Enhancing Dental Pulp Stem Cell Viability for Treatment of Spinal Cord Injury *via* Immune Cell Preconditioning

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Abstract

Spinal cord injury (SCI) is a devastating condition that affects more than 15,000 people in Australia. As there is no treatment or cure available, patients are often burdened with a lifetime of disability and co-morbidity. Growing evidence supports the use of stem cells to repair the spinal cord following injury, with dental pulp stem cells (DPSCs) in particular showing superior regenerative capacity and modulation of immune responses following injury. However, studies demonstrate that only approximately 1% of transplanted DPSCs survive due to harsh inflammatory conditions during SCI, thus limiting their full therapeutic benefits. Therefore, attempts to enhance the survival and viability of DPSCs should be investigated. Utilising fluorescence activated cell sorting, the present study characterised the inflammatory responses of peripheral blood mononuclear cells (PBMCs) in a clinically relevant rat model of contusion SCI, showing phenotypic variations between SCI, Sham and non-injured animals. Moreover, a novel approach was used to precondition DPSCs with PBMCs taken 3 days post-injury. Cytotoxicity analysis revealed that cytotoxicity is significantly higher following 7 days of culture than 3, particularly following pro-inflammatory TNF-α conditioning ($p<0.05$), but less cytotoxic than when DPSCs were cultured alone. Interestingly, following pro-inflammatory conditioning, co-cultured DPSCs were found to release significant amounts of IL-6 ($p<0.0001$), which may be involved in modulating the pro-inflammatory environment. This data suggests that preconditioning of DPSCs and PBMCs in co-culture for 3 days is a suitable *ex vivo* strategy to enhance the viability and immunomodulatory behaviour of DPSCs.

Introduction

Spinal cord injury (SCI) is a debilitating condition caused by direct insult to the spinal cord. It results in the long-term loss of functional mobility and/or sensation due to impaired conduction of descending motor and ascending sensory signals, and greatly increases the chance of morbidity and mortality.[1, 2] It is estimated that there are 15,000 Australians currently living with SCI[3] with 374 new cases in 2016-2017 alone.[4] Despite a wealth of knowledge on the pathophysiology of SCI, there is still no treatment to stop or revert the neurological deficits within the spinal cord following injury.

Central nervous system (CNS) injuries elicit secondary injury cascades (Fig. 1) within minutes that exacerbate neurological damage, creating a non-permissive environment for physiological regeneration.[5] This includes acute haemorrhagic swelling,[6] excitotoxicity,[7] oxidative stress, ionic imbalances,[8, 9] inflammatory cell recruitment and neuroinflammation,[10] all of which lead to neural and glial apoptosis and axon demyelination,[7, 11] thus worsening functional outcomes.

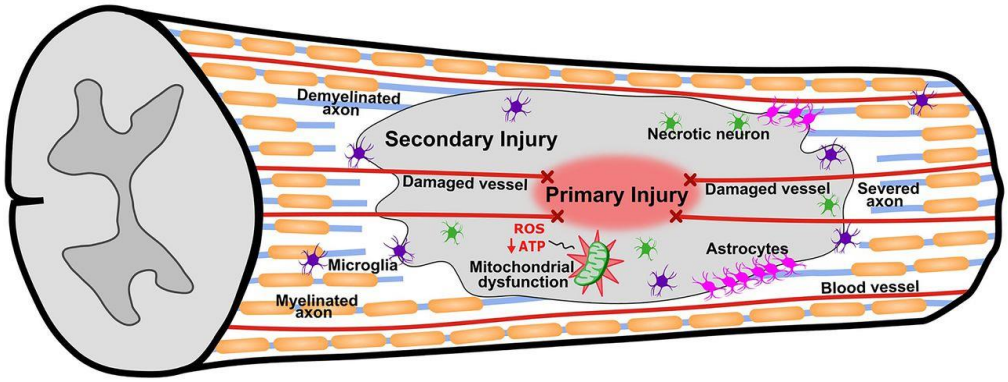

Figure 1. Multiple secondary injury cascades (including inflammation and cell death) follow the primary injury and exacerbate damage. Schematic extracted from Scholpa and Schnellmann.[12]

Inflammation is upstream of many secondary injury cascades and is both a driver and consequence of excitotoxicity, oxidative stress and ionic imbalances.[13] However, immune cells can change their

phenotype based on environmental cues (Fig. 2) and thus have varying effects on CNS tissue. Pro-inflammatory M1 polarised cells are activated by interferon-gamma (IFN-γ), lipopolysaccharide (LPS) and tumour necrosis factor (TNF),[14, 15] releasing cytokines that contribute to pathogen destruction and phagocytosis, but cause collateral damage to healthy neurons and glia.[15, 16] Conversely, anti-inflammatory M2 cells are activated by interleukin-4 (IL-4), IL-13 and IL-10[17] and work to promote tissue protection and repair[18, 19] through functions such as angiogenesis, axon growth, tissue remodelling[20] and suppression of pro-inflammatory effects.[15]

These inflammatory responses involve immune cells including lymphocytes (T-cells, B-cells), neutrophils and monocytes (microglia, macrophages). In the spinal cord following injury, the first responders are resident microglia which clear debris and fight infection.[21, 22] Neutrophils appear in the SCI lesion within 4-6h[23] and release proteases[6] and oxidative factors that injure neurons and glia and cause demyelination.[24] Monocytes then infiltrate the spinal cord, reaching maximal numbers within 7-10 days and persist for weeks to months after injury.[23] Microglia/macrophages, which are indistinguishable within the CNS, exhibit mainly pro-inflammatory phenotypes in SCI, leading to excessive oxidative factor release and neurotoxicity.[20] Microglia/macrophages also function as antigen presenting cells, recruiting T and B-cells to the injury site which release further pro-inflammatory cytokines.[25]

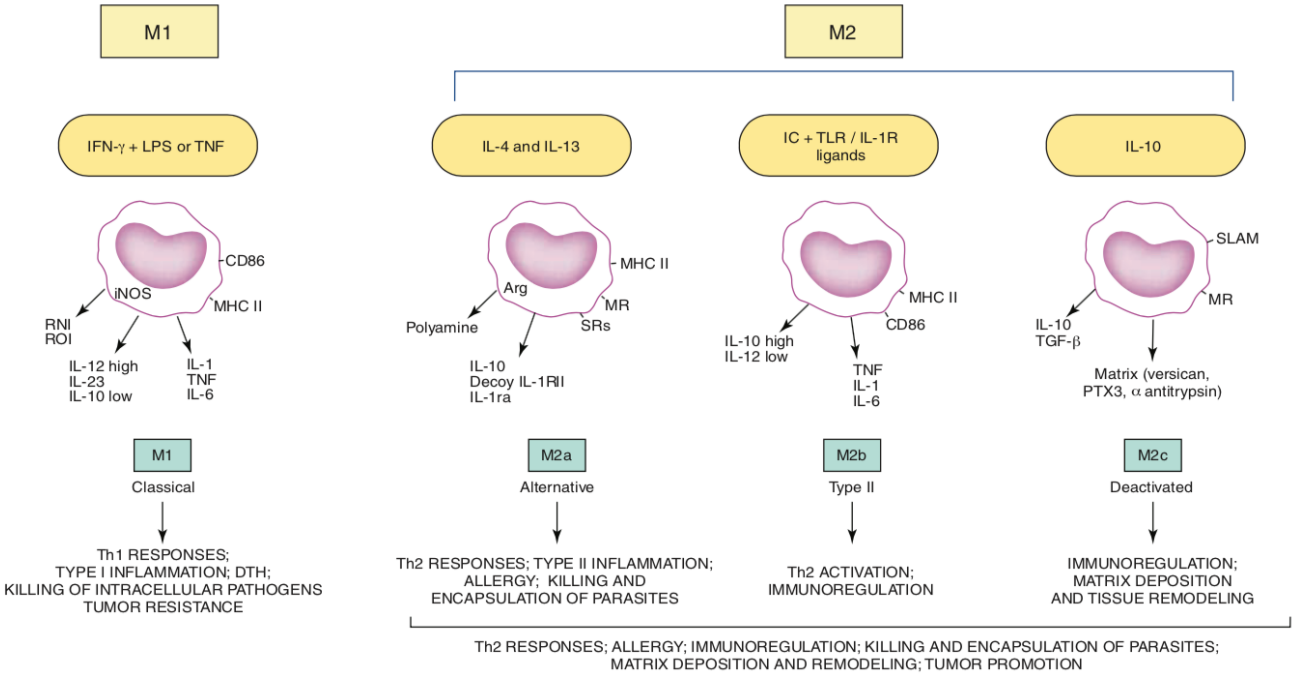

Figure 2. Pro-inflammatory (M1) and anti-inflammatory (M2) immune cell polarisation and their respective cell products (cytokines) and responses. Schematic extracted from Martinez and Gordon.[15]

Furthermore, immune function becomes dysregulated in chronic SCI due to insufficient dampening of the pro-inflammatory response, leading to increased neural and glial degeneration and death.[21, 23, 26] Blocking TNF-α[27] and the pro-inflammatory IL-1β cytokine[28] has been shown to reduce apoptosis and demyelination in the spinal cord, highlighting the detrimental effects of pro-inflammation in SCI.[23, 24] Therefore, inflammatory responses within SCI greatly impact functional outcomes,[29, 30] supporting the premise that targeting pro-inflammatory responses acutely would aid in SCI recovery and prevent chronic dysregulation.[13]

Despite inflammation and immune cells having a notorious reputation in injury and disease, studies have demonstrated the efficacy of activated immune cell engraftment as a potential targeted treatment strategy for SCI which circumvents the adverse effects of pharmaceuticals,[31, 32] directly promoting recovery whilst encouraging appropriate inflammatory responses. Prewitt et al. first showed that

implantation of microglia/macrophages into transected nerves in rodents significantly promoted axon regeneration.[33] Following this, studies engrafted preconditioned anti-inflammatory peripheral macrophages following SCI, which increased wound healing and nerve regeneration in rodent studies[13, 34] and showed safety in human trials.[35] Contradictory to long-standing knowledge of immune cell populations, studies have shown that unlike microglia, infiltrating peripheral macrophages perform anti-inflammatory roles,[36, 37] including wound healing and decreasing lesion size, and improve locomotor function following SCI.[38] However, treatment with anti-inflammatory macrophages alone has limitations, supported by findings describing a 20-40% phenotype loss of transplanted cells, favouring pro-inflammatory phenotypes once within the harsh acute SCI microenvironment.[20] Additionally, low functional recovery within Phase 1 and 2 human clinical trials limits translation from bench-to-bedside.[35, 39] Therefore, further studies are needed to develop novel strategies to increase the viability of anti-inflammatory immune cell populations, particularly blood-derived macrophages, following SCI.

Mesenchymal stem cells (MSCs) have been investigated within the field of neuroregeneration for decades, and, importantly, interact with immune cells to modulate inflammatory responses.[40-44] This includes decreasing apoptosis and lesion size, reducing the effects of pro-inflammatory cytokines such as IL-1β and TNF-α, inhibiting immune cell proliferation[45, 46] and releasing anti-inflammatory cytokines,[42] peripherally and within the spinal cord.[47, 48] Notably, a bidirectional relationship exists in which anti-inflammatory immune cells support the viability of stem cells[18] by providing trophic effects,[49] inducing cell renewal[50] and promoting growth.[51]

Neural crest derived dental pulp stem cells (DPSCs) are multipotent, self-renewing stem cells with MSC-like characteristics[52, 53] (Fig. 3), that exert similar modulatory effects on immune cells as MSCs.[54-58] These cells have a high regenerative capacity and higher proliferation rate than other MSC

populations,[59] and can be extracted in a non-invasive and autologous manner from dental tissue,[60, 61] thus limiting considerations of ethics and cell rejection.

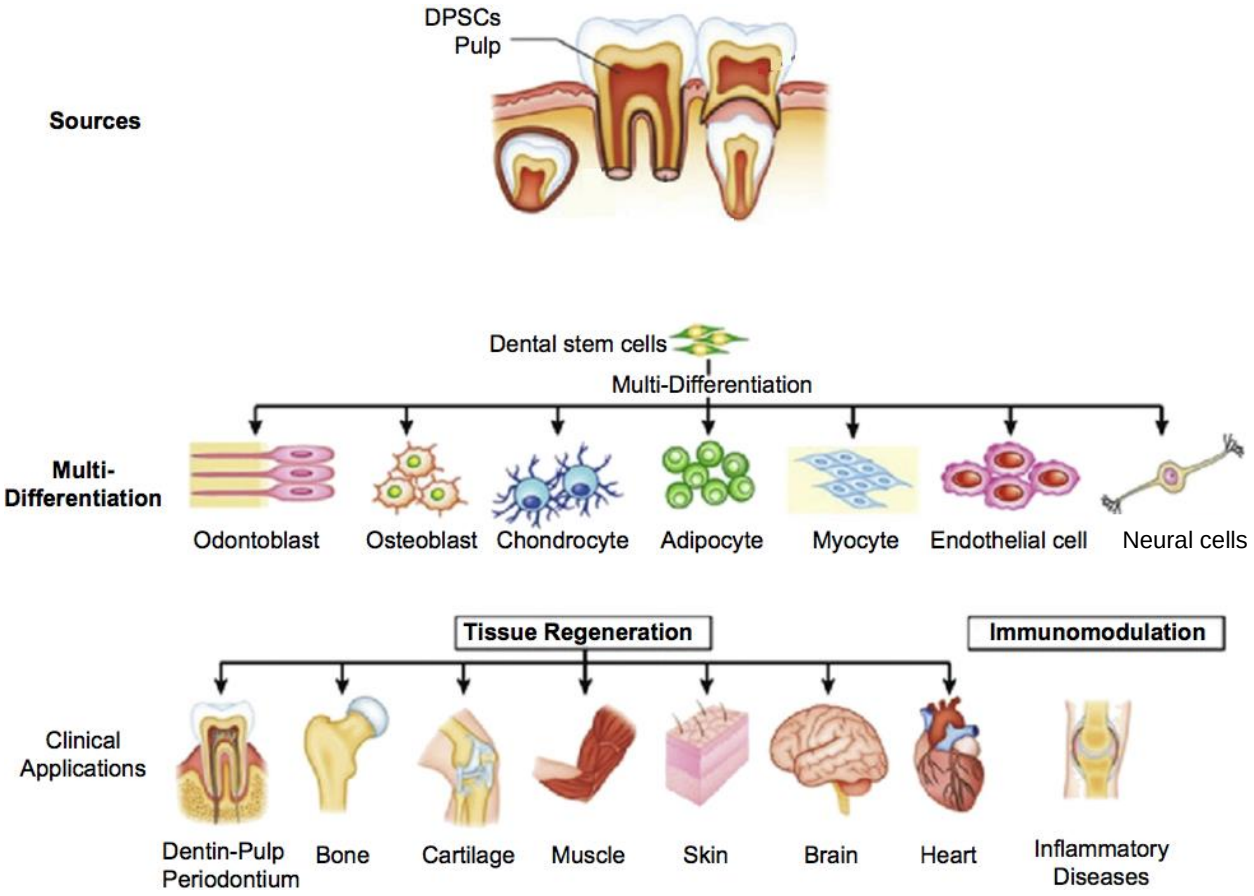

Figure 3. DPSCs can be extracted non-invasively from dental tissue and have various differentiation potentials and clinical applications. Schematic extracted from Li et al.[62]

As in MSC populations,[47, 63] studies utilising DPSCs show survival rates of approximately 1-2% following CNS engraftment,[57, 64] validating further investigation. Despite low survival, DPSCs have shown significant regeneration within the CNS and following SCI. In a rat stroke model, DPSCs promoted neuro-behavioural improvement with a ~2% survival rate.[64] Similarly, DPSCs engrafted into SCI rat models significantly increased regeneration of nerve tracts and improved locomotor function.[65, 66]

The independent administration of the two aforementioned preclinical SCI therapies (immune cell administration and DPSC administration) has great potential; however, issues concerning phenotype loss and low survival create gaps in knowledge. An increasing number of studies have outlined the potential of combinatorial treatment strategies for SCI that overcome the harsh inflammatory microenvironment whilst preserving neural and glial function.[9, 67, 68] Therefore, the present study employed a novel approach of merging the two therapies into one, multifaceted treatment mechanism. This strategy takes advantage of the symbiotic relationship between DPSCs and immune cells in order to increase their viability and immunomodulatory behaviour *in vivo*, and could enhance their neuroregenerative and neuroprotective potentials following engraftment. However, little is known regarding DPSC and immune cell co-culture and its effects on cell viability and immunomodulation *ex vivo*. Additionally, a limited understanding of the behaviour of peripheral immune cell populations following SCI limits the knowledge of when these cells can be extracted and administered as combinatorial therapies.

Hypothesis and aims

We hypothesise that preconditioning DPSCs and peripheral blood mononuclear cells (PBMCs), comprised of T-cells, B-cells and monocytes, will reveal immunomodulatory activities and enhance the viability of both DPSCs and PBMCs for future engraftment into a SCI model.

The aims of this project are, therefore, 1) to characterise how SCI changes the proportion of PBMC populations at different time-points following injury; 2) to identify a time-point for PBMC extraction and utilisation for novel preconditioning paradigms; and, 3) to investigate the effects of DPSC and PBMC preconditioning on cytotoxicity and immunomodulation *ex vivo.*

By showing that DPSCs and PBMCs can be preconditioned through co-culture to induce immunomodulatory activity and increase viability, their regenerative potential can then be further investigated *in vivo.* Knowledge gained from these studies could be used to develop a novel SCI

treatment mechanism that not only boosts cellular regeneration, but also limits the negative effects of inflammation. On a wider scale, this project could aid in the development of a viability model for DPSC survival, which may be used in a range of chronic pathologies both within the CNS and peripherally, particularly in those linked to an imbalance of inflammatory responses.

Methods

Animal surgeries. Adult female Sprague-Dawley rats (10-12 weeks; Bioresources, SAHMRI) were housed in a 12h light/dark cycle and fed *ad libitum*. Animals were sedated with Diazepam (1.75 mg/kg) and anaesthetised using isoflurane (2.0-2.5% v/v). Surgical sites were triple swabbed with ethanol and betadine solution and a longitudinal incision was made through the skin to reveal the spinal column. Partial laminectomy at T9-T11 was conducted to expose the spinal cord without disrupting the meninges. Using an Infinite Horizon impactor device (Precision Systems), animals received a moderate-severe T10 contusion (200kDyne) to the dorsal surface of the spinal cord.[69] The incision site was closed with nylon sutures and wound clips. Surgical control (Sham) animals received laminectomies only, whilst non-injured animals served as negative controls. Saline and Buprenorphine (0.01mL/100g bodyweight; 0.0324mg/kg) were administered post-operatively and then twice daily until 5 days post-injury (dpi), then once daily until 7 dpi. Benacillin (0.02mL/100g bodyweight; 64mg/kg [150mg/mL procaine penicillin, 150mg/mL benzathine penicillin, 20mg/mL procaine hydrochloride]) was administered immediately after and at 2, 4 and 6 dpi. Bladders of SCI rodents were manually expressed twice daily until normal bladder function returned (7-10 dpi). All procedures performed complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council (8th edition, 2013) with approval from the SAHMRI Animal Ethics Committee (SAM247; SAM413.19).

PBMC isolation. Animals were deeply anaesthetised with isoflurane (5% v/v) and humanely killed *via* cardiac puncture: whole blood samples (4-8mL) from non-injured (n=5), Sham (n=30) and SCI animals (n=30) were harvested using a 25-gauge needle and split into two heparinised vacutainers (Greiner Bio-One) per animal, at 1, 2, 3, 7, 14 and 28 dpi. PBMCs were isolated *via* density gradient centrifugation using Lymphoprep (STEMCELL Technologies), according to the manufacturer instructions. Briefly, blood samples were diluted 1:1 with Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher), centrifuged at 1200***g*** for 10min, washed with DPBS and centrifuged at 500***g***

for 10min twice and resuspended in 50% (v/v) foetal bovine serum (FBS) (Thermo Fisher; Australian Origin), 40% (v/v) RPMI 1640 (Thermo Fisher) and 10% (v/v) dimethyl sulfoxide (Sigma). Each animal sample was split into four cryotubes, placed in Mr Frosty freezing containers (Thermo Fisher) overnight at -80°C, and stored until further processing.[70]

Fluorescence activated cell sorting (FACS). FACS analysis utilising a modified 9-colour staining panel[71] was conducted to sort PBMC populations and obtain proportions of each as percentages of the total PBMC sample, to investigate aims 1 and 2. PBMC samples were thawed at 37°C and washed with RPMI and 20% FBS and centrifuged at 500***g*** for 10min twice. After incubating with viability assay and blocking with bovine serum albumin and CD32 (0.5μg/μL) for 15min at room temperature, $1x10^6$ cells/100μL were incubated with conjugated antibodies obtained from BD Biosciences against CD45 (BUV 395 OX-1; 0.1μg/μL), CD4 (Horizon V450; 0.025μg/μL), CD3 (BV605 1F4; 0.2μg/μL), CD45R (BUV 737 HIS24; 0.1μg/μL), RT1D (BV480 OX-17; 0.2μg/μL), CD161a (Alexa 647 10/78; 0.1μg/μL), CD8a (BV786 OX-8; 0.2μg/μL), and from BioLegend against CD43 (PC7; 0.2μg/μL), CD11b (FITC RUO; 0.5μg/μL), and CD172a (PE OX-41; 0.5μg/μL), as well as Brilliant Stain Buffer Plus (1:10; BD Biosciences) for 30min at 4°C. Cell sorting was conducted using a FACS Aria Fusion cytometer (BD Biosciences). The gating strategy involved first removing debris, non-living cells, cells stuck together and cells not positive for the hemopoietic marker CD45. PBMCs were then sorted sequentially, first using CD3 to sort T-cells, which were also separated by CD4 and CD8 expression. B-cells were sorted by CD45R expression and monocytes by CD43 and CD11b expression.

PBMC and DPSC co-culture. Human DPSCs were donated by the Mesenchymal Stem Cell Laboratory (University of Adelaide) and isolated as previously described.[59, 72] DPSCs were cultured in α-MEM (Thermo Fisher) supplemented with 10% FBS, 2mM glutamine (Thermo Fisher), 100 units/mL penicillin plus 100μg/mL streptomycin (Thermo Fisher) and 100μM L-ascorbate (Sigma)

and incubated at 37°C in 5% CO_2. 24h before co-culture, DPSCs at passage 6 were transferred to 48-well plates at $5x10^3$ cells/well resuspended in RPMI culture medium supplemented as above and incubated further. After 24h, 3d SCI (n=3) and 3d Sham (n=3) rat PBMC samples were thawed, washed twice with RPMI and seeded at $5x10^3$ cells/well, on top of DPSC cultures or as monocultures, and supplemented with fresh culture medium. Culture medium only wells served as negative controls. Culture media was changed 24h following PBMC addition, and then every 48h, thus leaving adherent monocytes[73] and DPSCs within wells.

Cell culture preconditioning and cytotoxicity analysis. To investigate aim 3, cytotoxicity analysis was conducted after conditioning with inflammatory cytokines to determine the effects of co-culture, as well as DPSC-only and rat PBMC-only culture, on cell viability, using the CytoTox96® Non-Radioactive assay (Promega) according to manufacturer instructions. Wells were stimulated with pro- (TNF-α, IFN-γ, granulocyte-macrophage colony-stimulating factor; GM-CSF) or anti-inflammatory (macrophage colony-stimulating factor; M-CSF) rat recombinant proteins (25ng/L) (STEMCELL Technologies) after 48h of culture. Non-stimulated wells served as negative controls. Half of the culture media was replaced with fresh recombinant protein media every 48h. Cell culture supernatants (n=3/condition) were collected and used to determine the level of lactate dehydrogenase release in each well as a measure of cytotoxicity[74] after 3 and 7 days of culture, measuring absorbance at 490nm on a GloMax® Discover (Promega) plate reader.

Measurement of DPSC cytokine release (Cytometric Bead Array). To determine the effect of preconditioning on DPSC inflammatory cytokine release as part of aim 3, supernatants collected from 3 day co-culture wells were analysed using a Cytometric Bead Array - Human Soluble Protein Master Buffer Kit (BD Biosciences), according to manufacturer instructions. Briefly, 50μL of each sample supernatant (n=3/condition) was added to a 96-well plate, followed by 50μL of mixed IL-6, IL-1β and TNF-α Capture Bead solution and incubated for 1 hour. 50μL of the corresponding mixed

Detection Reagent solution was then added and incubated for 2 hours. Fluorescence of each sample was analysed using a BD LSRFortessa™ X20 Flow Cytometer (BD Biosciences).

Data and Statistical analysis. FACS cytometric data were analysed using FlowJo v10.7.1 (Becton Dickinson & Company). PBMC population proportions at different time-points and between Sham and SCI groups were analysed using mixed ANOVA. Cytotoxicity following inflammatory conditioning after 3 and 7 days and DPSC cytokine release were analysed using two-way ANOVAs. Sidak's *post hoc* tests were utilised for multiple comparisons. Statistical analyses were conducted using GraphPad Prism v8.0 and were expressed as group mean $\pm$ SEM. Results were considered statistically significant at $p \leq 0.05$. All data assumes normality unless otherwise stated.

Results

Characterisation of PBMC populations reveals trends within Sham and SCI responses, and identifies a novel monocyte sub-population.

Quantification of the proportion of PBMC samples utilising FACS analysis (Fig. 1) revealed no significant differences between non-injured, Sham or SCI groups in the proportion of B-cells (CD45R+) at any time-point over 28 dpi (p=0.15; Fig. 2A). Similarly, no significant differences were observed within total T-cell (CD3+) proportions (p=0.12) or cytotoxic (CD8+; p=0.41) and helper (CD4+; p=0.070) T-cell sub-populations (Fig. 2B-D). However, trends were noticed whereby proportions of B-cells were consistently reduced when comparing SCI to Sham animals up to 14 dpi. At 28 dpi, the proportion of B-cells in SCI increased (27.12% ± 11) compared to Sham (10.78% ± 0.77). T-cell proportions trended higher at 2 (44.92% ± 8.0) and 3 dpi (37.28% ± 4.4) in SCI compared to Sham (24.15% ± 3.0 and 26.43% ± 4.7, respectively), with helper T-cell proportions increasing at 3 dpi. Conversely, cytotoxic T-cell proportions were reduced at 2, 3 and 7 dpi following SCI.

Although there were no significant differences between non-injured, Sham or SCI groups in total monocyte proportions (p=0.77), or $CD43^{high}CD11b^{high}$ (p=0.10) and $CD43^{low}CD11b^{high}$ (p=0.35) monocyte sub-populations at any time-point, notable trends were observed (Fig. 2E-G). At 1 and 2 dpi, $CD43^{low}CD11b^{high}$ (44.53% ± 13) and $CD43^{high}CD11b^{high}$ (35.77% ± 6.8) monocyte sub-populations increased following SCI when compared to Sham (28.70% ± 8.4 and 10.79% ± 6.8, respectively). Notably, a novel monocyte sub-population ($CD43^{mid}CD11b^{low}$) not previously reported in SCI literature in rats was identified, which made up the greatest proportion of the total monocyte population (54.95% ± 8.6 in non-injured animals). $CD43^{mid}CD11b^{low}$ sub-populations were consistently lower in SCI animals when compared to Sham at 1, 2, 3, 14 and 28 dpi (Fig. 2H).

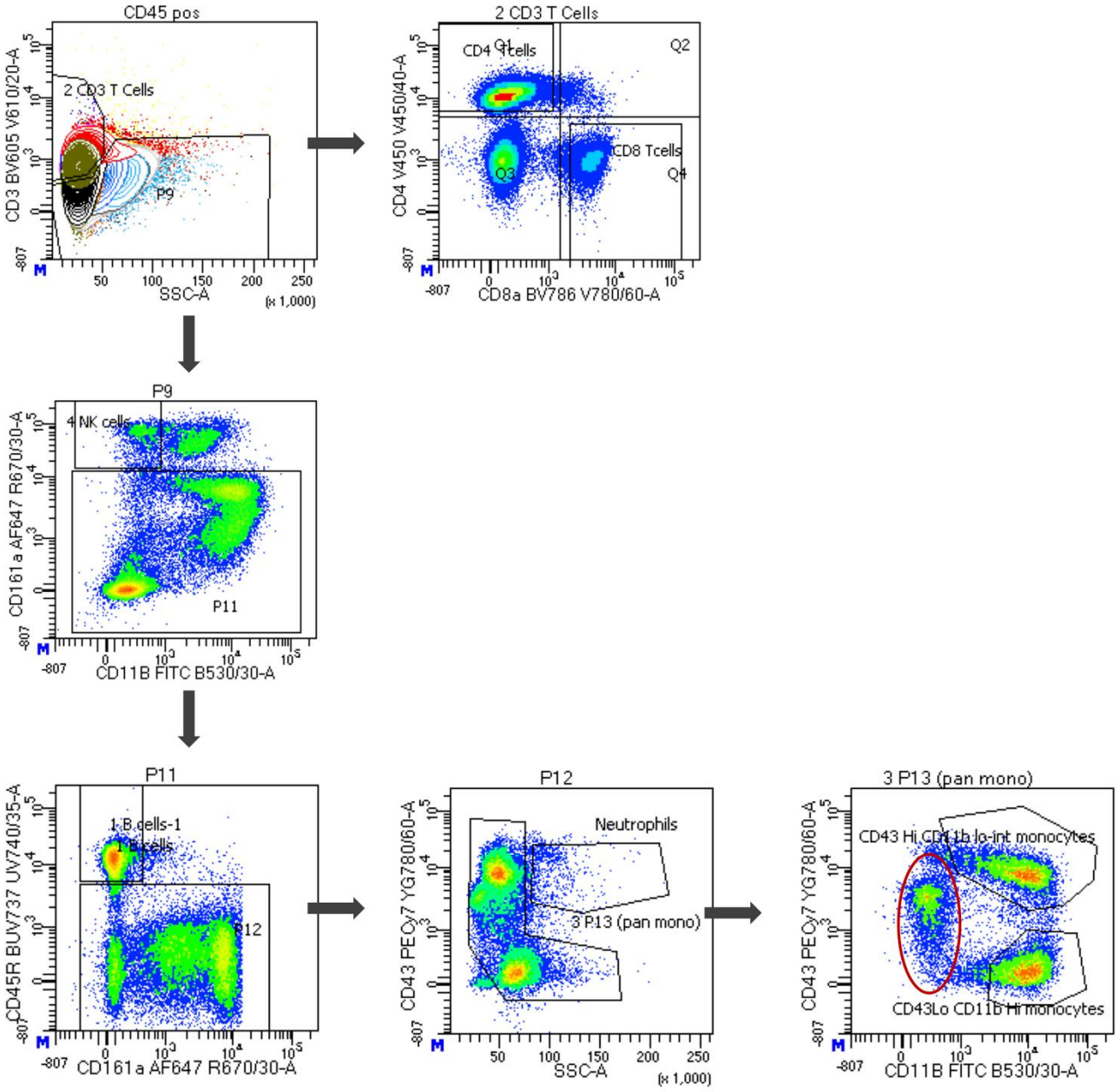

Figure 1. *FACS analysis identified distinct immune cell populations. Representative gating strategy utilised to sort and identify CD3+ and CD4+ or CD8+ T-cells, CD45R+ B-cells, CD43+/SSC-A monocytes and* $CD43^{high}CD11b^{high}$ *and* $CD43^{low}CD11b^{high}$ *monocyte sub-populations. Red ellipses indicate a novel monocyte sub-population (*$CD43^{mid}CD11b^{low}$*) not previously identified in rats.*

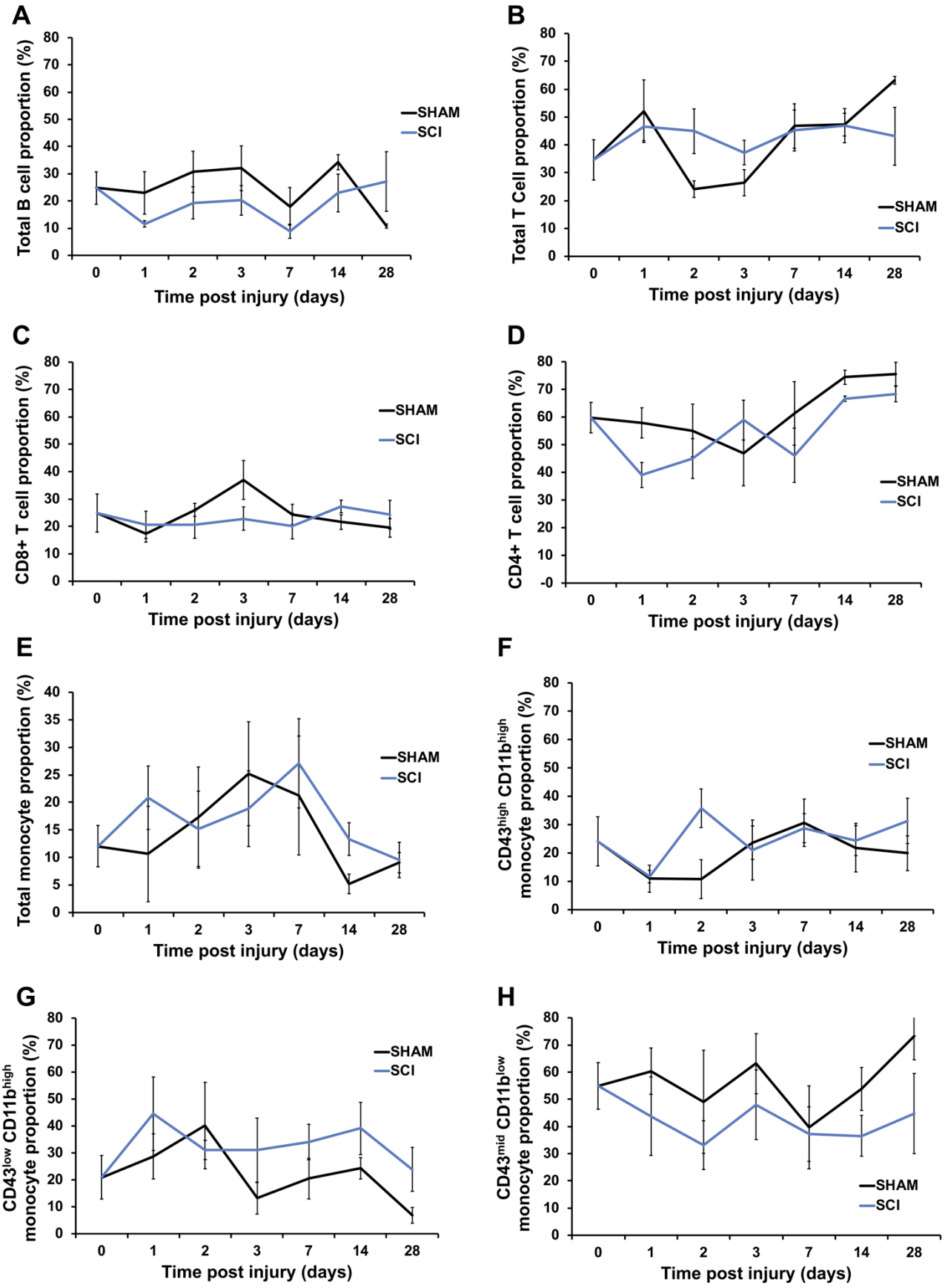
A
Total B cell proportion (%)
SHAM
SCI
Time post injury (days)
B
Total T Cell proportion (%)
SHAM
SCI
Time post injury (days)
C
CD8+ T cell proportion (%)
SHAM
SCI
Time post injury (days)
D
CD4+ T cell proportion (%)
SHAM
SCI
Time post injury (days)
E
Total monocyte proportion (%)
SHAM
SCI
Time post injury (days)
F
CD43high CD11bhigh monocyte proportion (%)
SHAM
SCI
Time post injury (days)
G
CD43low CD11bhigh monocyte proportion (%)
SHAM
SCI
Time post injury (days)
H
CD43mid CD11blow monocyte proportion (%)
SHAM
SCI
Time post injury (days)

Figure 2. *FACS analysis reveals non-significant trends between SCI and Sham PBMC proportions at different time-points.* ***A, B, E*** *Total B-cell, T-cell and monocyte populations, respectively, as proportions of the total PBMC sample.* ***C, D*** *CD8+ and CD4+ T-cell sub-populations as proportions of the total T-cell amount.* ***F-H*** *$CD43^{high}CD11b^{high}$, $CD43^{low}CD11b^{high}$ and $CD43^{mid}CD11b^{low}$ sub-populations as proportions of the total monocyte amount. Time-point 0 indicates the mean of non-injured animal PBMC proportions (n=5). Values expressed as mean ± SEM; p>0.05. n=5.*

Conditioning of PBMCs, DPSCs and DPSC+PBMC co-cultures ex vivo with various rat inflammatory cytokines for 7 days increases cytotoxicity.

Following conditioning with TNF-α, IFN-γ, TNF-α+IFN-γ, GM-CSF and M-CSF, PBMCs within both Sham ($p<0.0001$) and SCI groups ($p<0.0001$) showed significantly increased cytotoxicity following 7 days of conditioning when compared to 3 days (Fig. 3A-F). However, no significant differences were recorded between conditions at day 3, in both Sham and SCI groups ($p=0.86$), nor were Sham conditions significantly different from SCI conditions ($p=0.29$).

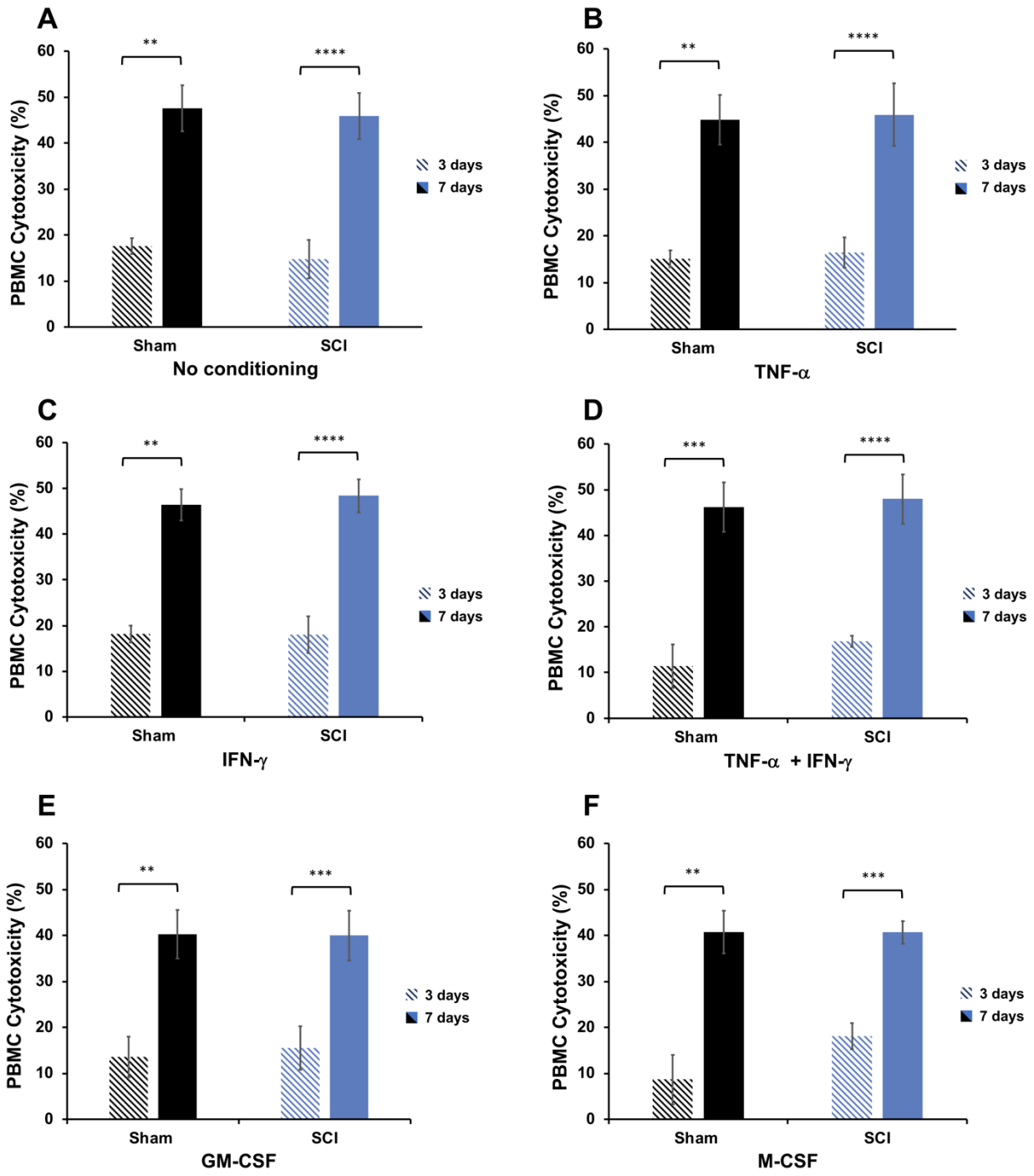

Figure 3. *Conditioning of PBMCs from SCI and Sham animals with rat inflammatory cytokines has higher cytotoxicity following 7 days of conditioning.* ***A*** *No conditioning and conditioning with* ***B*** *TNF-α,* ***C*** *IFN-γ,* ***D*** *TNF-α+IFN-γ,* ***E*** *GM-CSF and* ***F*** *M-CSF is significantly more cytotoxic following 7 days of culture than 3 days, within both Sham and SCI PBMCs. Values expressed as mean ± SEM; **p<0.01, ***p<0.001, ****p<0.0001. n=3.*

Following conditioning with TNF-α, IFN-γ, TNF-α+IFN-γ, GM-CSF or M-CSF, cytotoxicity of DPSCs was significantly higher after 7 days of TNF-α ($p<0.0001$), IFN-γ ($p=0.0022$) and TNF-α+IFN-γ ($p=0.0071$) stimulation, compared to 3 days. Following 7 days of conditioning, TNF-α stimulated DPSCs had significantly higher cytotoxicity than those stimulated with GM-CSF ($p=0.0068$) or M-CSF ($p=0.0053$), and non-conditioned DPSCs ($p=0.014$) (Fig. 4).

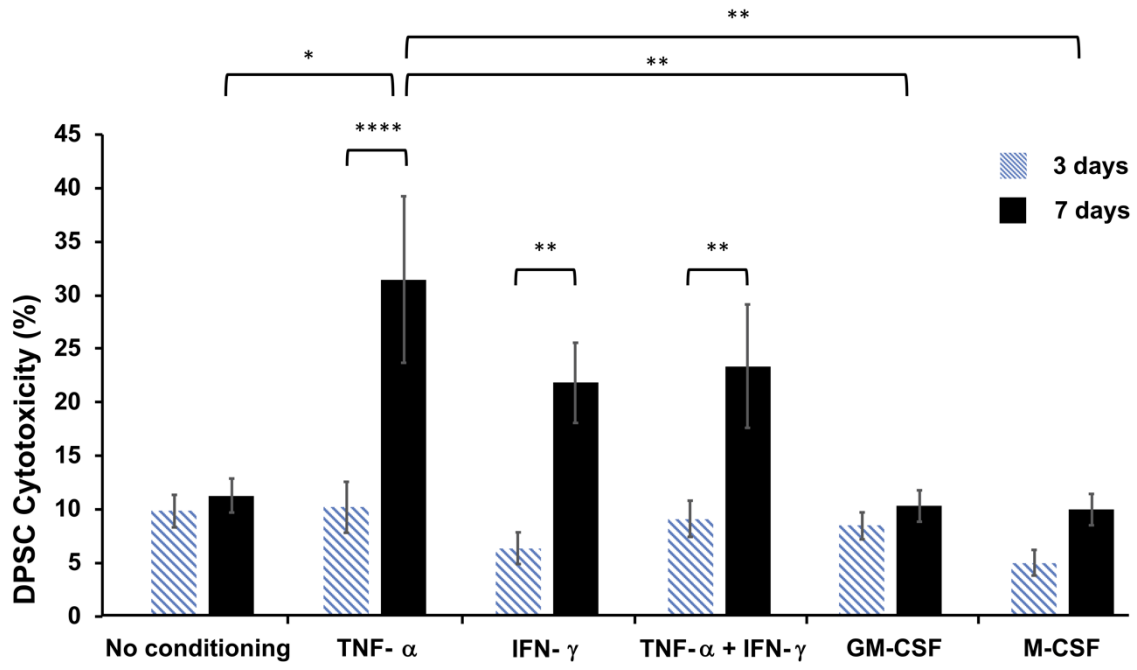

Figure 4. *Conditioning of DPSCs with rat pro-inflammatory cytokines has higher cytotoxicity following 7 days of conditioning. Conditioning with the pro-inflammatory cytokines TNF-α, IFN-γ and TNF-α+IFN-γ is significantly more cytotoxic at 7 days of culture than 3. Values expressed as mean ± SEM; *p<0.05, **p<0.01, ****p<0.0001. n=6.*

Cytotoxicity analysis was conducted following 3 and 7 days of co-culture of DPSCs and PBMCs. Following 7 days of conditioning, cytotoxicity was significantly increased in non-conditioned Sham co-cultures ($p=0.007$), and non-conditioned ($p=0.005$) and TNF-α stimulated ($p=0.02$) SCI co-cultures, compared to 3 days (Fig. 5A-F). No statistical significance was observed between different stimulation conditions following 3 days of conditioning ($p=0.28$) in either Sham or SCI groups.

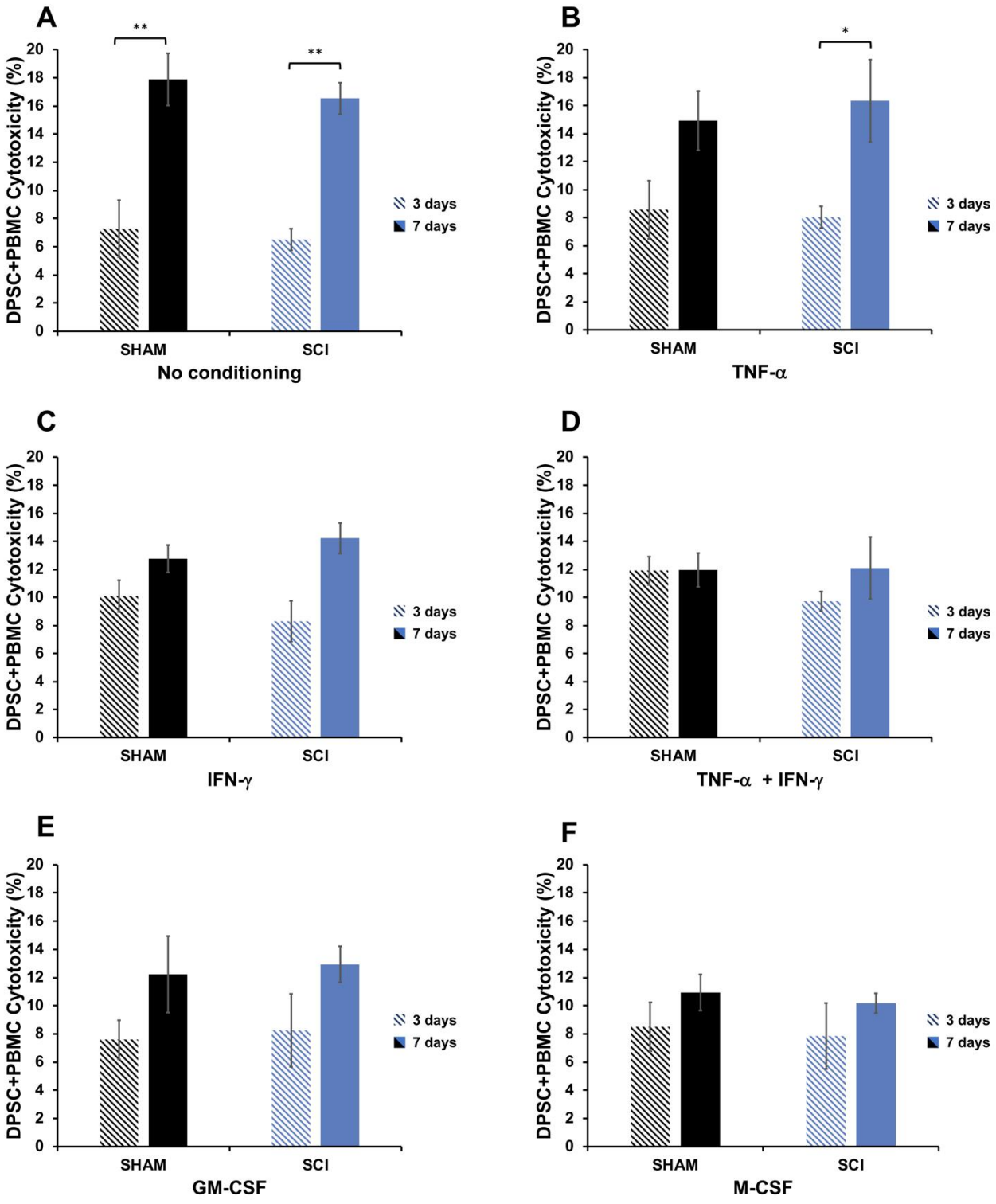
A
B
C
D
E
F
DPSC+PBMC Cytotoxicity (%)
3 days
7 days
SHAM
SCI
No conditioning
TNF-α
IFN-γ
TNF-α + IFN-γ
GM-CSF
M-CSF
**
*

Figure 5. *Conditioning of DPSC+PBMC co-cultures with rat inflammatory cytokines reveals increased cytotoxicity following 7 days in TNF-α and control conditions only.* ***A*** *No conditioning and conditioning with* ***B*** *TNF-α is significantly more cytotoxic at 7 days of culture than 3 in co-cultures containing SCI PBMCs. No conditioning has greater cytotoxicity following 7 days of culture in co-cultures containing Sham PBMCs. Conditioning with* ***C*** *IFN-γ,* ***D*** *TNF-α+IFN-γ,* ***E*** *GM-CSF and* ***F*** *M-CSF reveals no statistical differences between culture time-points. Values expressed as mean ± SEM; *p<0.05, **p<0.01. n=3.*

Ex-vivo conditioning of DPSC+PBMC co-cultures with rat pro-inflammatory cytokines increases the release of IL-6 protein from DPSCs.

Quantification of cytokines released from DPSCs co-cultured with PBMCs from Sham and SCI animals following 3 days of conditioning was performed utilising Cytometric Bead Array assays. Stimulation of DPSCs with TNF-α and TNF-α+IFN-γ significantly increased the release of IL-6 cytokine by DPSCs, within both Sham ($p<0.0001$) and SCI co-culture groups ($p<0.0001$), when compared to no conditioning (Fig. 6A). Additionally, IL-6 release was significantly higher following TNF-α+IFN-γ stimulation in SCI co-cultures compared to Sham ($p<0.0001$). No significant differences in IL-1β release were observed between Sham and SCI animals ($p=0.93$; Fig. 6B) or within any stimulation condition ($p=0.95$). No changes in TNF-α were detected due to the abundance of protein being below the lower detectable limits of the array assay (data not shown).

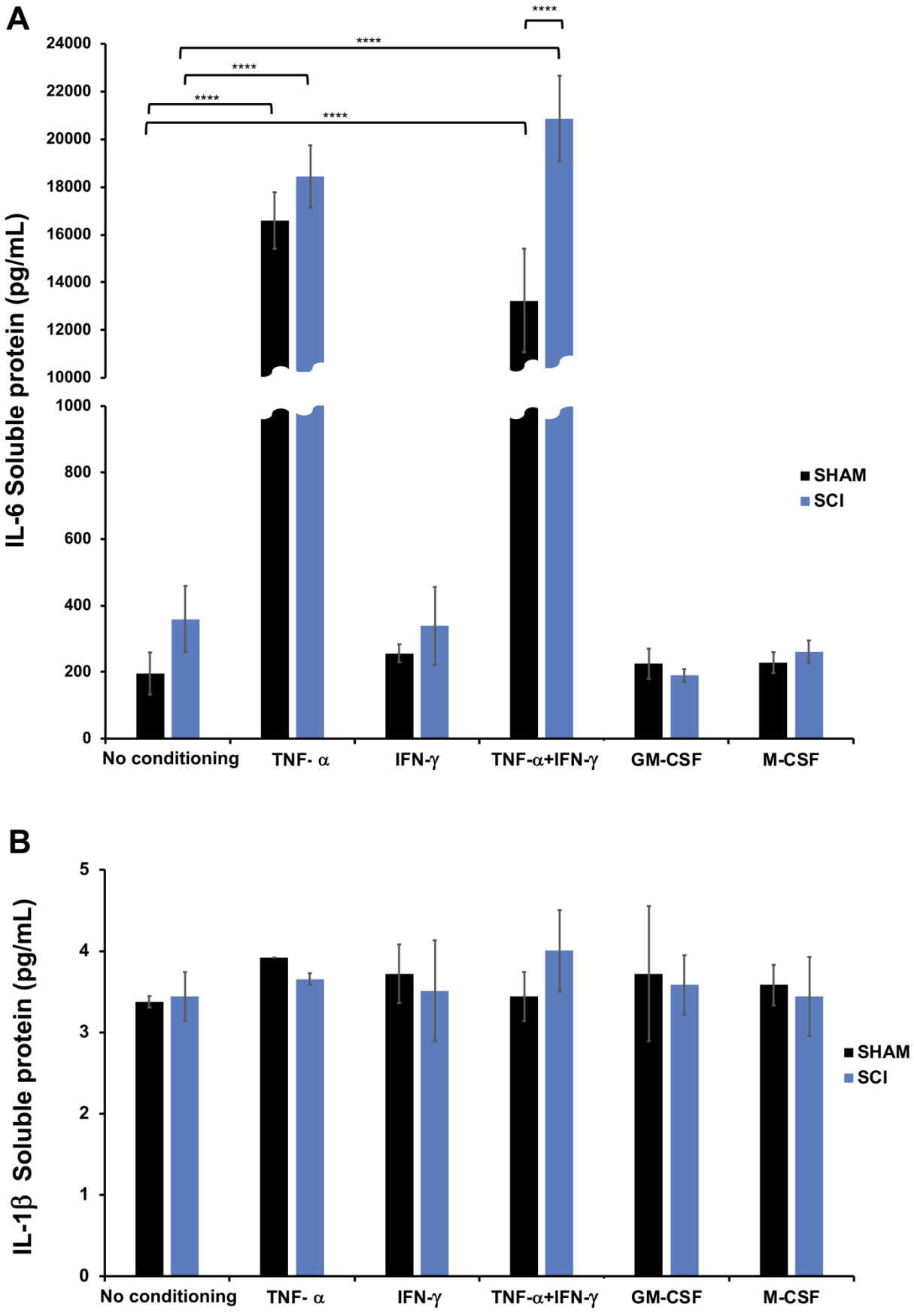
A
IL-6 Soluble protein (pg/mL)
24000
22000
20000
18000
16000
14000
12000
10000
1000
800
600
400
200
0
****
****
****
****
****
SHAM
SCI
No conditioning
TNF- α
IFN-γ
TNF-α+IFN-γ
GM-CSF
M-CSF
B
IL-1β Soluble protein (pg/mL)
5
4
3
2
1
0
SHAM
SCI
No conditioning
TNF- α
IFN-γ
TNF-α+IFN-γ
GM-CSF
M-CSF

Figure 6. *DPSC+PBMC co-culture with rat inflammatory cytokines increases the release of IL-6 protein from DPSCs.* ***A*** *IL-6 release is significantly increased following conditioning with TNF-α and TNF-α+IFN-γ. TNF-α+IFN-γ conditioning in SCI co-culture has significantly higher IL-6 release compared to Sham co-culture.* ***B*** *Changes in IL-1β release were not significant within any condition. Values expressed as mean ± SEM; ****p<0.0001. n=3.*

Discussion

DPSC engraftment has previously been investigated as a strategy to regenerate the injured spinal cord[65, 66] and address the lack of effective SCI treatment. However, low cell survival rates and viability within this hostile tissue environment limits translation from bench-to-bedside. Similarly, immune cell engraftment following SCI regulates pathological pro-inflammation following SCI,[13, 34] but is limited in its regenerative efficacy. Therefore, this study aimed to characterise how SCI changes PBMC populations in a clinically relevant contusion model of SCI, and to identify critical timepoints for PBMC extraction and utilisation in novel DPSC preconditioning co-culture models that could increase survival and viability of engrafted cells. Additionally, co-culture was conducted to further develop the limited understanding of the immunomodulatory effects of DPSCs and PBMCs *ex vivo.*

Immune cells infiltrate the spinal cord within the first week following SCI, persisting at upregulated numbers for weeks to months[75] and contribute to the exacerbation of secondary damage. FACS analysis showed acute, biphasic trends within each PBMC population following SCI, suggesting that PBMCs are recruited at these time-points, which could correspond to the biphasic opening of the blood-spinal cord-barrier at 1-2 and 6-10 dpi.[76, 77] In parallel, the increases in T-cell and monocyte proportions at 1 and 7 dpi within our study coincide with T-cell and monocyte influxes within the spinal cord at the same time-points.[26, 75, 78] Conversely, the decreased proportion of other PBMCs following SCI, such as B-cells, may be explained by immunodeficiency following SCI that limits the recruitment of immune cells to the spinal cord.[79]

Acute trends in Sham responses may be useful to decipher a functional immune response that, if mimicked, could promote healing following SCI, addressing the gap of no viable SCI treatment. In particular, because studies have shown that peripheral monocyte/macrophage activation is beneficial following SCI,[13, 33, 38] their responses within Sham peripheral blood could be pivotal. In our study, $CD43^{low}CD11b^{high}$ and $CD43^{mid}CD11b^{low}$ monocyte sub-populations varied most between Sham and

SCI groups. $CD43^{low}$ monocytes have previously been described as 'classically activated,' and are involved in pathogen clearance and more pronounced pro-inflammatory functions.[71, 80, 81] Contrastingly, $CD43^{high}$ 'non-classically activated' monocytes are smaller and less readily recruited within pro-inflammatory responses.[82, 83] $CD43^{low}CD11b^{high}$ proportions remained higher in SCI compared to Shams, particularly at 3 dpi, thus corroborating $CD43^{low}$ descriptions. $CD43^{mid}$ monocytes have been suggested to be a transitional sub-population between the two subsets in humans and mice, but have not previously been identified in rats.[83, 84] Additionally, low CD11b expression indicates an earlier state of monocyte/macrophage maturation.[81, 85] Therefore, the novel $CD43^{mid}CD11b^{low}$ sub-population may be a developing subset more amenable to modulation. However, further studies are needed to determine which level of CD43/CD11b expression is most beneficial for SCI recovery. Co-culture of monocytes with DPSCs within specific inflammatory conditions could then be a viable mechanism to achieve this expression. Additionally, further studies are needed to identify whether monocyte sub-populations exhibit anti- or pro-inflammatory phenotypic genes[20, 86] following SCI utilising real-time quantitative polymerase chain reaction, in order to support the selection of a time-point for their extraction that is most viable for SCI immune cell administration therapies.

As no statistical significance was recorded, 3 dpi was chosen as the PBMC extraction time-point for culture experiments, due to consistent variations between Sham and SCI trends at this time-point in multiple PBMC populations. Additionally, this was done to provide ample time for co-culture whilst still remaining clinically relevant, in terms of administration of PBMC+DPSC cell therapy during a sub-acute time-point, shown to be most beneficial for stem cell engraftment.[87] Within PBMC monoculture, 7 days of culture significantly increases cytotoxicity in all conditions. Interestingly, however, following 7 days of culture, only pro-inflammatory treatment with TNF-α, IFN-γ and TNF-α+IFN-γ significantly increased cytotoxicity in DPSC monoculture. This is in line with previous studies that have highlighted the detrimental effects of TNF-α and trophic effects of anti-

inflammatory cytokines on DPSC viability,[88] and suggests that GM-CSF and M-CSF conditioning may limit cytotoxicity of DPSCs beyond 3 days of culture.

DPSC+PBMC co-culture was conducted to address the limited understanding of DPSC and PBMC interactions on cytotoxicity and immunomodulation. Once again, 3 days of culture was less cytotoxic than 7, particularly following TNF-α conditioning. Compared to DPSC monoculture, cytotoxicity following TNF-α, IFN-γ and TNF-α+IFN-γ conditioning was reduced during the co-culture experiment, highlighting symbiotic immunomodulatory interactions between PBMCs and DPSCs reported previously.[51, 54] This provides evidence that preconditioning of DPSCs and SCI PBMCs through co-culture may be beneficial before engraftment into the injured spinal cord. Similarly, 3 day culture is most effective at maintaining cell viability, answering one of the main gaps addressed in this study. However, further studies are necessary to confirm the exact mechanisms by which cytotoxicity is reduced following co-culture.

The cytokine assay showed, for the first time, that within TNF-α and TNF-α+IFN-γ pro-inflammatory conditions, DPSCs significantly upregulate their secretion of IL-6, particularly following co-culture with SCI PBMCs. IL-6 protects DPSCs and MSCs from apoptosis, aiding wound healing *in vitro* following inflammation[89] and increasing proliferation.[90] Importantly, IL-6 is released during DPSC and MSC immunoregulatory functions,[91] and works to inhibit the synthesis of pro-inflammatory factors such as IL-1β and TNF-α.[92] Additionally, DPSC IL-6 secretion aids in anti-inflammatory macrophage polarisation.[93] These findings support the premise that co-culture of SCI PBMCs with DPSCs under pro-inflammatory conditions induces anti-inflammatory functional behaviour of DPSCs, which may modulate PBMCs to acquire anti-inflammatory phenotypes, thus answering a key gap in knowledge. However, conflicting studies suggest that IL-6 release is detrimental to recovery following SCI, due to its involvement in exacerbating pro-inflammation,[94] recruiting inflammatory cells,[95, 96] and neuropathic pain.[97] Therefore, further *in vivo* studies are

needed to identify whether engraftment of DPSCs secreting high levels of IL-6 into a SCI model worsens recovery, or whether the immunomodulatory properties of DPSCs inhibit the negative effects of IL-6. In parallel, investigation should be conducted into whether particular time-points following SCI, during which TNF-α levels are highest, should be avoided for DPSC engraftment.

This study has characterised changes in PBMC populations following SCI, and has shown that PBMCs extracted 3 dpi are suitable to achieve co-culture of PBMCs and DPSCs that has low cytotoxicity when cultured for 3 days, thus supporting our main hypothesis. Furthermore, we have shown that co-culture within pro-inflammatory conditions may be beneficial to induce anti-inflammatory functions of DPSCs. Future *in vivo* studies are necessary to elucidate the effects of this preconditioning on DPSC and PBMC survival and viability following engraftment into a SCI model, which may lead to the development of a novel neuroregenerative and immunomodulatory SCI treatment.

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