocytes \( (F_{(1, 22)} = 10.26, p < 0.01; \) Fig. 4I) and Ly6C Hi monocytes \( (F_{(1, 22)} = 4.61, p < 0.05; \) Fig. 4J). There was no main effect of treatment, nor an injury x treatment interaction, for all immune cells, however there were non-significant trends of an interaction for total leukocytes \( (F_{(1, 22)} = 3.84, p = 0.075) \) and myeloid cells \( (F_{(1, 22)} = 3.81, p = 0.064) \). There was no effect of injury or treatment found for macrophage/microglia expression at this time point (data not shown).

**Discussion**

Previous research has indicated that a single systemic treatment with hAECs is able to reduce neuroinflammation and infarct size, and improve functional recovery in the acute stages following experimental ischemic stroke, in at least two different animal models/species [17]. In the current
study, we aimed to determine whether treatment with the same preparation of hAECs would demonstrate similar therapeutic efficacy in the acute stages following experimental TBI in mice. We observed sensorimotor deficits in TBI mice at 24 h, along with a significant loss of healthy cortical tissue volume 48 h post-injury; findings indicative of a moderate severity TBI. Although we found that hAECs penetrated the injured brain and appeared to home to the site of injury, treated animals displayed comparable functional and structural deficits to their vehicle-treated counterparts. Given the similarities in treatment regime and outcomes assessed with the previous study on ischemic brain injury in mice [17], these surprising differences in findings between TBI and stroke models may highlight important distinctions in their pathogenesis, specifically regarding the contribution of the peripheral immune system.

Systemic hAEC administration has been shown previously to have profound effects on immune cell infiltration in experimental models of stroke and multiple sclerosis [17, 20]. With increasing evidence that excessive leukocyte infiltration may be a major contributor to neuropathology in TBI [31–33], and therefore a potential target for treatments, we hypothesized that hAECs would likewise mitigate infiltration in this context, and thereby improve acute TBI outcomes. As expected, our results indicated that the ipsilateral hemisphere of TBI mice contained significantly increased total leukocytes, myeloid cells, T cells, neutrophils and monocytes when compared to sham-operated mice at 48 h post-injury. These findings add to the growing literature suggesting a significant infiltration of immune cells during the acute stages of TBI [30, 34–37]. However, treatment with hAECs was not sufficient to prevent such infiltration. We observed no TBI-induced increases in microglia/macrophage numbers at this time point, a finding that is consistent with our previous studies that reports of increased numbers at a later time point of 3–7 days post-TBI [25, 36, 38]. Accordingly, it is possible that hAEC treatment used in this study may have indeed modified microglia/macrophage number or phenotype if assessed at later time points post-injury.

The finding that CCI increased splenic mass at 48 h post-injury adds to the growing pre-clinical literature that this model of TBI results in acute splenomegaly [34, 39]. Notably, this finding is in contrast to that seen in ischemic stroke, where splenic atrophy and loss of splenocytes is commonly
reported [40]. The underlying mechanisms of splenic atrophy following stroke are not well understood, and appear to differ between rats and mice; however, it is likely that sympathetic activation as a result of brain injury promotes both apoptosis and mobilization of splenocytes [40]. Although we did not assess splenocytes in the current study, our findings closely mimic those of Ritzel and colleagues who recently reported splenomegaly at 72 h post-CCI in mice, likely attributed to an increase in splenic leukocyte number [39]. However, we recently found that splenic leukocyte numbers were not altered at 24 h nor at 1 week following fluid percussion injury in both young adult and aged rats [30], indicating that the impact of TBI on the spleen may differ between TBI models, or between species.

We also found a main effect of hAEC treatment on spleen mass, likely attributed to either hAEC migration to the spleen or changes in splenocyte number. Notably, the neuroprotective effect of stem cell administration in both ischemic and hemorrhagic stroke has been shown to be prevented by prior splenectomy [17, 41], indicating that such effects are mediated by the spleen. Several studies have found that splenectomy can reduce number of circulating monocytes, the extent of neuroinflammation, and functional deficits following experimental stroke [40]; however, findings to date from the relatively few studies to investigate the impact of splenectomy on TBI are mixed [38, 42]. It is possible that the spleen may not exacerbate the neuroinflammatory response in TBI induced by CCI to the same extent as in the middle cerebral artery occlusion (MCAO) model of stroke; and as such, we speculate that the lack of hAEC-induced neuroprotection in the current study may be explained by the differential splenic contributions to neuropathology between these models.

Nevertheless, the mechanisms and consequences of altered splenic mass in various forms of traumatic and ischemic brain injury, and the potential interactions with hAEC treatment, requires further investigation.

Limitations and future directions

In the current study, we did not find a neuroprotective effect of single dose i.v. hAEC treatment on a range of immunological, pathological and neurobehavioral outcomes after experimental TBI in adult mice. However, we recognize that there are some limitations that should be considered, and that
future investigations are required to fully explore the potential of these cells as a treatment for TBI. Firstly, assessments of behavior, immune cell filtration and lesion size were all conducted at or within the first 48 h of injury, a deliberate experimental design choice to closely mirror the recent MCAO hAEC study. However, it is possible that treatment efficacy may have been apparent at later stages post-TBI. Furthermore, although no beneficial effects of hAEC treatment were observed on immune cell infiltration post-TBI, it is possible that hAECSs may have altered other pathophysiological aspects of TBI not assessed in the current study. In addition, although the hAEC treatment regime closely resembled that successfully used to improve acute outcomes following ischemic injury (i.e. same source, dose, route and similar timing of cells post-insult), and we observed hAEC migration to the injured brain regions, alternative dosing or local application to the site of injury may ultimately prove to yield some efficacy. It is also important to recognize that this study featured male mice only. Therefore, further studies are required to evaluate the efficacy of hAEC in females. Finally, as the TBI model utilized in this study induces a primarily focal injury to the cortex and underlying corpus callosum, it is possible that hAECSs may be more effective following diffuse TBI, a focal injury involving alternative brain regions, or a milder injury severity.

Conclusions
This study has demonstrated that immune cell infiltration, lesion volume and functional deficits in the acute stages following a moderate severity TBI in adult male mice are not reduced by a single intravenous injection of hAECSs. Nonetheless, this cell therapy has proven efficacious in other models of brain insult such as stroke, warranting further investigation in the future.

List Of Abbreviations
hAECSs Human amnion epithelial cells
TBI Traumatic brain injury
h Hours
CCI Controlled cortical impact
s.c. subcutaneous
m/s Meters per second
HSA Human serum albumin
PBS Phosphate buffered saline
PFA Paraformaldehyde
BSA Bovine serum albumin
MCAO Middle cerebral artery occlusion

Declarations
Ethics approval and consent to participate
All procedures were approved by the Animal Ethics Committee at La Trobe University (AEC #18-032).
Consent for publication
All authors consent to publication of this manuscript
Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
Competing interests:
The authors declare no competing interest.

Declarations

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**Consent for publication:** All authors consent to publication of this manuscript

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**Competing interests:** The authors declare no competing interest.

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**Authors’ contributions:** HK, BDS, CGS and SJM conceptualized the study. HK, BDS, LP and SJM conducted animal surgeries. RL isolated and quantified hAECs. SJM and LP conducted behavioral testing. LKD, SD, SZ and BDS conducted histology. HK conducted flow cytometry. All authors contributed to data interpretation, manuscript preparation and finalization.

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Figures
Sensorimotor ability, locomotion and anxiety-related behavior at 24 h post-TBI. A) A significant main effect of injury was found for accelerating rotarod performance (relative to pre-injury baseline) at 24 h post-injury (**p<0.01). Treatment with hAECs did not improve sensorimotor ability. B-C) Neither TBI nor hAEC treatment significantly altered measures of locomotion (distance traveled) and anxiety-related behavior (time in center) in open field testing conducted at 24 h post-injury. n=8-12/group. Data presented as mean + SEM.
Spleen and thymus weights at 48 h post-TBI. A) Both TBI (**p<0.01) and hAECs (*p<0.05) resulted in increased spleen weight at 48 h post-injury. B) No change in thymic weights was observed. n=7-12/group. Tissue weight / total body weight data expressed as mean + SEM.

Detection of hAECs in the brain at 48 h post-TBI. Immunofluorescence staining for human HLA-G (green) revealed hAEC cells within the cortical parenchyma of TBI brains (e.g. perilesional, as indicated by the black box in the coronal section schematic). Nuclei are identified by DAPI counter-staining (blue). Scale bar = 50 µm.
Lesion volume at 48 h post-TBI. Representative cresyl violet staining of (A) vehicle-treated and (B) hAEC-treated TBI brains. Healthy versus damaged tissue was delineated and volumes calculated (C), revealing a significant main effect of injury (****p<0.0001), but no effect of hAEC treatment. Scale bar = 1000 µm. n=2-5/group. Data presented as mean ± SEM.
Figure 5

Immune cell profiles of ipsilateral hemisphere at 48 h post-TBI. Representative flow cytometry plots demonstrate gating of leukocytes (A; CD45+) and myeloid cells (B; CD45+CD11b+) from the ipsilateral hemisphere of brains collected at 48 h post-injury. Quantitative analysis revealed significant TBI effects for total leukocytes (C), myeloid cells (D), neutrophils (E), and T cells (F). There were no effects of injury on helper T cells (G) and...
regulatory T cells (H). Ly6C Lo monocytes (I) and Ly6C Hi monocytes (J) were both increased by TBI. There were no significant effects of hAEC treatment found for any cell type.

**indicates main injury effect p<0.01, *indicates main injury effect p<0.05. n=5-8/group.

Data presented as mean + SEM.