Sample Research Fellowship Application
Goals for Fellowship Training and Career

Having been born and raised in Burma, I went to the UK to study at age 16, when I received a scholarship. I was selected to undertake MD/PhD studies at University of Cambridge, which provided an exceptional opportunity for combined clinical and academic training. I undertook my PhD in Transplantation Immunology, examining the role of donor T cells in chronic allograft rejection. For my research, I received a number of accolades, including the Young Investigator Award from the Transplantation Society, the Sir Roy Calne Award by the British Transplantation Society and the President’s Medal from the British Association of Plastic Reconstructive and Aesthetic Surgeons. Importantly, the combined clinical and research training during the MD/PhD program instilled in me the significance of research in medical advancement.

I have completed 3 years of a 6-year residency program in London Plastic Surgery Training Program. In October 2015, I began a full-time postdoctoral research fellowship at Brigham and Women’s Hospital in Boston. During the fellowship, I plan to extend my PhD studies in experimental transplantation to clinical face transplantation and examine the role of donor T cells in rejection using state-of-the-art immunological techniques.

I am fully committed to a career in Academic Plastic Surgery, with specific interest in vascularized composite allotransplantation, which will allow me to combine my two passions – plastic surgery and transplant immunology. At present, there is no facial transplant program in the UK. Therefore, this fellowship will not only provide me with a unique research opportunity, it will also allow me first-hand insights of setting up and maintaining a face transplant program. I plan to bring these insights to the UK and contribute to establishing a facial transplant program.

After completing my research fellowship at Brigham and Women’s Hospital and upon return to the UK, I plan to apply for a Clinical Lectureship in plastic surgery, which will allow me to develop an active research program with a view to becoming an independent investigator. In the long term, I intend to become a principal investigator of my own research group, ultimately applying for a Chair of Surgery.

I have had a number of discussions with my sponsor, Dr. Pomahac, and have developed a structured research training plan for my research fellowship. This includes i). a global objective detailing the knowledge and skills that I will focus on attaining during the fellowship ii). learning and task agreements for each 3 monthly periods, with specific activities and tasks for completion, iii). plan for monthly one-to-one meetings to review progress and identify areas for improvement.

My global objective for the research fellowship is to attain the knowledge and skills required to transition to an independent investigator. Under Dr. Pomahac's guidance, I have identified the followings as the areas to focus on during the fellowship, in addition to undertaking the proposed research study: grant writing, project management, leadership and management skills. Accordingly, we have agreed on a number of activities to complete within specific time-frames to achieve this (please see Activities Planned Under This Award for details).

Through this agreed structured research training plan, I am confident that I will develop and enhance the knowledge and skills, which are crucial for the attainment of my ultimate career goal as an established Academic Plastic Surgeon.
RESOURCES

Identify the facilities to be used as listed below. If appropriate, indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Describe only those resources that are directly applicable to the proposed work. Use continuation pages if necessary.

Laboratory:

For this research study, I will have full access to both Dr. Pomahac’s (the sponsor and mentor) and Dr. Clark’s (our collaborator) laboratories at Brigham and Women’s Hospital (BWH). Dr. Pomahac’s lab and Dr. Clark’s lab are 5 minutes walk from each other within the BWH campus.

The equipment available for use includes: laminar flow hoods, CO₂ incubators, freezers (liquid nitrogen, -80°C and -20°C), refrigerators, cold rooms (4°C), standard light and phase contrast as well as a state-of-the-art PerkinElmer Mantra Imaging microscope for analysis of multiplex immunostaining, centrifuges (ultra, super and low speed) and rotors, ABI 7700 real-time PCR machine, electrophoresis apparatus, BL2++ tissue culture suite, vacuum dryer, bacteria incubators and shaking incubators for growing cultures, microwave ovens, high temperature ovens for baking glassware, a fully equipped darkroom, BioRad GelDoc XR digital gel documentation, a Cellular Technologies Immunospot ELISPOT reader/analyzer, a Molecular Devices Versmax ELISA plate reader and an automated 96 well plate washer. Histology equipment available includes: cryostats and microtomes to section tissues, paraffin embedding equipment, and appropriate microscopes for analysis of histology slides.

Clinical:
Not applicable as there will be no direct patients contact for this research study.

Animal:
Not applicable as there is no animal work involved for this research study.

Computer:
All personnel in this study will have access to state-of-the-art network-based computers with up-to-date Windows®, Microsoft Office software and analytical applications, as well as full support from Partners Information Systems.

Office:
Dr. Pomahac, the sponsor and mentor for this study, has a dedicated office space (13×7 ft) in a suite off the main offices of the Division of Plastic Surgery at BWH.

Dr. Win, the principal investigator for this study, has a shared office space (15 x 9 ft) on the 15th floor of the Thorn Building at BWH.

Dr. Clark, the collaborator for this study, has a dedicated office space (13 x 7 ft) at Harvard Center for Skin Disease on the BWH Campus.

Within their office environments, all personnel in this study will also have full access to computers, telephones, fax machines, printers, photocopiers and scanners.

Other:
Also available to the investigators in this study is the conference room at the Division of Plastic Surgery offices at BWH, which will be used to conduct all study-related meetings. The conference room is a private room with a meeting table that can accommodate up to 10 people. The conference room is fitted with audiovisual equipment and a telephone.
Scientific Environment: Describe how the scientific environment in which the research will be done contributes to the probability of success (e.g., institutional support, physical resources, and intellectual rapport). In describing the scientific environment in which the work will be done, discuss ways in which the proposed studies will benefit from unique features of the scientific environment or subject populations or will employ useful collaborative arrangements.

The scientific environment at BWH is most propitious for this study. BWH is a 777-bed teaching affiliate of Harvard Medical School located in the Boston’s renowned Longwood Medical Area. BWH is recognized internationally for outstanding patient care, biomedical research, education and training of physicians, research scientists and other health care professionals.

BWH’s commitment to research is demonstrated by its inclusion of over 3,300 researchers among its ranks, $485M of research funds in FY09, a #2 ranking among independent hospitals in the USA for NIH funding, and its having housed three Nobel Prize laureates. BWH has all of the physical facilities, instrumentation, equipment and resources required to carry out this research project.

Early Stage Investigators ONLY: Describe institutional investment in the success of the investigator, e.g., resources for classes, travel, training; collegial support such as career enrichment programs, assistance and guidance in the supervision of trainees involved with the ESIs project, and availability of organized peer groups; logistical support such as administrative management and oversight and best practices training; and financial support such as protected time for research with salary support.

As a BWH employee and an affiliate of Harvard University, I have significant organized support and resources through the following facilities, in addition to my mentor and collaborator.

1. Center for Clinical Investigation (CCI), BWH
   CCI is the home for clinical research at BWH and offers a number of resources (including peer support and mentorship), services (including biostatistics consultation services) and organized educational programs free-of-charge to BWH employees. I have enrolled in a number of the educational programs offered by CCI (please see ‘Activities Planned Under This Award’ document for details).

2. The Harvard Clinical and Translational Science Center
   The Harvard Clinical and Translational Science Center offers more than a dozen courses and training programs free-of-charge to Harvard affiliates. I have enrolled in a grant writing course offered (please see ‘Activities Planned Under This Award’ document for details).
Respective Contributions

I have had multiple discussions with my sponsor, Dr Pomahac, in the development, review and editing of the structured research training plan. Under the supervision of both Dr Pomahac and our collaborator, Dr Clark, I have written and prepared this grant application, from initial conceptual development to experimental planning.

We have agreed on the following respective roles in accomplishing the proposed research, and achievement of the agreed structured training plan.

Dr Thet Su Win M.D., Ph.D. (Applicant)
I will be responsible for planning and carrying out all experiments. I will collect data and perform analysis, prepare presentations and manuscripts. I will perform the aforementioned tasks under the supervision of Drs Pomahac and Dr Clark.

In addition to the research project, I will take responsibility to actively participate in the other activities as agreed in the training plan. This includes enrolling and attending continuing education courses, including grant writing and project management courses, leadership and management trainings and active involvement in the weekly multidisciplinary vascularized composite allotransplant (VCA) team meetings.

Dr Bohdan Pomahac M.D. (Sponsor and Mentor)
Dr Pomahac will supervise all aspects of the study, lead weekly laboratory meetings to discuss the progress and exchange ideas, and supervise in preparing manuscripts for submission to peer-reviewed journals with the findings of this proposed research. He will oversee that study documentation is kept up to date, and reports are generated to the sponsor timely.

In addition, he will review the progress of the planned structured research training at monthly intervals, via one-to-one meetings, to ensure the achievement of agreed objectives.

Dr Rachel Clark M.D., Ph.D (Collaborator)
Dr Clark will be actively involved in many phases of this research study, including scientific oversight, assistance with infrastructure support and providing expertise on processing of precious tissue samples from face transplant patients, including cutting-edge high throughput T-Cell receptor (TCR) sequencing and 5-color multiplex immunostaining/spectral imaging. In addition, she will provide guidance and advice in preparing manuscripts for submission to peer-reviewed journals.
Selection of Sponsor and Institution

Brigham and Women’s Hospital has one of the largest and most successful vascularized composite allotransplant (VCA) programs, which has performed 7 clinical facial transplants, representing the largest cohort at a single center in the world. It has established a dedicated tissue repository, which prospectively collects tissue and blood samples from VCA recipients. This allows for a unique opportunity to carry out the proposed research study. This, combined with the fact that Brigham and Women’s Hospital is a well-established facility which has all the organizational resources to comprehensively support the proposed research, are the main reasons for selecting this institution as the performance site.

Dr. Pomahac, the sponsor for this study, is the founder and Principal Investigator of the face, hand and lower extremity transplantation programs at Brigham and Women’s Hospital. In 2008, he developed a multidisciplinary team and obtained IRB approval for face transplantation at Brigham and Women’s Hospital. In 2009, he led the team to perform the second face transplantation in the United States, and has subsequently gained approval to perform upper extremity transplantation and more recently lower extremity and abdominal wall transplantation. His team has developed multidisciplinary protocols for the pre-, peri- and post-operative area of the VCA recipients and fueled the progress of VCA in the United States and wider in the world, by providing a body of clinical and scientific evidence and methodology supporting the development of several other VCA programs.

Dr. Pomahac is the Principal Investigator of 5 current funding grants, totaling over $10 million of direct cost and has his own independent laboratory within Brigham and Women’s Hospital. He has a proven track record of supervising and training of students and postdoctoral fellows. Dr. Pomahac has active collaborations with multiple outstanding partners at Brigham and Women’s Hospital, Harvard Medical School and Massachusetts Institute of Technology. He has authored over 100 peer-reviewed publications, has presented more than 60 times at national and international venues on the topic of VCA, and is recognized as one of the leaders of reconstructive transplantation in the world.

In summary, I believe that the opportunity to undertake the proposed research at Brigham and Women’s Hospital under the sponsorship of Dr. Pomahac represents a unique opportunity and a true privilege.
Activities Planned Under This Award

<table>
<thead>
<tr>
<th>Activities</th>
<th>Percentage of time devoted</th>
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<tbody>
<tr>
<td>Conceptual development</td>
<td>10%</td>
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<tr>
<td>Experimental planning</td>
<td>10%</td>
</tr>
<tr>
<td>Experimental execution</td>
<td>40%</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>15%</td>
</tr>
<tr>
<td>Dissemination of study results</td>
<td>10%</td>
</tr>
<tr>
<td>(including presenting at local, national and international meetings and preparing manuscripts for peer-reviewed journals)</td>
<td></td>
</tr>
<tr>
<td>Continuing education (including attending courses, conferences and seminars)</td>
<td>10%</td>
</tr>
<tr>
<td>Teaching and training others</td>
<td>2.5%</td>
</tr>
<tr>
<td>Others (including administrative work)</td>
<td>2.5%</td>
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</tbody>
</table>

Activities planned other than research

In addition to the proposed research project, I have enrolled in a number of activities, courses and conferences to achieve the objectives of my structured research training plan (Table 1). In addition to these activities, I will actively participate in the weekly multi-disciplinary VCA team meetings and review VCA recipients at follow-up clinics (for research purposes) to gain an in-depth insight of an active VCA program.

<table>
<thead>
<tr>
<th>Activities</th>
<th>Location</th>
<th>Dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstructive Microsurgery Course</td>
<td>New York University Langone Medical Center, USA</td>
<td>20-21 November 2015</td>
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<tr>
<td>Art &amp; Anatomy of Writing a Clinical Research Career Development Grant</td>
<td>Center for Clinical Investigation, Brigham and Women’s Hospital, Boston, USA</td>
<td>8-9 December 2015</td>
</tr>
<tr>
<td>A practical guide to biobanking for biomarker and personalized medicine research</td>
<td>Center for Clinical Investigation, Brigham and Women’s Hospital, Boston, USA</td>
<td>12 January 2016</td>
</tr>
<tr>
<td>Observership in Plastic Surgery</td>
<td>Memorial Sloan Kettering Cancer Center, New York, USA</td>
<td>18-22 January 2016</td>
</tr>
<tr>
<td>Intermediate Biostatistics for Medical Researchers</td>
<td>Center for Clinical Investigation, Brigham and Women’s Hospital, Boston, USA</td>
<td>22 March 2016</td>
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<tr>
<td>Successful Grant Writing Strategies</td>
<td>The Harvard Clinical and Translational Science Center, Boston, USA</td>
<td>28 March 2016</td>
</tr>
<tr>
<td>British Association of Plastic Reconstructive and Aesthetic Surgeons Meeting</td>
<td>Bristol, UK</td>
<td>29 June – 1 July 2016</td>
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<td>American Transplant Congress</td>
<td>Boston, USA</td>
<td>11-15 June 2016</td>
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<tr>
<td>Clinical Trial Design</td>
<td>The Harvard Clinical and Translational Science Center, Boston, USA</td>
<td>15 July 2016</td>
</tr>
<tr>
<td>Plastic Surgery The Meeting</td>
<td>Los Angeles, USA</td>
<td>23 – 27 September 2016</td>
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Specific Aims

Introduction

More than 7 million people in North America every year can benefit from vascularized composite allotransplantation (VCA) secondary to oncologic surgery, traumatic injuries and congenital anomalies. The uptake of these life-changing procedures have, however, been restricted by our limited understanding of the mechanisms underlying the rejection process and the compulsory need for life-long immunosuppression.

Although the effector role of T cells in acute rejection is well established, the contribution of donor versus recipient T cells in rejection is unexamined. This project, using tissue samples from the largest cohort of face transplant patients at a single center in the world and multiple state-of-the-art techniques, will investigate, firstly, if the pathogenic T cell clones implicated in acute rejection are of donor or recipient origin, and secondly, if these pathogenic T cell clones are measurable in blood during acute rejection.

If we find that donor T cells persist within facial allografts and contribute to rejection, selective depletion of donor T cells from the allografts before transplantation will represent a novel approach to reducing rejection. If pathogenic T cell clones, which are unique in each patient, are detectable in peripheral blood during rejection, testing blood samples for these T cells will serve as non-invasive and personalized rejection biomarkers. Lessons learned will be applicable to other VCAs and will have clear translational impact in patients care.

Specific Aim 1: To identify the origin of pathogenic T cell clones

We will investigate if pathogenic T cell clones implicated in acute rejection are donor or recipient-derived.

Rationale

Previous studies have suggested that facial transplantation can transfer a significant number of donor T cells. It is important to examine whether these transferred donor T cells contribute to rejection because their removal from the allograft before transplantation can represent a novel strategy to reduce rejection.

Hypothesis

The pathogenic T cell clones identified within the allograft are both donor and recipient-derived.

Specific Aim 2: To determine if pathogenic T cell clones are detectable in blood during acute rejection

We will investigate if pathogenic T cell clones are measurable in blood during acute rejection and could, therefore, serve as a non-invasive and personalized biomarker of rejection.

Rationale

It is important to examine if we can detect pathogenic T cells in blood during rejection but not when the transplant is in a stable non-rejection state. If this is the case, testing blood samples for these pathogenic T cell clones, which are unique in each patient, will serve as non-invasive and personalized biomarkers to aid the diagnosis of rejection, allowing for individualized post-transplant management.

Hypothesis

Pathogenic T cell clones will be detectable in blood during acute rejection but will not be measurable at stable non-rejection time-points.
Research Strategy

Significance
Rejection is the single most formidable barrier to wider implementation of face and other vascularized composite allografts (VCAs) that hold great promise for restoring function for those with devastating injuries\(^1\). Unlike solid organ transplants, VCAs have a unique immunological characteristic – the presence of skin. The skin of a healthy adult human contains approximately 1 million T cells/cm\(^2\)^2, extrapolation of which suggests that the skin of a normal adult contains a staggering 20 billion T cells, nearly twice the number present in the entire blood volume\(^3\).

Our collaborator’s group (Dr. Rachel Clark, Associate Professor of Dermatology, Harvard Skin Disease Research Center, Boston) has recently categorized skin T cells and reported that human skin is populated by non-recirculating resident memory T cells (TRM) as well as recirculating T cells, both with potent effector functions\(^3\)^4. Given that a clinical full face transplant is on average 600-700cm\(^2\) in size\(^5\), it contains ~600–700 million donor T cells. An important and as yet unanswered question is whether these highly immunocompetent donor T cells persist within the allograft long-term after transplantation and contribute to rejection.

This study, utilizing tissue and blood samples from the largest cohort of face transplant patients at a single center in the world, represents the first comprehensive analysis of the role of donor versus recipient T cells in rejection. If we find that donor T cells persist within facial allografts and contribute to rejection, selective depletion of donor T cells from the grafts before transplantation, either by mechanical separation or pharmacological depletion, will represent a novel approach to reducing rejection.

If pathogenic T cell clones are increased in the blood during rejection, the study of blood samples for these T cells could be developed as a biomarker of acute rejection. Diagnosis of acute rejection in VCAs is challenging because both clinical and pathological changes of skin rejection are non-specific, and closely resemble several inflammatory dermatoses\(^6\)^7. Using a combination of high throughput T-cell receptor sequencing (HTS) and multiplex immunostaining of allograft samples, as described in this study, can identify pathogenic T cells that are unique to each transplant recipient. Monitoring these unique T-cell receptors (TCRs) over time in peripheral blood will provide a personalized and non-invasive rejection biomarker, enabling individualized post-transplant management and fill a long-term need in patients’ care.

Innovation
This study, using tissue collected from the largest number of face transplant patients at a single center in the world, represents the first comprehensive analysis of the role of donor T cells in rejection. It will challenge the current paradigm that recipient T cells are exclusively responsible for rejection and instead explore the role of donor T cells. In doing so, it will add new perspectives to the mechanisms underlying rejection in VCAs.

This study will use multiple cutting-edge techniques, including HTS and tyramide-based multiplex immunostaining. HTS provides a comprehensive and quantitative analysis of how many distinct T cell clones are present within a tissue sample, the relative frequency of each clone and the exact unique nucleotide sequences of each clone’s CDR3 regions\(^8\). Importantly, HTS provides the ability to track pathogenic T cell clones across different tissue (e.g. skin biopsy and blood) in the same individual and to track them longitudinally over time in a given patient\(^9\).

HTS of the TCR\(\beta\) allele allows identification of the TCR V\(\beta\) subunit used by each T cell clone. Commercially available antibodies exist that recognize approximately 60-70% of all human T cell V\(\beta\) subunits. Therefore, by immunostaining the T cell clones that show clonal expansion during rejection with commercially available TCR V\(\beta\) antibodies, together with antibodies to IFN-gamma\(^10\) and IL-17\(^11\), which are key cytokines in rejection, allows for identification of T cell clones are pathogenic. Although the combined use of HTS and multiplex immunostaining has been described in other clinical settings, including detection of pathogenic T cell clones in cutaneous T cell lymphoma by our collaborator’s group\(^9\), the use of this technique has not been reported in clinical transplant settings. Monitoring of these pathogenic T cell clones, which are unique to each patient, will
provide a novel, personalized and non-invasive rejection diagnostic, with the potential for individualized immunosuppressive regimens and post-transplant management.

**Approach**

**Preliminary Data**

Between 2009 and 2014, our group has performed 7 clinical facial transplants (Table 1).

<table>
<thead>
<tr>
<th>Year of Transplant</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
<th>Patient 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cause</td>
<td>Electrical burn</td>
<td>Electrical burn</td>
<td>Electrical burn</td>
<td>Animal attack</td>
<td>Chemical burn</td>
<td>Gunshot</td>
<td>Gunshot</td>
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<tr>
<td>Number of acute rejection episodes</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

*Table 1: Details of face transplant recipients at Brigham and Women’s Hospital, Boston, USA.*

We have recently reported, for the first time in clinical face transplantation, that donor T cells persist within the allograft up to 23 months following transplant\(^{12}\). In this study, we analyzed serial skin biopsies from 3 face transplant patients using dual-labeling immunofluorescence by staining for donor-recipient mismatch HLA antigens, and demonstrated that the majority (>90%) of lymphoid cells found at the site of target cell injury during acute rejection were donor resident memory T cells (Figure 1). Sequestration within the skin may offer the explanation for how donor TRM cells are protected from being destroyed by the recipient immune system. This is supported by the experimental finding that when male T cells were adoptively transferred to female recipients, only the male T cells that migrated into the skin survived in a murine model\(^{13}\).

![Figure 1. Confocal micrograph of 5µm skin biopsy from a facial allograft at 23 months following transplantation, showing donor T cells (donor HLA-B antigen = red; CD3-positive = green; co-expression = yellow-orange)\(^{12}\).](image)

The precise role donor T cells play in allograft survival remains unknown. My previous PhD studies have demonstrated the role of donor CD4 T cells in chronic cardiac allograft rejection\(^{14,15}\), but whether donor T cells play a similar role in VCA rejection is unknown. To date, no clinical graft-vs-host disease (GVHD) has been reported in face transplant patients, even in those that have received donor bones (e.g. mandibles) as part of their transplants, or those who received donor bone marrow transfusion to promote graft acceptance\(^{16}\). Facial transplant recipients are subject to life-long immunosuppression and it is likely that transferred donor T cells are also susceptible to these immunosuppressant, which likely limit their capacity to cause clinical GVHD. Instead, they may cause localized intragraft graft-vs-host alloimmune response, contributing to allograft damage.

In collaboration with Dr. Rachel Clark, I have performed a series of experiments to optimize the techniques of extracting DNA from skin punch biopsies of facial allografts as well as from peripheral blood mononuclear cells (PBMCs), and analyzing the extracted DNA using HTS (Adaptive Biotechnologies, Seattle).

1). HTS identifies T cell populations present within facial allograft at non-rejection and rejection time-points DNA extracted from punch biopsies taken from facial allografts transplanted to Patient 2 and Patient 5 at non-rejection and during acute rejection, respectively, were examined using HTS. Diverse populations of T cells were present in non-rejecting skin (Figure 2) whereas expanded populations of clonal T cells were identified in rejecting skin (Figure 3).
2). HTS of allograft and blood during acute rejection demonstrated an identical T cell population in facial allograft that was also detected in lower numbers in peripheral blood.

Evaluation of facial allograft biopsy and peripheral blood collected at the same acute rejection time-point demonstrated the presence of a specific clonal T cell population in allograft that was also present in lower but detectable numbers within the peripheral circulation (Figure 5).
Experimental Methodology
Our institution, Brigham and Women’s Hospital, has a dedicated tissue repository, which prospectively collects tissue and blood samples from patients who have received VCAs. Allograft biopsies (4mm punch skin biopsies) and cryopreserved viable PBMCs are available at the following time-points: before transplantation, at weekly during the first month after the transplant, then at 3, 6, 9, 12, 18 and 24 months post-transplant (protocol samples) or when clinically indicated (at time of rejections). Tissues (lymph nodes and spleen) and PBMCs from the donors obtained at the time of allograft procurement are also available.

This study will utilize existing banked donor and recipient tissue and blood samples from 7 face transplant patients at Brigham and Women’s Hospital. Data regarding their demographics, reason for transplant, presence of donor-specific antibodies and panel reactive antibodies, immunosuppressant regimes and rejection history will be collected.

i). Establishment of donor and recipient T cell repertoires
For each donor-recipient pair, donor T cell repertoire will be established by examining donor skin and PBMCs at the time of allograft procurement using HTS (Figure 6A). Recipient T cell repertoire will be established by examining recipient skin and PBMCs collected prior to transplant using HTS (Figure 6B). For each sample, DNA will be extracted as previously described by our collaborator’s group and the extracted DNA examined by HTS. The re-arranged V(D)J segments will be amplified using bias-controlled V and J gene primers for high throughput sequencing. After correcting sequencing errors via a clustering algorithm, CDR3 segments will be annotated according to the International ImMunoGeneTics collaboration.

![Figure 6. Schematic of high throughput TCR$\beta$ CDR3 sequencing (HTS) analysis strategy to identify donor and recipient T cell repertoires. For each donor-recipient pair, DNA will be extracted from skin biopsy and PBMCs of both donor and recipient. The extracted DNA will then be used for HTS to establish the donor and recipient T cell repertoire.](image)

ii). Determination of turnover of donor vs. recipient-derived T cells within facial allografts following transplant
HTS analysis will be performed (as described above) on serial punch biopsies taken from 7 facial allografts (1, 3, 6, 12, 24 months post-transplant in 5 patients and 1, 3, 6, 12 months post-transplant in 2 patients). By comparing the sequences of T cell clones to the known T cell repertoires of the donor and recipient, I will directly quantify the total number and relative percentages of donor vs. recipient-derived T cells within the facial allograft to determine the persistence of donor vs. recipient T cells within facial allografts and their turnover over time.
iii). Identification of pathogenic T cell clones within facial allografts using a combination of HTS and multiplex immunostaining

By comparing HTS studies from non-rejection and rejection allograft biopsies, individual T cell clones that expand during rejection will be identified. To investigate if these expanded T cell clones are pathogenic, state-of-the-art five color tyramide-based multiplex immunostaining\(^9\) and spectral imaging (Perkin-Elmer Mantra Quantitative Pathology Imaging System) will be performed in conjunction with HTS as previously described\(^9\). HTS of the TCR\(\beta\) allele allows identification of the V\(\beta\) usage of the T cell clone of interest. This enables tissue-based selective immunostaining of skin allografts of these putative pathogenic T cell clones by commercially available TCR V\(\beta\) antibodies, together with antibodies to IFN-gamma and IL-17, key cytokines in rejection. Co-localization of inflammatory cytokine production within the same T cell clones identified by HTS will indicate that these clones are major drivers of tissue destruction (refer from here as pathogenic T cell clones).

iv). Identification of the origin of pathogenic T cell clones

Once pathogenic T cell clones are identified, I will assign these T cell clones as either of donor or recipient origin by comparing the TCR sequences of these clones to the known T cell repertoires of the donor and the recipient (Figure 7).

![Combined HTS & multiplex immunostaining](image)

**Figure 7.** Schematic of analysis strategy to identify the origin of pathogenic T cell clones. After pathogenic T cells clones are identified using a combination of HTS and multiplex immunostaining, these T cells will be assigned as either donor or recipient-derived by comparing their unique CDR3 nucleotide sequences to the known T cell repertoires of donor and recipient.

v). Determining if pathogenic T cell clones are measurable in blood during acute rejection

HTS of the TCR\(\beta\) CDR3 regions will be performed to investigate if pathogenic T cell clones identified by HTS and immunostaining/spectral imaging could be detected in peripheral blood during periods of acute rejection. I will analyze DNA extracted from PBMCs collected from 7 face transplant patients at periods of no rejection (1, 3, 6, 12, 24 months post-transplant in 5 patients and 1, 3, 6, 12 months post-transplant in 2 patients) and at rejection episodes, using the techniques as previously described\(^9\). If pathogenic T cell clones are detected in peripheral blood, it will enable the study of blood samples by HTS as a personalized and non-invasive biomarker of acute rejection.

**Data and Statistical Analysis**

Analyses of HTS studies will be done in an investigator-blinded fashion. Multiplex immunostaining studies will be performed without blinding. The results will be expressed as mean ± standard deviation. Differences between two sample groups will be detected using the one-tailed Wilcoxon-Mann-Whitney test. Expansion of T cell clones over time in the same patient will be examined using paired t-test. \(p<0.05\) will be considered significant. Analysis of HTS data, including statistical analysis, will be performed in consultations with Dr. David Williamson (Project Biostatistician at Adaptive Biotechnologies, Seattle). Analysis of the remaining data will be performed in consultations with Dr. Shelley Hurwitz (Biostatistics Director, Center for Clinical Investigation, Brigham and Women’s Hospital).
Potential Pitfalls and Alternative Approaches
1. The output of HTS studies includes thousands of T cell CDR3 sequences and comparison of repertoires may appear daunting. However, the ImmunoSeq tools available online via Adaptive Biotechnologies provide one-step rapid overlap analyses and free biostatistical support. Our collaborator’s laboratory is experienced in carrying out HTS repertoire overlap analyses and clone tracking.

2. Identification of pathogenic T cell clone in this study will be carried out using multiplex immunostaining for the TCR Vβ in T cell clones that demonstrate clonal expansion during rejection as identified by HTS. However, commercially available antibodies exist only for approximately 60-70% of all TCR Vβ subunits, and therefore, not all top clones may be trackable by immunostaining. If the five most frequent clones in rejecting allograft are not recognizable by commercially available Vβ antibodies, we will evaluate the next five most frequent clones. Our pilot studies demonstrate that multiple T cell clones are up regulated in rejecting skin (Figure 4) and over half of these will be recognizable by the available antibodies.

Data and Research Resources Sharing Plan
All data and research resources derived from the proposed study will be shared with the research and clinical community and the public at large, while safeguarding the privacy of study subjects, protecting confidential and proprietary data and third-party intellectual property. We will present the findings of this study at academic conferences and plan for publication in peer-reviewed journals.

Potential Hazard and Precautions
This study will involve analyzing tissue samples collected from patients. Standard precautions will be taken in handling the samples and all discarded tissues will be disposed off, according to institutional regulations.

Anticipated Results and Potential Future Research
If our findings indicate that donor T cells contribute to rejection, this will prompt a prospective randomized trial in VCA recipients at our institution to determine if therapies directed at selectively eliminating donor T cells prior to transplantation will represent a novel therapy to reduce rejection. If our results indicate that donor T cells do not play a role in rejection, this will underscore the importance of recipient T cells in allograft rejection and prompt further studies to elucidate the fate of the transferred donor T cells (for example, if they are clonally deleted following transfer to the recipient).

If our findings indicate that monitoring specific pathogenic TCRs in peripheral blood provides a non-invasive and personalized rejection biomarker, it will prompt a prospective clinical trial in VCA recipients at our institution in order to validate this method, followed by multi-center prospective validation in collaboration with other VCA centers, prior to clinical use.

Time Plan for the Research Project and Other Research Training

<table>
<thead>
<tr>
<th>Specific Aims to be Achieved for the Research Project</th>
<th>Other Training</th>
<th>Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Establishment of donor and recipient T cell repertoires using HTS</td>
<td>Biostatistics</td>
<td>Month 1</td>
</tr>
<tr>
<td>Determination of donor vs. recipient T cells turnover within facial allografts using HTS</td>
<td>Clinical Trial Design</td>
<td>Month 2-3</td>
</tr>
<tr>
<td>Identification of pathogenic T cell clones within allografts using HTS and multiplex immunostaining</td>
<td>Grant Writing</td>
<td>Month 4-6</td>
</tr>
<tr>
<td>Determination of origin of pathogenic T cell clones identified within allograft</td>
<td>Communicating research data effectively</td>
<td>Month 7</td>
</tr>
<tr>
<td>Detection of pathogenic T cell clones in peripheral blood</td>
<td>Leadership and Management</td>
<td>Month 8-9</td>
</tr>
<tr>
<td>Data analysis and dissemination of study results</td>
<td>Leadership and Management</td>
<td>Month 10-12</td>
</tr>
</tbody>
</table>
Human Subjects

This study will examine banked tissue and blood specimens from 7 patients who had received facial transplants at Brigham and Women’s Hospital, Boston. There will be no direct interaction with patients.

This study will be added as amendment to the existing departmental Institutional Review Board (IRB) Approval (Protocol Number 2010P000743, Partners Human Research Committee).
Vertebrate Animals

N/A

There is no plan to use vertebrate animals in the proposed research.