Title: Ex-Vivo Flush Of The Limb Allograft Reduces Inflammatory Burden Prior To Transplantation

Article Type: Article

Keywords: Hand Transplant; Leukocytes; Acute Rejection; Preservation

Abstract: Background
Passenger leukocytes and inflammatory debris transferred from the donor limb to the recipient can induce allorecognition, which activates the host immune response. This is the first study to evaluate whether the transfer of this inflammatory burden can be reduced via post-preservation flush prior to revascularisation, and whether this is influenced by ischaemia.

Methods
Bilateral forelimbs from the same pig were procured and infused with preservation flush and stored on ice. Each limb from the same pig underwent a post-preservation intravascular flush with isotonic solution at either 2 or 6hrs. Venous effluent underwent flow cytometry to phenotype leukocyte populations, with additional quantification of cytokines and cell-free DNA.

Results
We identified large populations of viable leukocytes in the flush effluent (8.65x10^8 ±3.10x10^8 cells at 2hrs and 1.02x10^9 ±2.63x10^8 at 6hrs). This comprised T cells, B cells, NK cells and monocytes. Post-preservation flush yielded significant concentrations of pro-inflammatory cytokines including IL-6, IL-18, GM-CSF, IL-1β, IL1α and CXCL-8 and mitochondrial DNA. The regulatory cytokine, IL-10 was undetectable.

Conclusions
This study supports the finding that a post-preservation flush removes leukocytes and inflammatory components that are responsible for direct presentation. This study also gives an indication of how ischaemia impacts on the inflammatory burden transferred to the recipient upon reperfusion.
Although minor points, I think the following small amendments should be made:

The title suggests that an allotransplant has been conducted when in fact it has not. I would change the emphasis of the title slightly perhaps to 'Ex-Vivo Flush Of a Limb reduces Inflammatory Burden in A porcine limb vascularised allotransplant model'.

We think the recommended title is more suggestive that a transplant has been performed. We have removed the word ‘vascularised’, and we were eager to keep the statement ‘prior to transplantation’ in the title as we think this clarifies that a transplant has not been performed.

In several places in the text, the word graft, transplant, or allograft follow the acronym VCA. As the ‘A’ in VCA stands for allotransplant, these additional nouns are not required and should be deleted.

We have now corrected this throughout the manuscript.

I would replace the word graft in the text with transplant as the term graft, as used by plastic surgeons has a specific meaning which is not consistent with the meaning used by transplant surgeons.

We agree with this and have amended the text throughout or removed the word graft and used allograft when referring to transplantation.

Reviewer 4:

1. While they have quantified the leukocyte, cytokine and free-DNA content of effluent at two post-procurement time points, they do not report on the content of effluent time zero immediately following procurement and perfusion with preservation solution. Considering the lack of statistically significant differences between 2 and 6 hours of ischaemia in many of their measures, inclusion of zero time data would aid interpretation.

We chose not to collect effluent samples at retrieval during flush with UW to avoid blood contamination. We did consider performing an additional flush immediately after UW preservation flush as a baseline but it was felt this did not represent the majority of current limb transplant procedures and would obscure flushes at later times points. For this to apply revascularisation would need to occur almost immediately. Therefore timings were selected based upon acceptable cold static storage times.

Furthermore, in this entirely ex-vivo study they make no assessment of the impact of this "inflammatory burden" following revascularization: this would clearly be a much more complex study, with it's attendance regulatory and animal welfare issues, but I nonetheless find reference to revascularization in the first paragraph of the summary misleading I would suggest this passage be edited.
We have replaced ‘revascularisation’ in the summary with ‘transplantation’ since it is correct that no transplant has taken place.

Methods: "Six porcine bilateral forelimbs" is a grammatically awkward construct, but this is a minor point.

We have amended this to read as: Bilateral forelimbs from the same pig were procured.

Results: "This comprised alloreactive T cells, B cells, NK cells and monocytes with populations increasing with prolonged cold storage". The authors present no data demonstrating alloreactivity. Furthermore, while the data presented suggest an increase in most populations between the 2 hour and 6 hour time points, statistical significance is the exception rather than the rule and I would hesitate to state this so confidently.

We have removed the word alloreactivity and stated which cell populations were eluted to act from a more observational standpoint.

Conclusions: I would agree with the authors conclusion that this study supports the hypothesis that a post-preservation flush removes donor leukocytes from the graft. However, I find that the data provide only minimal indication of the impact of ischemic time on the composition of flush effluent, or for that mater how this translates to the "inflammatory burden" experienced by a recipient.

We now state that we have identified a residual immune population that can be removed via flush and that more prolonged ischaemia may enable us to more confidently see the true impact of cold storage on leukocytes, cytokine concentrations and cell free DNA.

Introduction:

Page 4 Line 3: "Reference to the "donor hospital" and "recipient hospital" seems unnecessary and distracting - transplants may occur within a single institution.

Reference to the donor and recipient hospital has been removed.

Materials / Methods:

I commend the authors choice of porcine tissue for this study, as a well established and useful model of human limb transplantation. While I am mindful of the cost and effort involved in such studies, I can't help but wonder if larger sample sizes might have been achievable in the context of an ex-vivo
study such as this, which may have helped with significance of some of the data.

Our primary objective was to demonstrate which populations of cells would be transferred within a time frame relevant to clinical practice. We therefore believe that the sample size is appropriate to generate this observational data, though no power calculation was performed.

Page 6 Line 3: Sample collection and preparation: Please check figures in this paragraph - "1000ul was samples from the total volume….1000ul aliquots of supernatant were cryopreserved". It seems unlikely that the entire sample volume was supernatant as this line suggests. It would be useful to include mention of sample duplication (if any) in this section - were individual aliquots taken at each time point, or were samples collected in duplicate/triplicate etc?

This was an error to suggest the whole sample was supernatant. We have amended this in the text.

Page 6 Line 11: Flow Cytometry: Identification of the specific antibodies used (by clone number and source) would be helpful.

This has been added to supplementary data in addition to manufacturer and isotype.

Page 6 Line 22: The author's nomenclature regarding T-regulatory cells is somewhat unconventional - I would suggest "T-regulatory cells (FoxP3)+ CD4+ (Treg)" might be edited to reflect the fact that the use of phenotypic markers alone is widely considered insufficient to define regulatory cell populations. Perhaps something to the effect of "cells of the potentially regulatory phenotype CD4+FoxP3+ were identified by intracellular expression of FoxP3" would be more appropriate.

We acknowledge this and the text has been amended accordingly.

To this end, it would be helpful if the authors could include examples of their gating schema within the figures, or as supplementary material, as expression of FoxP3 alone as a marker of Treg status has the potential to overestimate this population, as transient expression of FoxP3 will also be detected during T cell activation. Based on the inclusion of anti-CD25PE in the panel, can I assume that the CD25hi population was selected? I would direct the authors to Abbas et al, Nature Immunology (2013) 14;307-308, and related publications by Abbas, Sakaguchi and colleagues for further recommendations should they be required.

Our gating strategy has been included in the supplementary data. We based our gating on the CD45+ population and identified CD3+ cells and those identified as CD4+ were then sub-populated using FOXP3+ and CD25+. We believed this was the most conservative method to identify Tregs within the effluent. We have amended our gating to select CD25 bright FOXP3+ cells. As a result the number of Tregs within the flush has reduced using the
reviewer’s recommendations. We have amended the table and graphs accordingly.

Page 7 Line 3: The description of cytokine analysis methods is rather brief, and I find insufficient to thoroughly interpret the results presented. Further details, including control samples used, would be of benefit.

More information for this has been provided. The controls were provided by the manufacturer to enable serial dilutions for the standard curve. We have now removed reference to controls from the manuscript.

Results:
Page 8 Line 10: "This made limbs from the same pig comparable for ischemic interval and volume of effluent." This statement suggests direct comparison was made between each limb from the same donor, while the methods section indicates that limbs were randomized rather than subjected to paired analysis - in which case comparability across the whole sample set, not individual pairs, would be of greater importance.

The words from ‘the same pig’ have been removed as the main comparison is between the two randomised time points.

Page 8 Line 17: "There were differences in the leukocyte repertoire between groups". The authors follow this statement with a series of results for which p values are not stated, but which appear to fail to reach significance. In the absence of demonstrable statistical significance I would encourage a more cautious description of these results. If the intention is to highlight differences in relative population size, rather than differences between experimental groups, this should be clarified, and discussion included of how their data compare to, for instance, these populations in blood or tissue.

We have attempted to clarify this throughout the manuscript. A table of all p-values and cell numbers has been included within the main manuscript. Within the supplementary data relative cell percentage populations in healthy pigs is provided. We wanted to avoid comparing flush effluent with blood so that the reader has no confusion in interpretation since all blood should have been removed during preservation flush, but we have included some reference to this in the discussion by including the reference range of leukocytes found in whole blood and how our data compares to this.

Page 8 Line 22: "Treg (n=2) formed a small subpopulation...". The inclusion of n=2 would benefit from further explanation in the methods section. Was Treg analysis performed on samples from two limbs only? Again the data comparing 2 and 6 hour time points do not appear to reach statistical significance. Inclusion of p values would be of value.

The decision to perform Treg came after we initially saw which populations were being eluted and recognised the significance of flushing out Tregs. Therefore, the data for Treg came from two experiments and have written (4
Page 9 Line 4: Are these data for classical or non-classical monocytes? Figure C looks like non-classical monocytes may be the major population, which is at odds with text.

This has now ben corrected as non-classical monocytes formed the major population at 6 hours. This is in keeping with other published work from our lab when cells are eluted during lung perfusion.

Page 9 Line 9: Cytokine Profile: I think this section would benefit from more graphical representation of the data, including control samples (at least in the form of supplementary data) to aid interpretation.

Graphical representation has now been added between 2hr and 6hr time points. We used a number of standards as per the manufacturers protocols using serial dilution for standards as per the plate designed for porcine cytokines Merckmillepore (Cat. PCYTMG-23K-13PX). Our initial reference in the text to controls was for serial dilutions for the standard curve and therefore the word control has been removed.

Discussion:
Page 9 Line 22: The authors state that their study attempts to characterize the residual immune compartment of the limb, and that they have demonstrated removal of large numbers of viable marginal donor leukocytes from the vasculature. While I would agree that they have demonstrated the removal of large numbers of leukocytes in effluent flush fluid, they have not presented any data directly demonstrating the source of these cells, or indeed quantifying tissue-resident leukocytes before and after flushing. While the numbers of cells presented seem large, without a suitable comparator the neither the biological significance cannot easily be interpreted.

We do not describe the source of these cells. In addition we have minimised our reference in the text to suggest the source or fate of these cells and attempted to stay observational. Reference has been made to whole blood and our reasons to avoid baseline samples have been mentioned previously.

Page 10 Para 2: Have the authors considered making comparison to porcine blood? Is there any data to suggest differential release of certain populations into effluent?

Aside from the current study we have performed a series of continuous ex-vivo limb perfusions, and routinely analysed cellular diapedesis up to 12 hours. In these studies the cellular repertoire significantly changes over time. This is true of other tissue perfusions we currently perform including lung, heart and kidney. As such we are confident that the flush content is from the limb and is not residual blood. Furthermore, given that we are simply replicating the limb flush protocol used in clinical practice, we provide observational data describing the immune compartment that is transferred into
the recipient, based on current practice. Therefore attempting to describe the immune content of pig blood would be confusing and unnecessary.

Similarly, analysis of tissue biopsies for tissue resident cell populations would be of value in determining the degree to which the "inflammatory burden" of the limb is reduced by flushing.

We agree with the reviewer’s comment. However multiple biopsies would be needed from vessel, muscle, skin, and at multiple sites across the graft. The interpretation of this data would be very difficult, and still remain observational. Our view is that a randomised transplant model is needed to truly evaluate the impact of an additional flush on alloreactivity and recipient T cell infiltration. As such we believe this is outside of the remit of the current study.

As a general point, given the lack of statistical significance demonstrated for many of the parameters investigated, some discussion of this, and potential methodological limitations of this study would be of benefit.

We have now included the statements that there is an increasing trend with immune cells and cytokines with ischaemia and though not significant warrants further consideration.

Other limitations include lack of transplant model and unknown significance of the immune cells eluted and also the true quantifiable immune content within all tissues.

Many of the parameters measured do not differ significantly between the two time points measured, and in the absence of a zero time point, and/or a more extended series of time points, trends cannot reliably be identified. Related to this, was the study adequately powered? Similarly, I find the data presentation quite reductionist in style, and feel that inclusion of representative flow cytometry plots would aid confident interpretation of these data.

No power calculation was performed as this is the first study of its kind, and although we compare 2 with 6 hours, it is ultimately descriptive. We arbitrarily selected 2 hours and 6 hours as these are within the realms of clinical practice.

Including flow histograms is a difficult choice as some reviewers/journals consider them unnecessary. Our view is that they are of use so we are pleased with this comment. We have now incorporated flow cytometry data as part of the supplementary data.
Ex-Vivo Flush Of The Vascularised Limb Allograft Reduces Inflammatory Burden Prior To Transplantation

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Summary

Background Passenger leukocytes and inflammatory debris transferred from the donor limb to the recipient can induce allore cognition, which activates the host immune response. This is the first study to evaluate whether the transfer of this inflammatory burden can be reduced via post-preservation flush prior to revascularisation, and whether this is influenced by ischaemia.

Methods Six porcine bilateral forelimbs from the same pig were procured and were infused with preservation flush and stored on ice. Each limb from the same pig underwent a post-preservation intravascular flush with isotonic solution at either 2 or 6 hrs. Venous effluent underwent flow cytometry to phenotype leukocyte populations, with additional quantification of cytokines and cell-free DNA.

Results We identified large populations of viable leukocytes in the flush effluent (8.65x10^8 ± 3.10x10^8 cells at 2hrs and 1.02x10^9 ± 2.63x10^8 at 6hrs). This comprised alloreactive T cells, B cells, NK cells and monocytes, with populations increasing with prolonged cold storage. Post-preservation flush yielded significant concentrations of pro-inflammatory cytokines including IL-6, IL-18, GM-CSF, IL-1β, IL1α and CXCL-8 and mitochondrial DNA. The regulatory cytokine, IL-10 was undetectable.

Conclusions This study supports the finding that a post-preservation flush removes donor leukocytes and inflammatory milieu components from the graft that are responsible for direct presentation. More prolonged ischaemia would be beneficial to identify the true impact of cold storage on inflammatory burden—This study also gives an indication of how ischaemia impacts on the inflammatory burden transferred to the recipient upon reperfusion.
Keywords

Hand Transplant; Leukocytes; Acute Rejection; Preservation
Introduction

Vascularised composite allotransplantation (VCA) offers the optimal treatment to replace defective or amputated limbs to replicate the look, function and feel of a normal hand. Over one hundred VCA procedures have been performed for the upper extremity, but the immunological constraints, namely that of acute and chronic rejection remain. A single acute rejection (AR) episode is estimated to occur in 85% of individuals within the first year, with 56% developing multiple episodes. The prevalence of VCA rejection is higher than any other solid organ transplant, and our understanding of the mechanisms involved are limited.

A potential contributing factor to AR is the transfer of donor derived passenger leukocytes from the alloVCA graft to the recipient following revascularisation. Donor leukocytes with antigen presenting capacity (APC) can migrate from donor tissues and home to recipient peripheral lymph nodes to self-present alloantigens to recipient T cells, a process referred to as direct presentation. This is a well-recognised initiator of allorecognition, the permanent recognition of donor antigens by the recipient immune system. Evidence from solid organ research comes from a lung transplant model whereby the mechanical removal of donor leukocytes via machine perfusion reduces T cell priming and therefore cell infiltration into the donor organ after transplantation. As such, donor leukocytes represent a major immunogenic factor in solid organ transplantation, yet minimal simple therapeutic interventions have been discussed in an attempt to minimise the transit of donor leukocytes upon reperfusion. Hypothetically, reducing the limb donor leukocyte population prior to transplantation may confer similar benefit.
The current clinical protocol involves flushing the VCA graft with preservation solution to remove erythrocytes and protect against ischaemia induced cellular damage prior to transplantation at the donor hospital. The graft is then stored on ice (cold static storage, CSS) in preparation for transplantation at the recipient hospital. The exact volume of preservation flush has varied more commonly from 500ml to unspecified with suggestions to continue until the venous effluent is clear. Not all procurement protocols mention a post-preservation flush, and those that do recommend it to washout clots and preservation solution. The actual resident immune compartment for the limb has never been quantified. This is complex taking into account graft variation in size and immunogenicity in response to tissue injury. For this purpose, the primary aim of this study was to examine whether post-preservation ex-vivo flush of the VCA-allograft is sufficient to remove donor leukocyte populations that contribute to allore cognition. Furthermore, ischaemia during CSS results in anaerobic cell metabolism and strongly correlates with the incidence of AR in solid organs. Thus, the secondary aim was to determine whether the length of CSS influences donor leukocyte diapedesis from the tissues into the circulation.
Materials and Methods

Animals and limb procurement: Pigs were chosen for their clinical similarities to humans owing to comparable muscle mass, vessel diameter, nerve calibre and ratio between limb surface and tissue volume. In addition to similar blood physiology, pig skin also shares similar blood perfusion patterns and immunological properties. Six landrace pigs with a mean weight of 80kg were sacrificed from a local abattoir after veterinary inspection with procurement of bilateral forelimbs. Pigs were sacrificed under Schedule 1 of the Home Office Scientific Act 1986 and in accordance with the EU Council Regulation (EC) 1099/2009 on the protection of animals at the time of killing. Briefly, abattoir animals were stunned via electrocution and exsanguinated by division of the carotid artery. An incision was made at the shoulder joint down to joint capsule. Care was taken to preserve the axillary neurovascular plexus. Both forelimbs from the same animal were disarticulated at the glenohumeral joint (shoulder) and were examined to ensure there was no evidence of injury or disease. After procurement, limbs were then cooled on ice during dissection.

The dominant brachial artery was cannulated and each limb was flushed simultaneously with 500ml of cold University of Wisconsin (UW) preservation solution, the most widely adopted preservation solution in limb transplantation (4°C, standard pH 7.45) mixed with 10,000iU of heparin via intravenous giving set at a height of 120cm hydrostatic pressure (equating to 88mmHg) to mirror descriptions from clinical transplant programmes. A pressure bag was avoided to prevent endothelial shearing. The extremity was wrapped in soaked gauze and placed in a bag, which itself was submerged in iced water for transportation. Direct contact with ice was avoided to minimise cellular damage secondary to ice crystal formation. The
start of cold flush marked cold ischaemia and a second intravascular flush (post-preservation) was performed on each limb from the same animal at 2 and 6hrs from this time point.

**Sample collection and preparation:** Each limb was randomised (Randomizer.org) and assigned to either a 2 or 6hr intravascular flush with two litres isotonic, physiologically normal Ringers solution. Venous effluent was collected in a plastic receptacle and the total volume recorded. *We sampled 1000µl was sampled* from the total volume after thorough mixing. All samples were centrifuged at 2000g for 4 minutes at 4°C and *1000µl aliquots of plasma (supernatant) were cryopreserved* at -80°C for cytokine profiling and later assessment of mitochondrial and genomic DNA. The cell pellet underwent leukocyte analysis via flow cytometry.

**Flow Cytometry:** Using a BD LSR II flow cytometer (Becton Dickinson, UK), CD45+ cells were identified as of leukocyte origin. Zombie viability dye was used to quantify which cells were viable (Biolegend, UK). A panel of antibodies was designed to cover a broad range of key leukocytes involved in innate and adaptive immunity including T cells (CD4+, CD8+, CD4+/CD8+), B cells (CD3-CD21+), immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+), mature eosinophils/basophils (6D10-2B2+), and natural killer cells (CD35+). Monocytes were also included in the panel to include classical (CD14+CD163-) and non-classical (CD14/CD163+) phenotypes. Cells were resuspended with PBS and divided into tubes for erythrocyte lysing (BD Biosciences, UK), washed twice and resuspended in 300µl staining buffer. T regulatory cells (FoxP3)+ CD4+ (Treg) Cells of the potentially regulatory phenotype CD4+FoxP3+ were determined via intracellular expression of FoxP3.
Leukocytes were stained with anti-CD4<sup>FITC</sup>, anti-CD25<sup>PE</sup> and anti-CD8-α-Texas Red. Leukocytes were then fixed and permeabilised before staining with anti-FoxP3<sup>APC</sup>-with a series of perm/wash buffer steps. To enable quantification of all leukocyte populations from flow cytometry events, 20µl e123count beads (eBioscience, CA, USA) were added to all samples.

**Cytokine Analysis**: Cytokine and chemokine expression was quantified using a porcine species reactive panel consisting of a magnetic bead-based multiplex assay (Merck Millipore, Billerica, MA, USA). Calibration standards were generated via dilution steps as per the manufacturers protocol. Plasma was thawed and a 96-well plate prepared using a 200µl assay buffer. 25µl of standards and controls were added to appropriate wells, together with 25µl of matrix solution. To sample wells, 25µl of assay buffer was added with 25µl of plasma sample. Pre-mixed antibody (25µl) solution was vortexed to ensure adequate separation of beads and added to each well. The plate was sealed and incubated for agitation overnight at 4°C. After 3 washes 50µl of detection antibody was added. The plate was further incubated for a further 2 hours. Streptavidin-Phycerythrin was added to each sample well and after incubation, 3 washes were performed. The sample was added to a biotinylated antibody and incubated with a reporter molecule followed by wash steps. Data was analysed using the Bio-Plex 200 system (Bio Rad, Hertfordshire, UK) and Bio-Plex Manager software.

**DNA Extraction and Quantitative PCR (qPCR)**: DNA purification using the QIAamp DNA Mini and Blood Mini kit (Qiagen, Germany) isolated flush DNA as per the manufacturers protocol. Qualitative PCR was carried out to quantify extracellular genomic (gDNA) and mitochondrial DNA (mtDNA). Suitable primers (Sigma Aldrich, Dorset, UK) specific to the PCR template using the Primer Express® Software v3.0.1
(Life Technologies, Paisley, UK) were selected against the nucleotide sequence database BLAST. To identify extracellular genomic DNA, primers to the housekeeping gene glyceraldehyde-3-phosphate (GAPDH), and primers specific for cytochrome B were used to quantify extracellular mitochondrial DNA. A MicroAmp Optical 384-Well Reaction Plate was used and qPCR was performed using a QuantStudio™ 12K Flex system (Life Technologies, Paisley, UK) with Power SYBR green PCR master mix (Life Technologies, Paisley, UK).

**Statistical analysis:** All statistical analysis was carried out using GraphPad Prism v.7.0. Data normality was assessed using the Shapiro-wilk test and an independent sample t-test was used to compare differences in leukocytes. Data is presented as the mean ± standard error of mean (SEM) and considered significantly different if a $p$-value of ≤0.05 was observed and presented using GraphPad Prism v.7.0. All gating strategies and analysis for flow cytometry was performed using FlowJo version 10.0.6 (TreeStar Inc).
Results

Procurement: Time to procurement (defined as time of death after exsanguination and complete limb detachment) did not differ between limbs flushed at 2hrs (9.2±1.2mins) versus 6hrs (8.5±1.5mins) (p=0.74). Warm ischaemia time until cold preservation was 17.8±1.4mins. There were no differences in volume of effluent between limbs flushed at 2hrs (1,753±32ml) versus 6hrs (1,655±59ml) (p=0.18). This made limbs from the same pig comparable for ischaemic interval and volume of effluent for both ischaemic time points.

Leukocyte extravasation: (n=6, Table 1) The numbers of CD45+ leukocytes between both groups was did not differ significantly different (8.65x10⁸ ±3.1x10⁸ cells per limb for 2hrs and 1.02x10⁹ ±2.63x10⁸ cells per limb for 6hr, p=0.70). Cold ischaemic time did not impact upon the proportion of non-viable leukocytes with (6.32x10⁸ ±4.23x10⁹) at of leukocytes non-viable at 2hrs compared with (6.31x10⁸ ±3.54x10⁸) at 6hrs (Figure. A). There were differences in the leukocyte repertoire between groups. With increasing cold storage there was an increase in the number of viable T cells in the flush effluent (6.94x10⁸ ±1.51x10⁸ 2hrs vs 9.64x10⁸ ±2.37x10⁸ 6hr, p=0.36). There was greatest an increase was seen in CD8+ T cells (3.37x10⁸ ±7.06x10⁷ 2hrs vs 4.44x10⁸ ±1.02x10⁸ 6hr, p=0.40) and CD4+ T cells (4.52x10⁷ ±1.27x10⁷ 2hrs vs 7.99x10⁷ ±2.90x10⁷ 6hr, p=0.31), though neither increased significantly. Treg (n=2, Table 1) formed a small subpopulation of the T cell
compartment at 2hrs (0.9% of T cells, 0.18% of leukocytes) and numbers decreased with prolonged cold storage (1.306.13 ± 8.29x10^6 5.27x10^6 - 2hrs vs 7.58x10^6 ± 6.16x10^6 1.16x10^6 - 6hrs, p=0.61) (Figure. B). Small populations of B cells were found (3.73x10^7 ± 1.36x10^7 2hrs vs 4.07x10^7 ± 1.11x10^7 6hr, p=0.85).

There was an increase in numbers of NK cells (1.60x10^8 ± 5.58x10^7 2hrs vs 2.90x10^8 ± 1.15x10^8 6hr, p=0.34). Non-classical monocytes comprised the major monocyte population at 6hrs and significantly increased with the prolonged length of cold storage (1.182±10^8 ± 7.742.94x10^7 2hrs vs 3.242.43x10^8 ± 2.294.94x10^8 6hr, p=0.4105) (Figure. C). Populations of viable granulocytes including neutrophils, basophils and eosinophils were found in all samples, although there were no differences with prolonged CSS (Figure. D).

**Cytokine Profile:** Almost all cytokine and chemokine concentrations showed no significant differences with length of CSS (cold storage). The limb effluent allograft secreted significant volumes of pro-inflammatory cytokines (Table. 24). IFN-γ, IL-1RA, IL-2, IL-4, IL-12 and TNF-α were minimal or undetectable (Figures. E, F). The regulatory cytokine, IL-10 was undetectable in all limb effluents. However, there was a significant decrease in CXCL-8 with prolonged cold storage at 6hrs (p=0.01).

**Mitochondrial & Genomic DNA:** Comparisons were made for free circulating gDNA and mtDNA. Mitochondrial DNA concentrations at 2hrs (38,662ng ±14,226ng) and 6hrs (31,046ng ±7,809ng) were not significant (Figure. G). Genomic DNA was not detected at 2 and 6hrs following 40 cycles using a QuantStudio™ 12K Flex system.
Discussion

Limb transplantation programmes are increasing in number worldwide, with tissue rejection the main complicating factor influencing VCA graft outcomes. This is the first study that attempts to characterise whether ex-vivo post-preservation flush removes any of the residual immune compartment of the limb prior to implantation transplantation in a large animal model. We have shown that performing a secondary post-preservation isotonic flush removes large numbers of viable marginal donor leukocytes from the vasculature prior to implantation. In the transplant setting this would equate to the rapid transfer of donor cells entering the recipient circulation following reperfusion. Given that a healthy limb is perfused with approximately 300ml of blood per minute (based on 70ml blood per kg in pigs), a flush volume of 2000ml represents less than 7 minutes following revascularisation. Using this framework it would take less than 7 minutes for over 1 billion viable donor leukocytes to enter the recipient circulation following transplantation after 6hrs of cold storage. The only published study quantifying donor leukocyte transfer-populations with using a similar methodology is from the studies in the kidney in solid organ research. In this study, 2L of isotonic solution after 2 hours cold ischaemia eluted almost half the number of donor leukocytes than this study. However, the volume
of post-preservation flush was double that of the volume used in this study. Given that a healthy pigs blood, compared with a healthy pigs peripheral blood, we had leukocytes range between $10\text{-}20 \times 10^9/L$ \textsuperscript{21}, at 6hrs we eluted approximately $9.5 \times 10^8/L$ viable and non-viable leukocytes. This remains a large proportion of cellleukocytes and warrants further review into selected volumes for preservation flush in the clinical setting.

\textsuperscript{21} an almost 100 fold increase in the number of leukocytes within the flush effluent\textsuperscript{20}.

The primary cell population found within the effluent were FoxP3- CD8+ and CD4+ T cells. Donor T cells have the capacity to drive inflammation, secrete pro-inflammatory cytokines and orchestrate alloantigen specific immune responses \textsuperscript{22}. Reassuringly, there were limited populations of Tregs within the effluent and IL-10 was undetectable. Treg and IL-10 have well reported roles in driving transplant tolerance, so hypothetically their removal would not be considered beneficial. It is worth noting that overestimation of this population occurs when immunophenotyping with flow cytometry, which would indicate that less cellleukocytes with a Treg phenotype that may confer suppressive activity are being eluted (<3% of the T cell content) and IL-10 was undetectable. Tregs and IL-10 have well reported roles in driving transplant tolerance, so hypothetically their removal would not be considered beneficial. In addition to donor T cells, NK cells were present in the flush effluent that are central in the recruitment of cytotoxic T cells during inflammation and interact with nucleated cells via self-MHC detection. This facilitates recipient antigen presenting cells to process shed donor antigen, which can amplify the immune response \textsuperscript{23}. However, the role of the donor NK cell is unknown \textsuperscript{24}. On the contrary, recipient NK cells have been implicated in allograft tolerance by killing donor APC \textsuperscript{25}. We also identified large populations of donor monocytes that have well reported
roles following transplantation. Donor monocytes migrate to recipient lymph nodes where they present antigen to recipient T cells. A significant proportion of monocytes differentiate into dendritic cells and continue to orchestrate T cell priming and clonal expansion.

Ischaemia caused by cessation of blood flow through the allograft augments an injurious response upon reperfusion. This drives recipient immune activation and correlates with primary graft dysfunction and failure. Human hand transplant cold ischaemia has ranged from 30 minutes to 13hrs (mean 5.3hrs). Cold ischaemia worldwide averages 6 hours and is the reason this study quantified flush effluent at this time point. We have demonstrated the detrimental impact of ischaemia, albeit within a narrow time interval, which demonstrates that the number of donor leukocytes increases with prolonged CSS. Interestingly, the proportion of viable leukocytes remained similar despite more dead cells with longer CSS. This in turn may increase alloantigen recognition and allograft immunogenicity. The evidence for allograft dysfunction and rejection secondary to ischaemia is not in the remit of this study and requires further investigation. However, increasing concentrations of extracellular mitochondrial DNA, released following donor leukocyte death supports this. Aside from representing a biomarker of cell death, extracellular mitochondrial DNA is a damage associated molecular pattern (DAMP), which activates the recipient immune system via toll like receptor (TLR) binding and can contribute to allograft immunogenicity. Furthermore, mitochondrial DNA is also a potent inflammasome activator that can amplify downstream immune responses initiating the release of IL-1β and IL-18.

Though we are unable to comment on the source of cytokines and chemokines, the flush effluent contained a significant pro-inflammatory milieu of other growth factors,
cytokines and chemokines including GM-CSF, IL1α, IL-6, and CXCL-8 with no detectable ‘anti-inflammatory’ or regulatory cytokines. The constituent components of this pro-inflammatory effluent are required for leukocyte survival, activation and proliferation, allore cognition, cell migration and cytotoxicity. GM-CSF inhibits leukocyte apoptosis and promotes maturation and proliferation of a range of leukocytes. It is used clinically as a salvage therapy in patients who are severely immunocompromised to drive leukocyte proliferation. Its release is stimulated by IL-1β during inflammation, which itself is released by the vascular endothelium, leukocytes or platelets after surgical trauma, signifying a pro-inflammatory environment. In addition, pro-inflammatory IL-6 was found at 2 and 6hrs cold ischaemia. This cytokine is secreted following TLR signalling, potentially in response to the extracellular mitochondrial DNA found in the effluent. The role of IL-6 following transplantation is complex, but in the immediate period following revascularisation it is likely to be involved in T cell activation and suppression of Treg pathways. Though we do not know the fate of these donor leukocytes, evidence from solid organ transplantation suggests that they may contribute to allore cognition and alloreactivity.

Our data demonstrate little statistical significance between both 2 and 6hrs cold ischaemia. However, across almost all cell types and cytokines there is an increasing trend which is likely to continue with ischaemia, though this warrants further investigation. The actual resident immune compartment for the limb has never been quantified and therefore the true impact of flushing on immune depletion...
remains unknown. This is complex taking into account variation in allograft size and immunogenicity in response to tissue injury. A major limitation of this study is the lack of a transplant model used and thus the fate of these immune cells leukocytes remains unknown. We propose future studies to evaluate whether post-preservation ex vivo flush of donor leukocytes, DAMPs and cytokines immediately prior to revascularisation impacts on transplant outcomes in a large-animal transplant model, and whether this is flush volume dependent. It would also be beneficial to characterise the true impact of ischaemia on immune activation. Also, although not a limitation, it should be noted that this study was performed using a circulatory arrest model, and differences between this and donor brain death may occur. To our knowledge limb transplantation has not been performed outside of a brain-dead donor setting.

Conclusion

Collectively, this data identifies a residual immune population within the donor VCA allograft, which using contemporary transplant protocols is could be transferred into to the the recipient. Performing a post-preservation flush can remove this donor immune burden that appears to manifest with prolonged CSS.

‘Conflict of interest statement’: There are no financial and personal relationships to disclose to influence or bias this work.

References

**Figure Legends**

**Figure. A** Total viable and non-viable CD45+ve leukocytes in flush effluent at 2 and 6hrs cold ischaemia. *Viable leukocytes increase with ischaemia.* (Plot of mean with SEM).

**Figure. B** Total viable T cells in flush effluent at 2 and 6 hrs cold ischaemia. Increase in CD4+ T cells and CD8+ T cells was seen. *Regulatory T-regulatory cells (FoxP3)+CD4+* comprise a minority of the T cell population (Plot of mean with SEM).

**Figure. C** Total viable classical and non-classical monocytes, NK, γδ T cell and B cells in flush effluent at 2 and 6 hrs cold ischaemia. *An increase in classical monocytes and NK cells was seen with prolonged CSS* (Plot of mean with SEM).
**Figure. D** Total viable (granulocytes) mature and immature neutrophils, eosinophils and basophils in flush effluent at 2 and 6 hrs cold ischaemia. (Plot of mean with SEM).

**Figure. E** Total cytokine concentrations at 2 and 6 hrs cold ischaemia detecting IL-6, GM-CSF and CXCL-8 (Plot of mean with SEM).

**Figure. F** Total cytokine concentrations 2 and 6 hrs cold ischaemia of IL1α, IL-1β, IL-18. Decreasing concentrations were seen in all cytokines with prolonged ischaemia other than IL-18 (Plot of mean with SEM).

**Figure. G** Total mitochondrial DNA (quantified from Cyto B concentration) at 2 and 6 hrs cold ischaemia determined from cytochrome B concentrations. (Plot of mean with SEM).

**Table. 1** Viable cell numbers at 2hr and 6hrs of flush effluent

**Table. 24** Cytokine concentrations at 2 and 6hrs identifying IL-6, IL-18, GM-CSF, IL-1α, IL-1β and CXCL-8 (Mean with SEM).
### Tables

<table>
<thead>
<tr>
<th>Viable cell population</th>
<th>Cells at 2 hours cold ischaemia</th>
<th>Cell at 6 hours cold ischaemia</th>
<th>P-Value</th>
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<tbody>
<tr>
<td>CD45+ leukocytes</td>
<td>8.65x10^8 ±3.10x10^7</td>
<td>1.02x10^9 ±2.63x10^8</td>
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<td>Immature Neutrophils</td>
<td>2.47x10^7 ±1.06x10^7</td>
<td>2.72x10^8 ±1.43x10^8</td>
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<td>Mature neutrophils</td>
<td>4.21x10^6 ±9.5x10^6</td>
<td>4.49x10^7 ±1.19x10^6</td>
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<td>Eosinophils &amp; Basophils</td>
<td>5.16x10^5 ±1.55x10^6</td>
<td>4.36x10^6 ±9.13x10^6</td>
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<tr>
<td>T cells</td>
<td>6.94x10^4 ±1.51x10^5</td>
<td>9.64x10^5 ±2.37x10^6</td>
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<tr>
<td>T-regulatory cells</td>
<td>1.30x10^7 ±8.29x10^6</td>
<td>7.58x10^8 ±6.16x10^8</td>
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<tr>
<td>CD4+ (T helper)</td>
<td>4.52x10^6 ±1.27x10^6</td>
<td>7.99x10^7 ±2.90x10^7</td>
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<td>CD8+ (Cytotoxic)</td>
<td>3.37x10^5 ±7.06x10^5</td>
<td>4.44x10^6 ±1.02x10^5</td>
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<td>CD4+CD8+</td>
<td>9.00x10^4 ±2.82x10^4</td>
<td>1.08x10^6 ±3.58x10^6</td>
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<td>γδ cell</td>
<td>1.20x10^5 ±6.15x10^5</td>
<td>1.73x10^6 ±9.38x10^6</td>
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<tr>
<td>Natural Killer cell</td>
<td>1.60x10^6 ±5.58x10^6</td>
<td>2.90x10^7 ±1.15x10^6</td>
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<tr>
<td>B cell</td>
<td>3.73x10^5 ±1.36x10^5</td>
<td>4.07x10^6 ±1.11x10^6</td>
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<td>Classical monocyte</td>
<td>1.21x10^6 ±2.91x10^6</td>
<td>2.43x10^8 ±4.94x10^6</td>
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<td>Non-classical monocyte</td>
<td>1.18x10^7 ±7.74x10^6</td>
<td>3.24x10^8 ±2.29x10^8</td>
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**Table 1.** Viable cell numbers at 2hr and 6hrs of flush effluent

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Concentration 2hrs (pg)</th>
<th>Concentration 6hrs (pg)</th>
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<tr>
<td>IL-6</td>
<td>83,133pg ±46876</td>
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<tr>
<td>IL-18</td>
<td>3,966pg ±12922</td>
<td>10,483pg ±16,862</td>
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<tr>
<td>GM-CSF</td>
<td>114,666pg ±24593</td>
<td>100,333pg ±16,862</td>
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<tr>
<td>IL1α</td>
<td>60,200pg ±26179</td>
<td>54,466pg ±21,944</td>
</tr>
<tr>
<td>IL1β</td>
<td>22,933pg ±3626</td>
<td>20,066pg ±2,866</td>
</tr>
<tr>
<td>CXCL-8</td>
<td>43,000pg ±5874</td>
<td>20,066pg ±5,285</td>
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**Table 2.** Cytokine concentrations at 2 and 6hrs detecting IL-6, IL-18, GM-CSF, IL-1α, IL-1β and CXCL-8 (Mean with SEM). Cytokine concentrations of flush effluent at 6 hours