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14. ABSTRACT **Vascularized composite allografts (VCA) are transplants containing multiple tissue types (including bone, muscle, skin, nerves and blood vessels), which offer patients restoration of function and form following severe, disabling and disfiguring injury or tissue loss, in circumstances where the results of conventional reconstructive surgery remain unsatisfactory. The high incidence of episodes of skin-targeted acute rejection, and the morbidity associated with current immunosuppression regimens, necessary throughout the life of the recipient to prevent rejection, remain significant areas in which improvement would enhance quality of life, improve the risk-benefit ratio of VCA and ultimately expand availability of these procedures to severely injured service men and women, and civilian victims of disabling and disfiguring trauma or disease. The objective of the VCA laboratory at the MGH is to develop a clinically-applicable strategy for the induction of immune tolerance of VCAs. The aim of the work supported by this award is to introduce and optimize a protocol for VCA tolerance based on the principle of delayed induction of mixed chimerism in a non-human primate (NHP) model. This approach, in contrast to protocols which have already reached clinical trials for kidney transplantation, permits induction of tolerance in the context of transplantation from deceased donors – a prerequisite for clinical application in VCA. Successful induction of tolerance for VCAs using this protocol in NHPs can be expected to lead to rapid translation into clinical trials.**

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INTRODUCTION

Vascularized composite allografts (VCA) are transplants containing multiple tissue types (including bone, muscle, skin, nerves and blood vessels), which offer patients restoration of function and form following severe, disabling and disfiguring injury or tissue loss, in circumstances where the results of conventional reconstructive surgery remain unsatisfactory. The high incidence of episodes of skin-targeted acute rejection, and the morbidity associated with current immunosuppression regimens, necessary throughout the life of the recipient to prevent rejection, remain significant areas in which improvement would enhance quality of life, improve the risk-benefit ratio of VCA and ultimately expand availability of these procedures to severely injured service men and women, and civilian victims of disabling and disfiguring trauma or disease. The objective of the VCA laboratory at the MGH is to develop a clinically-applicable strategy for the induction of immune tolerance of VCAs. The aim of the work supported by this award is to introduce and optimize a protocol for VCA tolerance based on the principle of delayed induction of mixed chimerism in a non-human primate (NHP) model. This approach, in contrast to protocols which have already reached clinical trials for kidney transplantation, permits induction of tolerance in the context of transplantation from deceased donors – a prerequisite for clinical application in VCA. Successful induction of tolerance for VCAs using this protocol in NHPs can be expected to lead to rapid translation into clinical trials.

KEYWORDS

Vascularized composite allograft, vascularized composite allotransplantation, restorative transplantation, transplant tolerance, mixed chimerism, delayed induction of transplant tolerance, non-human primate model.

OVERALL PROJECT SUMMARY

i. Progress against Current Objectives

Year	AIM	TASK	SUBTASK	MONTH(S)	% COMPLETE
Year 1	AIM 1. To optimize the delayed tolerance induction protocol for vascularized composite allotransplantation in a non-human primate model.	(1.1) TASK 1. Investigate version 1 delayed tolerance induction protocol (DTIP) for upper extremity transplantation in nonhuman primates. (Months 0-18)	(1.1.1) SUBTASK 1. IACUC and ACURO review and approval. (Month 0-4)	Month 0-4	100%
			(1.1.2) SUBTASK 2. Order and take delivery of first cohort of non-human primates. (Month 5-6)	Month 5-6	100%
			(1.1.3) SUBTASK 3. Orthotopic upper extremity transplants on 4 months SIS (n=4). (Months 6-9)	Month 6-9	100%
			(1.1.4) SUBTASK 4. Delayed tolerance induction protocol, wean immunosuppression. (Months 10-13)	Month 10-13	100%
			(1.1.5) SUBTASK 5. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 10-18)	Month 10-18	100%

			(1.1.6) SUBTASK 6. Summarize preliminary data/progress on DTIP transplants for inclusion in year 1 report (Month 12)	Month 12	100%
Year 2	AIM 2. To investigate the effect of T memory cell inhibition and in-vivo T regulatory cell up regulation on the delayed induction of VCA tolerance.	(2.1) TASK 1. Investigate effect of Tmem inhibition on delayed induction of VCA tolerance (Months 12-24)	(2.1.1) SUBTASK 1. Heterotopic partial face transplants on 2 months SIS (n=4) (Months 12-15)	Month 12-15	0%
			(2.1.2) SUBTASK 2. Delayed tolerance induction protocol + CTLA4-Ig/rapamycin (Months 14-17)	Month 14-17	0%
			(2.1.3) SUBTASK 3. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 14-23)	Month 14-23	0%
			(2.1.4) SUBTASK 4. Summarize and report data on effect of Tmem inhibition on delayed induction of VCA tolerance for year 2 report (month 24)	Month 24	0%
		(2.2) TASK 2. Investigate effect of Treg up-regulation on delayed induction of VCA tolerance (Months 16-24)	(2.2.1) SUBTASK 1. Heterotopic partial face transplants on 2 months SIS (n=4) (Months 15-18)	Month 15-18	100%
			(2.2.2) SUBTASK 2. Delayed tolerance induction protocol + a-IL-6R (Months 17-20)	Month 17-20	100%
			(2.2.3) SUBTASK 3. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 17-24)	Month 17-24	100%
			(2.2.4) SUBTASK 4. Summarize and report data on effect of Treg upregulation on delayed induction of VCA tolerance for year 2 report (month 24)	Month 24	100%
Year 2-3	AIM 3. To investigate the effect of combined T memory cell inhibition and T regulatory cell up regulation on the delayed induction of VCA tolerance	(3.1) TASK 1. Investigate effect of combined Tmem inhibition and Treg up regulation on delayed induction of VCA tolerance (Months 22-36)	(3.1.2) SUBTASK 2. Heterotopic partial face transplants on 2 months SIS (n=4) (Months 25-28)	Month 25-28	100%
Year 3			(3.1.3) SUBTASK 3. DTIP with combined Tmem inhibition/Treg upregulation. (Months 24-30)	Month 24-30	0%
			(3.1.4) SUBTASK 4. Investigate durability of chimerism, VCA survival, frequency of complications (eg GvHD) and in vitro immune status (Months 24-36)	Month 24-36	0%
			(3.1.5) SUBTASK 5. Summarize and report data on effect of combined Tmem inhibition/Treg upregulation on delayed induction of VCA tolerance for year 3 report (month 36)	Month 36	0%

			(3.1.6) SUBTASK 6. Complete data analysis, prepare final (year 3) report, prepare manuscripts for submission	Month 36	75%

Overall:

- Aim 1 – 14 animals used (7 donors, 7 recipients) in upper extremity VCA model
  - 5 of 7 technical failures
  - N = 4 required for statistical analysis
    - 1 euthanized due to steroid-resistant rejection on POD 51
    - 1 euthanized due to necrotizing fasciitis on POD 143
  - **Decision made to change to face VCA model** as no meaningful analysis was possible

- Aim 2 – 20 animals used (10 donors, 10 recipients) in facial VCA model
  - 2 of 10 technical failures
  - 2 of 2 recipients developed PTLD by POD 79 and 107 when the delay period was 4 months (i.e bone marrow transplantation (BMT) at 4 months post-VCA); subsequent 6 recipients were studied on a 2 month delay and none developed PTLD before POD 60
  - N = 4 required for statistical analysis for each of Task 1 and 2; only Task 2 performed however (reasons detailed below)

- Aim 3 – not performed due to budgetary constraints

ii. Results

The ensuing discussion is broken down into two parts (i) the development, identification, treatment and resolution (or not) of acute rejection episodes while recipient animals are maintained on immunosuppression prior to receiving conditioning and bone marrow transplantation (BMT); this mirrors clinical VCA patients who are all currently on maintenance immunosuppression, (ii) the clinical and chimerism outcomes after delayed BMT, to have a preview of the challenges that may be encountered in future clinical trials on tolerance induction in VCA.

- ***Investigation of delayed tolerance induction protocol for facial transplantation in NHPs***

Using our previously established screening and selection algorithm for donor and recipient NHP pairs, 6 face transplants (Barth et al, 2009) have been performed during the period covered by the current annual report for this award. All recipients were successfully maintained on standard triple immunosuppression for 2 months (instead of the previous 4 months) and avoided systemic complications such as steroid-resistant rejection and post-transplant lymphoproliferative disorder (PTLD), which would necessitate removal from study.

M6514 (Full MHC Mismatched Recipient)
At the time of the last annual report, the animal was POD 14. This animal subsequently developed acute rejection on POD 36 (Banff I) and 46 (Banff II) (Figure 1). As per our study protocol, in the event of strong clinical suspicion for clinical rejection, a biopsy will be taken and the animal started on a course of steroids (IV 40 mg bolus x 2 days, followed by gradual taper to 1 mg/day maintenance after 14 days) due to the turnaround time of histological samples in a laboratory setting (~1-2 weeks).

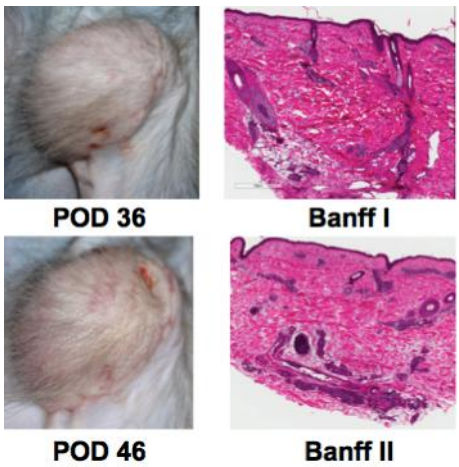

Figure 1. Development of acute skin rejection of VCA in M6514.

We then reviewed our earlier experience in M1413, M4213 and M6014 (all full MHC mismatched recipients) from Year 2 and observed that rejection first developed *clinically* around POD 30 with signs such as focal erythema, maculopapular rashes etc. Steroid treatment at this time point was often inadequate as demonstrated in M4213 (upper extremity VCA), which eventually lost the allograft due to steroid-resistant rejection and has previously been reported clinically in hand transplant patients (Schneeberger et al, 2004). In M6014, following clinical rejection on POD 28, it developed a second and third episode on POD 48 and 78. Indeed, M6514 had progression/recurrence of rejection from Banff I (POD 36) to II (POD 46) and required another bolus of IV steroids before subsequent taper, as per our protocol.

These observations led us to perform an additional, surveillance biopsy during the second post-operative week in the last four animals to investigate whether equivocal, sub-clinical appearance of rejection in the VCA would correlate to Banff rejection on histology.

M3815 (Haploidentical Recipient)
Similar to our previous haploidentical recipient (M6714) in the last annual report, this animal managed to go through 2 months on the same immunosuppression regimen without the development of acute rejection both clinically and on histology.

M4415, M4515 (Full MHC-Mismatched Recipient)
Introduction of the additional surveillance biopsy due to equivocal appearance of the VCA indeed, led to a diagnosis of Banff II and I rejection in M4415 and M4515 on POD 15 and 14 respectively (Figure 2). Interestingly, our treatment regimen of steroid bolus and subsequent taper at this time point successfully avoided both progression and recurrence of rejection episodes (clinically and on histology) up to POD 60.

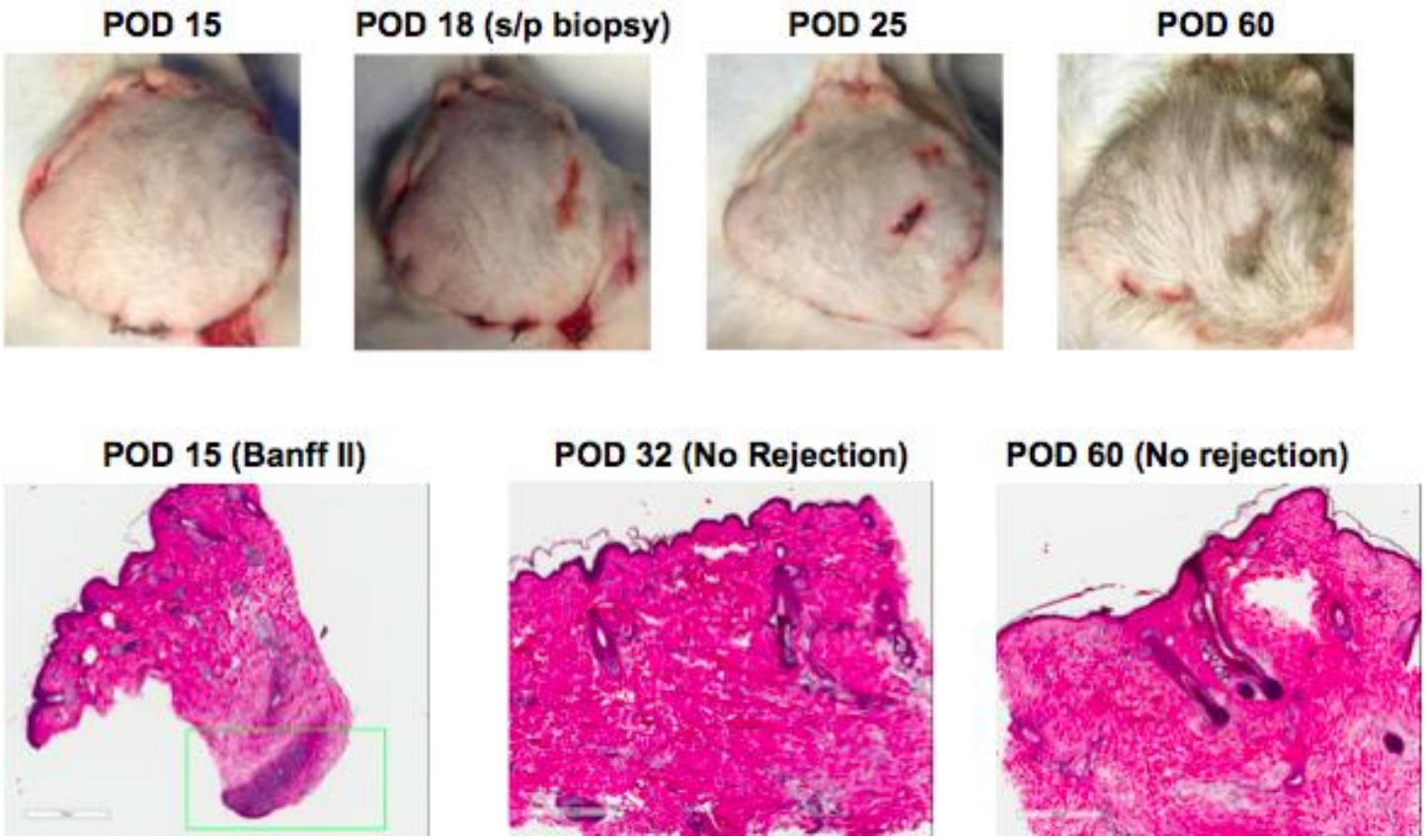

Figure 2. Clinical course of M4415 and corresponding histology. Slight hint of rejection at approximately 2 and 7 o'clock position on POD 15; 2 o'clock position biopsied and revealed Banff II rejection in the deep dermis. Steroid bolus and subsequent taper was sufficient in achieving both clinical and histological resolution, and avoidance of recurrence and/or progression up to POD 60.

M3516, M3316 (Haploidentical Recipients)

By this point of the study, we have established that acute skin rejection of VCAs appears to be inevitable by week 2 across full-MHC mismatch barriers, despite adequate levels of systemic immunosuppression. However, using the same regimen, previous haploidentical recipients (n=2) remained rejection-free up to POD 60-79.

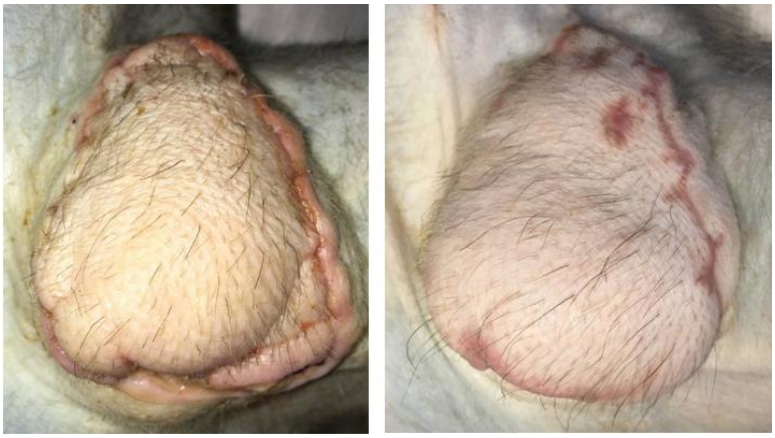

Figure 3. Clinical appearance of VCA in M3516. (*Left*) No clinical suspicion of rejection on POD 12 but histology returned as Banff I. (*Right*) POD 32 view.

M3516 developed Banff I rejection on POD 12 when systemic levels of FK506 were 15.1 (target 20-30) ng/mL and the clinical appearance of the VCA was not suspicious for rejection (Figure 3), and resolved with re-establishment of FK506 levels within our target range. M3316 developed Banff I rejection on POD 27 when systemic levels of FK506 were 23.9. This suggests that MHC sharing appears to confer a protective effect, on top of systemic immunosuppression, in preventing or delaying the early development of acute rejection in VCA. However, this effect does not allow successful rejection-free survival on lower maintenance immunosuppression.

- ***Analysis of acute skin rejection episodes during delay period on maintenance immunosuppression***

Building on our Year 2 work, we have now obtained *in vitro* data to suggest a possible mechanism responsible for acute skin rejection based on 10 recipient animals (both hand and face VCA) with adequate follow-up. We previously observed that by the first protocol biopsy on POD 30, the majority (> 90%) of CD4+ and CD8+ skin leukocytes within the dermis of a VCA were recipient-origin. The kinetics of leukocyte turnover in the epidermis was slower, with an average of ~60% CD207+ Langerhans cells of donor-origin at this time point. Our additional VCA biopsies have now shown that ~ 90% Langerhans cells were donor-origin at week 2 but by this time, there was already massive infiltration and turnover (also > 90%) of the leukocytes within the dermis to recipient-origin cells (Figure 4).

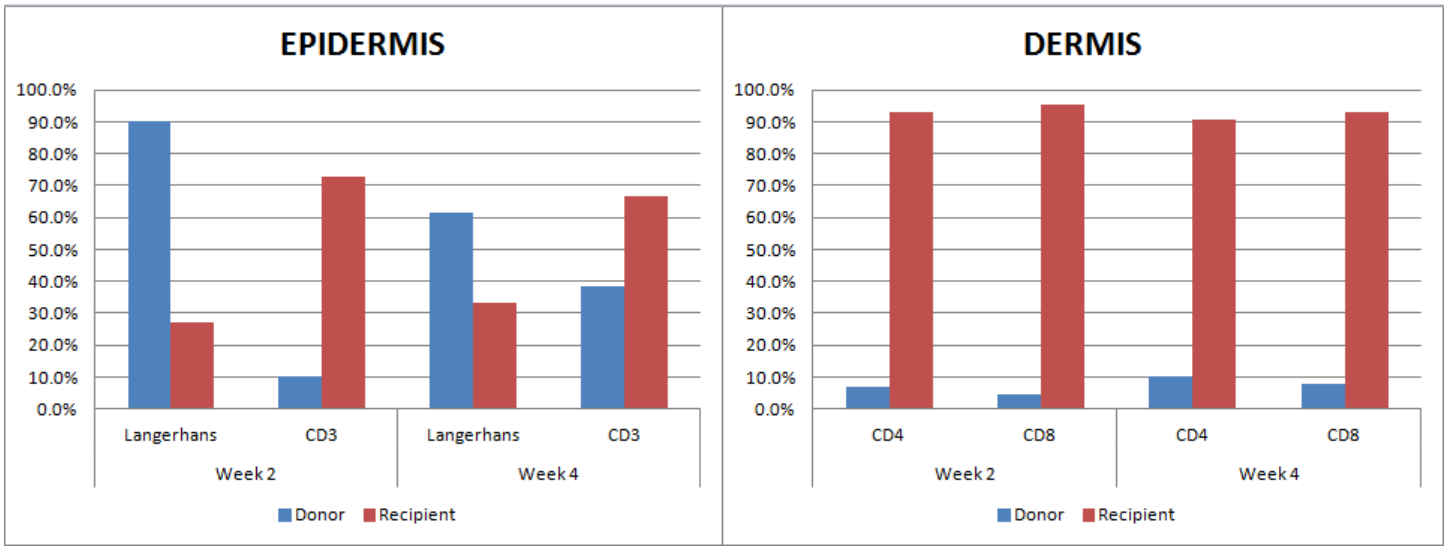

Figure 4. *In vitro* analysis of leukocytes from VCA skin biopsies. Donor-derived Langerhans cell populations are near 90% at week 2 in the epidermis, with corresponding CD4 and CD8 populations > 90% by this time in the dermis. By week 4, the donor-derived Langerhans cell population decreases to ~ 60% but the CD4 and CD8 dermal populations remain at > 90%.

In other words, we believe that the Langerhans cells in VCA epidermis are acting as antigen-presenting cells and stimulating an allo-immune response. This is evidenced by the recruitment of recipient-origin leukocytes into the dermis where the vascular network is more robust. Unfortunately, the cellular yield from the preparatory process of VCA skin biopsy samples does not allow further characterization of cellular subsets or mechanistic studies, but we have reason to believe that these recipient-origin leukocytes are skin resident, effector-type memory T cells based on a similar study on clinical samples of human face VCA patients (Lian et al, 2014).

As before, there was no evidence of allo-antibody formation (data not shown) in recipient animals that developed acute rejection episodes and received steroid treatment prior to delayed bone marrow infusion.

- ***Outcomes analysis following delayed tolerance induction***

We have focused our work on Aim 2 Task 2 in view of our institution's results in achieving tolerance of lung allografts through the same delayed tolerance induction protocol (Tonsho et al, 2015) because this regimen has proven successful in generating persistent mixed chimerism.

M6514 (Full MHC-Mismatched Recipient)
This recipient animal did not receive enough donor bone marrow (BM) cells (50 x 10^6 cells/kg; target dose 3 x 10^8 cells/kg) due probably to the storage process. At approximately 2 weeks after induction (i.e. BM infusion), it was found to have respiratory distress due likely, in part, to neutropenic sepsis from both the irradiation process and tocilizumab (known side effect). Corresponding WBC counts following conditioning were 1.5 → 1.5 → 2.0 → 0.6. Although fluoroquinolone-based antibiotic prophylaxis was given as per clinical practice (Simondsen et al, 2013), this was evidently inadequate coverage for NHPs. No evidence of mixed chimerism was detected but the VCA remained rejection-free at the experimental end point of POD 81.

In consultation with our transplant infectious disease experts at MGH, we revised our antibiotic prophylaxis regimen to IV vancomycin and IM cefepime.

M4415 (Full MHC-Mismatched Recipient)
This recipient animal received the target dose of donor BM cells and was progressing according to the protocol. However, around 2 weeks after induction, there was a noticeable growth in size of the VCA itself. Other pertinent clinical findings were scrotal and bilateral lower limb edema, poor appetite and melenic stools. Despite conservative treatment with loop diuretics and omeprazole, a rectal mass was palpated and identified on ultrasound examination under sedation. In consultation with the veterinarian, a clinical diagnosis of PTLD was made in view of our previous experiments and the animal had to be euthanized as the growth of the mass was rapid. Again, the VCA remained rejection-free at the experimental end point of POD 76 but no evidence of mixed chimerism had developed. Final histopathology was indeed PTLD with local invasion of the surrounding soft tissues and regional and distant metastases to the para-aortic and mesenteric lymph nodes.

M4515 (Full MHC-Mismatched Recipient)
This recipient animal received the target dose of donor BM cells and received all dosages of anti-CD8, 5c8 (co-stimulatory blockade), and tocilizumab. Infective complications were avoided with our new antibiotic prophylaxis regimen of vancomycin and cefepime. This animal was successfully weaned off immunosuppression and was completely off for 25 days before it also developed PTLD and had to be euthanized on POD 115.

Final histology at experimental end-point revealed Banff I rejection although there were no clinical signs of rejection. Similarly, *in vitro* analysis failed to detect any evidence of mixed chimerism throughout all time points following donor BM infusion. Of note, skin leukocyte analysis at POD 90 showed that in the epidermis,

93.7% of Langerhans cells and 96.5% of CD3+ cells were recipient-derived; in the dermis, 99.9% of CD4+ cells and 98.6% of CD8+ cells were recipient-derived.

M3815 (Haploidentical Recipient)
This recipient animal remained rejection-free during the 2-month delay period and completed tolerance induction successfully with no adverse events. Following the weaning of immunosuppression, all medications were withdrawn for a total of 36 days before clinical rejection developed on POD 126, which was confirmed as Banff II on histology. In view of the possibility of a waxing and waning course before tolerance was fully established, we decided to treat the rejection episode by reinstating FK506, and a steroid bolus and subsequent taper (as per our protocol for treating rejection). The VCA was salvaged and on follow-up biopsy on POD 143, both clinical and histological resolution was achieved. We then withdrew immunosuppression again but this time, only about 2 weeks had lapsed before rejection recurred on POD 172 (Banff II). Again, we treated with FK506 and steroids but on further biopsy on POD 194, rejection persisted at Banff II. After consultation with the veterinarian, we decided not to treat this animal further and to let the VCA reject completely before euthanizing on POD 224 (Figure 5).

In vitro analysis again, failed to detect any evidence of mixed chimerism. Of note, skin leukocyte contributions demonstrated a gradual turnover of epidermal Langerhans and CD3+ cells from donor- to recipient-origin. In the dermis, the percentage of CD4+ and CD8+ leukocytes were > 90% recipient-origin throughout the period of study. Corresponding MLR assays showed that following donor BM infusion, there was a reduction in anti-donor and anti-third party responses, which demonstrates the immunomodulatory effect of donor BM cells. However, in spite of such *in vitro* donor-specific unresponsiveness, clinical rejection still developed. Moreover, terminal samples of the VCA demonstrate on histology that the animal had developed chronic rejection with evidence of graft vasculopathy with both bony and muscular invasion and C4d deposition.

M3516, M3316 (Haploidentical Recipients)
Both M3516 and M3316 completed recipient conditioning and BMT uneventfully. M3516 was withdrawn from all immunosuppression for 8 days when it started to autophage its VCA. Despite operative intervention, the animal continued to attack its own VCA and had to be euthanized after consultation with the attending veterinarian. Final histology revealed no evidence of rejection. M3316 was similarly withdrawn from all immunosuppression but was noted to have developed a rapidly growing soft tissue mass from within the VCA; again, this animal had to be euthanized and removed from study and final histopathology indeed, was confirmatory for PTLD. Unfortunately, mixed chimerism could not be detected throughout all time points in both animals.

Summary Tables

Table 1. Study outcomes on initial cohort of animals on 4-month maintenance immunosuppression.

Recipient	VCA	MHC-Mismatch	Complications	Average FK506	Allo-Antibody	Survival	Creatinine Glucose	Reason for Euthanasia
M1413	Hand	Full	Banff II (POD 97)	28.8 ng/mL (16.7 – 40.2)	No	> POD 120	0.6 – 0.9 53 – 157	–
M4213	Hand	Full	Banff I (POD 30)	25.7 ng/mL (5.7 – 48.7)	No	POD 51	1.1 – 1.4 47 – 175	VCA loss (Banff IV)
M6014	Face	Full	Banff 0 (POD 19, 48, 78)	23.3 ng/mL (9.0 – 38.9)	No	POD 107	0.6 – 1.1 74 - 157	PTLD
M6714	Face	Haplo-identical	–	18.7 ng/mL (8.7 – 47.5)	No	POD 79	0.8 123	PTLD

MHC = major histocompatibility complex, POD = post-operative day, PTLD = post-transplant lymphoproliferative disorder, VCA = vascularized composite allograft.

Table 2. Study outcomes on subsequent cohort of animals on 2-month maintenance immunosuppression.

Recipient	VCA	MHC-Mismatch	Complications	Average FK506	Allo-Antibody	Survival	Creatinine Glucose	Reason for Euthanasia
M6514	Face	Full	Banff I (POD 36) Banff II (POD 46)	21.2 ng/mL (9.6 – 33.0)	No	Received BMT	0.6 – 0.8 59 – 151	–
M4415	Face	Full	Banff II (POD 14)	20.5 ng/mL (9.0 – 29.8)	No	Received BMT	0.6 – 0.8 94 – 131	–
M4515	Face	Full	Banff I (POD 15)	23.4 ng/mL (10.2 – 34.8)	No	Received BMT	0.7 – 0.9 73 – 140	–
M3815	Face	Haplo-identical	–	29.1 ng/mL (17.0 – 40.6)	No	Received BMT	0.7 – 0.9 73 – 115	–
M3516	Face	Haplo-identical	Banff I (POD 12)	21.2 ng/mL (10.2 – 33.7)	No	Received BMT	1.0 – 1.1 111 – 131	–
M3316	Face	Haplo-identical	Banff I (POD 27)	23.1 ng/mL (11.0 – 34.3)	No	Received BMT	0.7 – 0.7 114 – 119	–

BMT = bone marrow transplantation, MHC = major histocompatibility complex, POD = post-operative day, PTLD = post-transplant lymphoproliferative disorder, VCA = vascularized composite allograft.

Table 3. Study outcomes after delayed bone marrow transplantation

Recipient	MHC-Mismatch	Donor BMT (% of target dose)	Survival	Days Off All immunosuppression	Reason for Euthanasia
M6514	Full	16	POD 74	No	Neutropenic sepsis
M4415	Full	100	POD 76	No	PTLD
M4515	Full	67	POD 119	25	PTLD
M3815	Haploidentical	96	POD 224	36	Chronic rejection
M3516	Haploidentical	63	POD 100	10	Self-mutilation
M3316	Haploidentical	76	POD 93	4	PTLD

BMT = bone marrow transplantation, POD = post-operative day, PTLD = post-transplant lymphoproliferative disorder

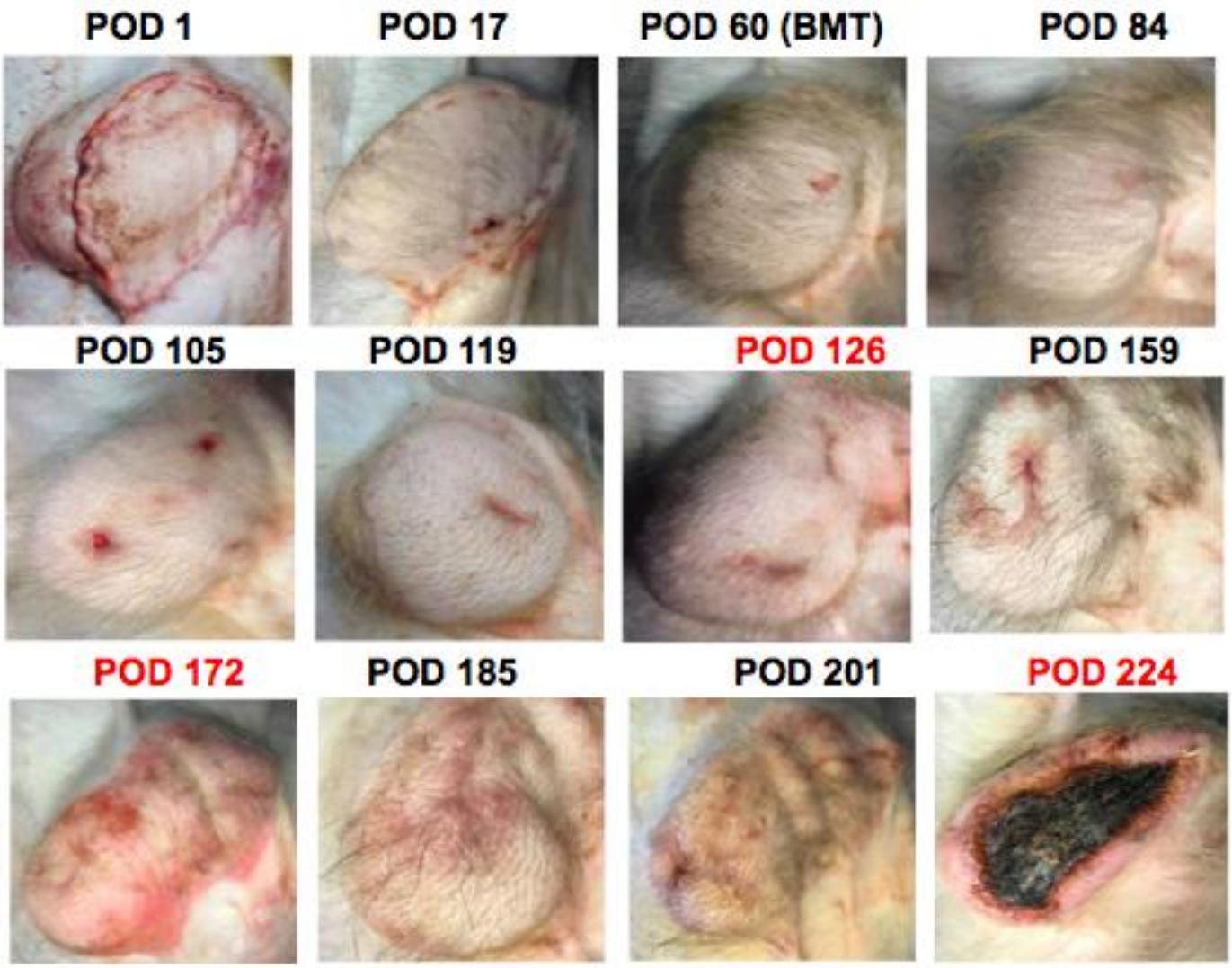

Figure 5. Clinical course of M3815.

iii. Changes/Problems

Development of PTLD After Bone Marrow Infusion

The high incidence of PTLD (5/10, 50%) in MHC-mismatched NHP recipients is especially concerning in this study. For the protocol to be translatable to the clinic, it should be safe when applied across full MHC-mismatch barriers. This is because clinically, the extent of HLA matching is unpredictable and allograft size and color currently take precedence. Previous studies in the field have attributed the high incidence of PTLD in NHP studies of VCA to simian lymphocryptovirus (LCV) which is genomically equivalent to human EBV (Barth et al, 2009). Unfortunately, testing of LCV status pre-transplant is not widely available and would presumably add on further direct experimental costs (Blossom 2007). Post-BMT, a bridging course of cyclosporine is administered to prevent early rejection of the VCA while allowing the HSCs to engraft and develop mixed chimerism. However, studies have since shown that in lymphodepleted recipients, cyclosporine prevents T cell activation and would inadvertently, allow replication of B cells infected with LCV/EBV, which probably accounts for the high incidence of PTLD seen in this study. Future studies ought to consider the use of MMF, an antiproliferative agent, additional rituximab for B cell depletion, or to replace ATGAM (largely T cell depletion) with alemtuzumab (depletes both T and B cells).

Failure to Develop Mixed Chimerism & Development of Chronic Rejection

Our laboratory has previously shown that stable mixed chimerism is required for long-term tolerance of VCA in a swine model across single haplotype full MHC mismatch barriers (i.e. haploidentical recipients) (Leonard et al, 2014). Using the same swine model, we have also shown that the alternative hypothesis of transient mixed chimerism does not allow tolerance of VCAs (Leto Barone et al, 2015). Therefore, the failure to develop stable mixed chimerism with resulting rejection in our NHP study is not unexpected. The infusion of donor BM cells would technically be equivalent to transient mixed chimerism because we were able to detect the presence of these cells during the time of BM infusion (Figure 6). However, the immunomodulatory effect of donor BM cells was not long lasting and most likely, resulted in rejection once its effect had worn off.

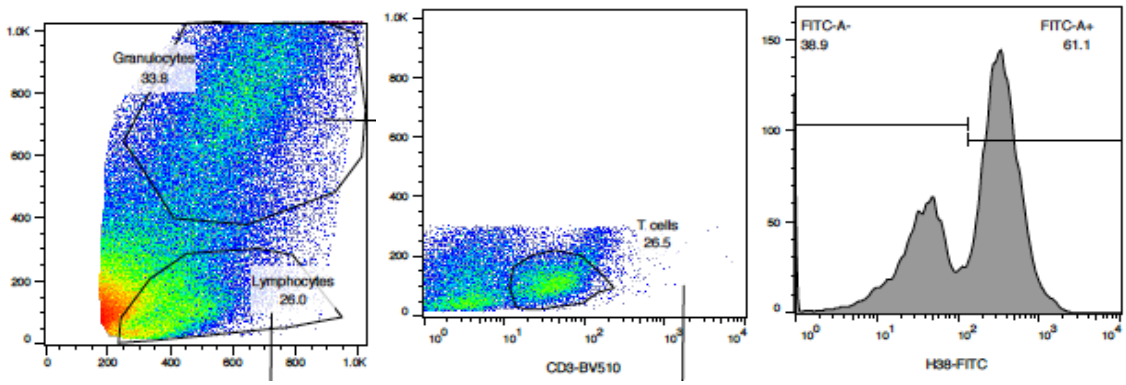

Figure 6. Evidence of donor-origin (H38-) cells within the circulation after infusion of donor bone marrow cells. Gating from left to right.

The identification and development of chronic rejection in our NHP model (Figure 7) is also timely with recent similar reports emerging from long-term follow-up of VCA patients (Kanitakis et al, 2016). The aggressiveness and velocity of rejection starting from deeper tissues mirrors the clinical experience and lends further support to the need for successful tolerance induction strategies.

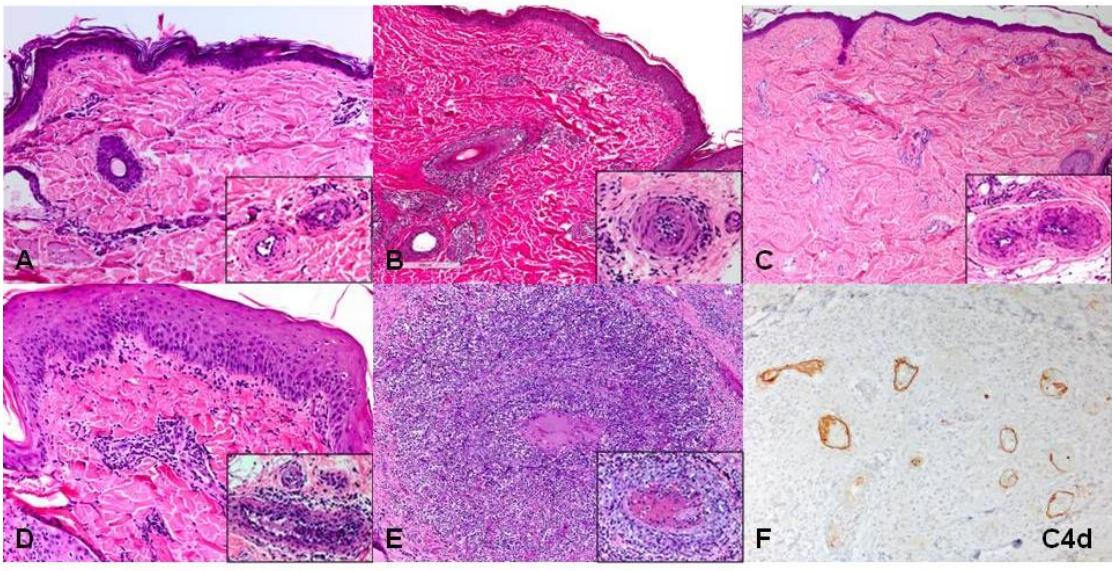

Figure 7. Serial VCA biopsies of M3815: (A) Biopsy at POD92 showing no evidence of rejection. (B) Biopsy at POD126 showing acute cell-mediated rejection characterized by moderate perivascular inflammation with mild epidermal involvement and endothelialitis (inset). (C) Resolution of rejection at POD143. Inset shows a subcutaneous artery with chronic allograft vasculopathy. (D) Biopsy at POD172 showing acute cellular

rejection with moderate perivascular inflammation, epidermal involvement and prominent endothelialitis in arterioles. (E) Graftectomy at POD224 showing severe transmural vasculitis and chronic allograft vasculopathy (not shown). Frank epidermal necrosis was also present (not shown). (F) C4d staining was positive.

iv. Discussion

The technical feasibility of performing VCA for restoration of upper extremity, craniofacial and most recently, genitourinary loss, is no longer in question. However, the requirement for and consequent sequelae of life long systemic immunosuppression remains unabated. Most recently, the development of chronic rejection has also come to attention, in contrast to early enthusiasm in the field when it was thought the presence of the vascularized bone marrow component as part of the VCA may mitigate or even prevent the development of chronic rejection while simultaneously, reducing overall immunosuppression requirements.

First, we have shown in Aims 1 and 2 that the sharing of MHC antigens confers additional protection through mechanism(s) that remain unknown, against the development of acute skin rejection of VCAs within a limited timeframe. We also demonstrated that recipient-derived CD4+ and CD8+ skin leukocytes are most likely responsible for rejection episodes, and have almost completely (>90% of biopsy samples) infiltrated the VCA from as early as the second post-operative week. This result, to the best of our knowledge, is the first to show that MHC sharing leads to predictable outcomes on the incidence and development of acute rejection of VCAs, and is in line with Joseph Murray's observations that skin transplants performed between closely related patients tend to survive longer. We have since secured funding for another grant to explore local delivery of FK506 to mitigate such graft infiltration and turnover of skin leukocytes with the aim of reducing the overall burden of immunosuppression in the recipient. Alternatively, future studies may be designed to reduce CD207 populations within the epidermis such as topical anti-CD207 formulations to prevent antigen presentation and the subsequent allo-immune response.

Second, through Aims 1 and 2, the incidence of PTLD both before and after tolerance induction remains unacceptably high in this NHP model of VCA. While LCV testing is not routine, an alternative strategy may be the use of alemtuzumab for induction rather than ATGAM because the latter is associated with a 71% increased risk of subsequent development of PTLD (Cherikh et al, 2003). Implementation of this change would however, require switching from Mauritian to Indonesian strain NHPs due to the propensity of Mauritian strain macaques to develop hemorrhage following alemtuzumab administration (Hale et al, 1983).

Third, the failure to develop delayed mixed chimerism in Aim 2 in the context of VCA was surprising. Perhaps this protocol based on *in vivo* upregulation of regulatory T cells is not as robust as previously reported (Tonsho et al, 2015). Alternatively, rejection episodes may have led to further proliferation of memory T cell populations that act in some way to prevent successful engraftment after donor BM cell infusion. We also note that there was barely any gross thymic tissue visible on necropsy. This suggests that age-related thymic involution may negate successful mixed chimerism-based tolerance induction strategies because of the failure to induce both central and peripheral tolerance.

Finally, techniques to further isolate and characterize the skin leukocyte sub-populations within a VCA are urgently required. It is possible that the infiltrating leukocytes become resident within VCA dermis and do not recirculate i.e. tissue resident memory T cells. If so, it is not inconceivable that even if stable mixed chimerism was established, circulating cells may not recognize these memory T cells as being allo-reactive and they may not become tolerized. On the flip side, identification of particular leukocyte subsets may provide insight into possible mechanism(s) and the culprit behind rejection episodes. Future strategies targeted at such particular leukocyte subsets may then negate the need for systemic tolerance approaches.

KEY RESEARCH ACCOMPLISHMENTS

The following represent key accomplishments of this research during this reporting period:

- *Optimization of the delayed tolerance induction protocol in non-human primates.*
    - By reducing the delay period from the original 4 months to 2 months, we have reduced the number of acute rejection episodes and were able to avoid rejection completely in haploidentical recipients. None of the recipient animals were lost to PTLD during this delay period either.
- *Short-to-medium term immunosuppression-free survival.*
    - We have shown, for the first time, successful short-to-medium term withdrawal of immunosuppression in a clinically relevant NHP model of VCA. Successful engraftment strategies would likely be required to achieve successful tolerance although alternative strategies such as a local tolerance approach through intra-graft delivery of FK506 may have to be considered.
- *Chronic rejection develops in the absence of mixed chimerism.*
    - Clinically, several VCA patients have received variable doses of donor BM cells but none have developed mixed chimerism. This may be attributed, in part, to the timing of BM infusion, and the lack of recipient conditioning due to concerns over the risks of myeloablation. We have shown that even when all of the above have been addressed, reliable engraftment of donor BM for mixed chimerism to develop remains a particular challenge and when unsuccessful, the immunomodulatory effect of donor BM and tocilizumab unsurprisingly, does not negate the subsequent development of chronic rejection. Our study has also shown that chronic rejection may result from a combination of acute cellular and antibody-mediated rejection (based on C4d staining) in the absence of circulating DSA; in other words, the ensuing graft vasculopathy may have developed in response to non-MHC antigens and represents a new avenue for further investigation in VCA.
- *Development of PTLD in VCA.*
    - The high, overall incidence of PTLD (5/10, 50%) was reported in MHC-mismatched NHP recipients. Analysis of a terminal tissue samples from M3316 demonstrated PTLD of recipient-origin (H38- by chimerism FACS), CD20+ B cells. Clinical risk factors for PTLD development have been previously reported in the literature and have, unsurprisingly, been similarly confirmed in the context of VCA in this study – prolonged immunosuppression (Schmidtko et al., 2002), T-cell depletion (ATGAM), calcineurin inhibitor therapy (cyclosporine, tacrolimus).

CONCLUSION

The induction of transplant tolerance for reconstructive transplantation would be of considerable benefit to civilian victims of disabling and disfiguring tissue loss, and of significant importance to military victims of upper extremity and/or craniofacial trauma. Currently, the necessity of life-long immunosuppression and regular medical monitoring would prevent recipients of restorative transplants (such as hand or face transplant) from returning to active duty, but a safe and effective protocol for induction of transplant tolerance holds the potential to fundamentally change this paradigm.

Introduction of novel protocols of this type to clinical practice clearly requires the highest degree of rigor during pre-clinical testing prior to translation to clinical trial. Consistent with this, research in large animal models is challenging, and unsurprisingly we have faced a number of significant challenges throughout this 3-year study. However, despite this, progress toward our aims has been steady, the challenges met have been carefully analyzed and corrective action plans determined and implemented. Furthermore, improvements in our ability to isolate and analyze immunologically active cells from small volume skin biopsies is an important development which can be expected to facilitate significant insights into the mechanisms operational in VCA acceptance under immunosuppression, rejection and tolerance, as this work is expanded on in future grants.

Overall, despite the obvious challenges encountered, we are encouraged by our overall progress as reported. Successful proof-of-concept of delayed tolerance induction in future studies would be a huge achievement in view of the recent setbacks to the field with the development of chronic rejection.

PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

1. Lay Press:
Nothing to report

2. Peer-Reviewed Scientific Journals:

Ng ZY, Read C, Kurtz JM, Cetrulo CL Jr. Memory T cells in vascularized composite allotransplantation. Vasc Compos Allotransplantation 2015;2(4):75-79.

Lellouch AG, Ng ZY, Kurtz JM, Cetrulo CL Jr. Mixed chimerism based regimens in VCA. Curr Transpl Rep 2016;3(4):390-4.

Ng ZY, Lellouch AG, Drijkoningen T, Chang IA, Sachs DH, Cetrulo CL Jr. Vascularized composite allotransplantation - an emerging concept for burn reconstruction. Accepted for publication in J Burn Care Res.

3. Invited Articles:

Cetrulo CL Jr, Ng ZY, Winograd JM, Eberlin KR. Current concepts in microsurgery: the advent of vascularized composite allotransplantation. Accepted for publication in Clin Plast Surg.

4. Abstracts:

Ng ZY, Lellouch AG, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Jr. Immunomodulation in pediatric vascularized composite allotransplantation – preliminary results in a non-human primate model with tocilizumab. Poster Presentation. 8th Annual MassGeneral Hospital for Children Research Day, Boston, MA; 29 March 2016

Ng ZY, Leonard DA, Defazio MW, Heroux ZW, Kurtz JM, Cetrulo CL Jr. Challenges of triple immunosuppression in non-human primate models of vascularized composite allotransplantation. Oral Presentation. 61st Annual meeting of the Plastic Surgery Research Council, New York, NY; 21 May 2016

Ng ZY, Lellouch AG, Defazio MW, Powell H, Leonard DA, Heroux ZW, Cetrulo CL Jr, Kurtz JM. Analysis of acute skin rejection in non-human primate models of face and hand allotransplantation. Oral Presentation. 57th Annual meeting of the New England Society of Plastic and Reconstructive Surgeons, Bretton Woods, NH; 11 June 2016 ***(Shortlisted for Joseph E. Murray, M.D. Award)***

Ng ZY, Lellouch AG, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Jr. Towards tolerance of facial allotransplantation – preliminary results in a non-human primate model with tocilizumab. Poster Presentation. Military Health Sciences Research Symposium, Kissimmee, FL; 17 August 2016

Ng ZY**,** Defazio MW, Powell H, Leonard DA, Heroux ZW, Lellouch AG, Cetrulo CL Jr, Kurtz JM. Analysis of acute skin rejection in non-human primate models of face and hand allotransplantation. Oral Presentation. 26th International Congress of The Transplantation Society, Hong Kong; 23 August 2016

Ng ZY, Lellouch AG, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Jr. Immunomodulation in vascularized composite allotransplantation – preliminary results in a non-human primate model with tocilizumab. Oral Presentation. American Society of Plastic Surgeons Annual meeting, Los Angeles, CA; 24 September 2016 ***(Awarded Outstanding Paper Presentation in Research & Technology Track)***

Ng ZY, Lellouch AG, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Jr. Delayed bone marrow infusion allows prolonged immunosuppression-free survival of vascularized composite allografts in a non-human primate model. Oral Presentation. Northeastern Society of Plastic Surgeons Annual meeting, Baltimore, MD; 14 October 2016

Ng ZY, Defazio MW, Powell H, Leonard DA, Heroux ZW, Lellouch AG, Schol I, Kurtz JM, Cetrulo CL Jr. Acute skin rejection in non-human primate models of face and hand allotransplantation: before and after tolerance induction. Oral Presentation. 5th Biennial meeting of the American Society for Reconstructive Transplantation, Chicago, IL; 4 November 2016

Ng ZY, Lellouch AG, Defazio MW, Heroux ZW, Schol IM, Kurtz JM, Cetrulo CL Jr. Towards tolerance of facial allotransplantation – preliminary results in a non-human primate model with tocilizumab. Oral Presentation. 5th Biennial meeting of the American Society for Reconstructive Transplantation, Chicago, IL; 4 November 2016

INVENTIONS, PATENTS AND LICENSES
Nothing to report.

REPORTABLE OUTCOMES

1. Determined that acute skin rejection develops in a predictable manner in full MHC-mismatched recipients despite adequate immunosuppression levels

2. Demonstrated that acute skin rejection, when diagnosed early, can be treated successfully with avoidance of early recurrence with a steroid bolus and subsequent taper regimen

3. Identified that sharing of MHC antigens (i.e. haploidentical recipients) can successfully avoid the development of acute skin rejection using the same immunosuppression regimen; however, this does not allow maintenance immunosuppression at lower dosages

4. Failure of tolerance induction (i.e. no mixed chimerism) leading to rejection can be salvaged with reinstatement of tacrolimus and steroids, but does not mitigate chronic rejection

OTHER ACHIEVEMENTS

Our team has received the following awards:

- Royal College of Physician and Surgeons of Glasgow College Traveling Fellowship (Ng ZY)
- American Society for Surgery of the Hand Annual Meeting Scholarship (Ng ZY)
- American Society of Plastic Surgeons Best Abstract (Ng ZY, Lellouch AG)
- Assistance Publique des Hopitaux de Paris (Lellouch AG)
- Medical Z (Lellouch AG)
- American Society of Plastic Surgeons Outstanding Paper Presentation in Research & Technology Track (Cetrulo CL Jr)

Post-doctoral research fellow Ng ZY was also invited as a guest speaker at the 1st Face and Hand Transplant Update back in his home country of Singapore.

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APPENDICES

- Draft manuscripts (x 2) prepared for submission to *American Journal of Transplantation*

Delayed Tolerance Induction Protocol to Vascularized Composite Allografts in Non-Human Primates: First results after by bone marrow transplantation

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Running Title: Induction of tolerance to VCAs in Non-Human Primates

Keywords: Vascularized composite allograft, bone marrow transplantation, transplant tolerance, mixed chimerism, post transplant lymphoma disease, delayed induction protocol.

This work was presented, in part, at the 61st annual meeting of the Plastic Surgery Research Council, New York, NY, 21 May 2016; 57th annual meeting of the New England Society of Plastic and Reconstructive Surgeons, Bretton Woods, NH, 11 June 2016; Military Health Sciences Research Symposium, Kissimmee, FL, 17 August 2016; 26th international congress of the Transplantation Society, Hong Kong, 23 August 2016; annual meeting of the American Society of Plastic Surgeons, Los Angeles, CA, 24 September 2016; and the 5th biennial meeting of the American Society of Reconstructive Transplantation, Chicago, IL, 4 November 2016. Surgical Academy, Paris, 16 December 2016.

Abbreviations:

ATG	anti-thymocyte globulin (equine)
DTIP	delayed tolerance induction protocol
FK506	tacrolimus
GvHD	graft versus host disease
IM	intra-muscular
IV	intra-venous
MHC	major histocompatibility complex
MGH	Massachusetts general hospital
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
NHP	non-human primate
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
POD	post-operative day
PTLD	post-transplant lymphoproliferative disorder
SBT	steroid bolus and taper
SOT	solid organ transplantation
VCA	vascularized composite allograft
WBC	white blood count

ABSTRACT

Functional and aesthetic reconstruction after an important defect non-treatable by conventional surgery can now be achieved through vascularized composite allografts (VCAs). For almost 20 years, a large panel of VCA was performed worldwide (ranging from hand to face and penile transplantation). Although initial clinical successes have been achieved with standard immunosuppressive strategies, the first cases of chronic rejection in VCA were reported. In addition, the long-life immunosuppression therapy in healthy recipients to maintain a graft survival increase the risk of developing metabolic disorder, diabetes, malignancy and infection. Therefore, the development of tolerogenic protocols or regimens with minimal maintenance immunosuppression constitutes an indispensable step to extend indications for VCA surgery. Our laboratory is working on a tolerance protocol based on the induction of the mixed chimerism approach in the large animal. We herein report for the first time a clinically relevant non-human primate VCA model using the delayed tolerance protocol.

INTRODUCTION

Vascularized composite allografts (VCAs) constitute a relevant option for the reconstruction of complex trauma and soft tissue loss not amenable to conventional, autologous methods (1) . For almost 20 years, a large panel of VCA was performed worldwide (ranging from hand to face and penile transplantation(2)). The methods including standard immunosuppressive strategies were initially very promising with the success of controlling the early rejection and demonstrate improvement in function and quality of life (3) . Unfortunately it was counterbalanced by the first cases of chronic rejection of VCA (4) reported in France. In addition the prolonged immunosuppressive therapy in healthy recipients to maintain a graft survival increases the risk to develop various systemic disease including metabolic, infectious and malignancy complications. As reported by Lantieri and al (5), episodes of acute rejection become more and more resistant by the usage of conventional immunosuppression therapies and lead to deal with others therapeutic agent (i.e plasmapheresis, rituximab,etc) to attain the control of the immune rejection. However, the strategy to prevent the acute rejection increases the rate of infections and neoplasia in the posttransplant period. Indication of VCA surgery requires the agreement of multidisciplinary team and a perfect coordination between all different specialties involved (6). Indeed the duration of immunosuppression regimen, the related diseases, and the cost of this surgery in our society represent a real challenge nowadays (7). They are determined in specialized VCA centers on a case-by-case basis. Consequently, the development of tolerogenic protocols or regimens with minimal maintenance immunosuppression would reinforce the field of VCA. Thus, the induction of specific tolerance of the VCA in the absence of immunosuppression remains ideal. The mixed chimerism approach represents the most promising pathway because it is the only strategy working on animals (small (8) and large (9)) and humans (clinical trials (10)). It might potentially negate the specter of chronic rejection, and is a proven strategy to enable immunosuppression withdrawal. The chimerism state is defined as a co-existence between the donor and the recipient's derived lymphohematopoietic elements (11).

Recently, one group from our laboratory induced lung allograft tolerance in NHPs by the donor's bone marrow transplantation (BMT) 4 months after the organ transplants (9) .This protocol was proven successful in generating stable mixed chimerism and therefore reached the organ tolerance. Using the same protocol as aforementioned we herein report for the first time in a clinically relevant, non-human primate VCA model the delayed tolerant protocol.

MATERIALS AND METHODS

Animals

Mauritian strain, male cynomolgus monkeys (*Macaca fascicularis*) weighing approximately 5–10 kg were used (Charles River Primates, Wilmington, MA) as donor-recipient (1:1) transplant pairs based on ABO matching and MHC-mismatching. For MHC genotyping, genomic DNA was prepared from peripheral blood mononuclear cells (PBMCs) and panels of 17 microsatellite loci spanning ~5Mb of the MHC were amplified with fluorescent-labeled PCR primers. Fragment size analysis was performed and the microsatellite haplotypes were then converted to MHC genotypes based on previous cloning and sequencing work (12). Cynomolgus Macaques recipients were quarantined for 6 weeks before the surgery.

All surgical procedures and postoperative care of animals were performed in strict accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC) and the Animal Care and Use Review Office (ACURO) of the US Army Medical Research and Materiel Command (USAMRMC).

VCA Techniques

Facial VCAs were similarly performed as described by Barth et al (13). Both models consist of skin, inner gingival mucosa, muscle and bone (including vascularized bone marrow), and allow

investigation of the immune response to VCAs when transplanted under conventional immunosuppressive protocols.

Experimental Design and Immunosuppression Regimen

6 Cynomolgus Macaques monkeys received heterotopic face transplant. VCAs were transplanted in MHC-fullmismatched (n=3) and haploidentical (n=3). All 6 recipients (VCAs) receive induction with equine ATG (ATGAM; Pfizer, New York, NY; IV 50 mg/kg x 3 days on post-operative day (POD) 0, 1 and 2). Maintenance immunosuppression (up to POD 60) consisted of FK506 (IM 0.1 mg/kg BID on POD 0 and 1, then adjusted to keep plasma levels between 20-30 ng/mL), MMF (CellCept; Genetech, San Francisco, CA; IV 300 mg on POD 0, then given parenterally QD mixed into the animal's daily feed provided *ad libitum* with reduction to 100-200 mg QD by POD 14 and maintained to the end of experiment), and methylprednisolone (Solu-Medrol; Pfizer, New York, NY; IV 40 mg on POD 0 and 1, followed by gradual taper over 14 days to IM 1g QD maintenance to the end of experiment). All Monkeys received anti-IL-6R mAb (tocilizumab; Chugai Pharmaceutical Co.; Tokyo, Japan; 10 mg/kg IV on post-LTx Days 0, 7, 14, 21 and 28). Two months later, each monkey underwent BMT, as described below. They received one dose of anti-CD8 mAb (cM-T807, Centocor, Inc., Horsham, PA; 5 mg/kg IV on the day prior the BMT. Following BMT, the recipients were also treated with anti-CD154 mAb (h5C8; 20 mg/kg IV on post-BMT Days 0 and 2, and 10 mg/kg on post-BMT Days 5, 7, 9, and 12). Cyclosporine A (15 mg/kg IM initially, then tapering to 5 mg/kg by post-BMT Day 7) was provided for 28 days following BMT. No further immunosuppression was given. *Figure 1* depicts the overall protocol based on the lung group at MGH.

Non-myeloablative conditioning and bone marrow transplantation (BMT)

Two months after VCA transplantation, monkeys were conditioned with total body irradiation (TBI) (1.5 Gy 6 and 5 days prior to BMT), local thymic irradiation (TI) (7 Gy on the day prior to BMT) and ATG (50 mg/kg/day IV x 3 ending on the day of BMT). Three doses of Ganciclovir (5mg/kg) were given day -2, -1 and 0 of BMT. Also one dose of anti-CD8 5mg/kg was given. Donor bone

marrow cells (3 x 10^8 mononuclear cells/kg IV) were infused as previously described(14)(15). Ketorolac (3 mg, IM) was administered to prevent thrombosis on the day of BMT.

Rejection monitoring and clinical assessment

Clinical macroscopic manifestations of acute rejection can range from mild pink discoloration or erythema to lichenoid papules, edema, and onychomadesis (16). All VCAs were monitored twice daily for the first 72 hours and once daily subsequently. Any suspicion of an acute rejection led to a skin biopsy for histopathological confirmation and presumptive treatment with a steroid bolus and taper (SBT, similar to our maintenance regimen – starting from 40 mg before reduction to 1 mg/day over the ensuing 14 days). Any suspicious of acute rejection is considered as such until proven otherwise. We examined thoroughly on a daily basis the general condition of NHP. The signs of PTLD (17) were general condition deterioration, unusual behavior, curled up position, weight loss, decreased appetite, lying down. In addition, we can notice the appearance of local signs such as: firm, lymph node, bleedings, pitting edema, vomit, and diarrhea.

Histopathological Examination -> Ivy parts

Protocol skin biopsies of the VCA were performed using a standard 6 mm punch biopsy kit at approximately 30-day intervals. Biopsies were closed using running stitches with resorbable sutures Monocryl 3/0 and 4/0. Each animal received painkiller (IM Met Glutamine(R) 2mg/kg) after the procedure. All skin biopsy samples were fixed in formalin and stained with hematoxylin and eosin (H&E). Histopathological examinations were performed by transplant pathologists (I.A.R and R.B.C) blinded to the study. The severity of acute rejection was graded according to the 2007 Banff scale for composite tissues (18).

Identification of cases. Necropsies were performed immediately after euthanasia. All necropsy and biopsy samples from the VCA recipient animals were saved in routine histological sections (formalin fixed, paraffin embedded, stained with H&E) including lymph nodes, VCA, masses found (as well as extra visceral tumor). PTLD was morphologically classified into three categories based on previously published classification systems (19): *polymorphous* (small lymphocytes,

plasma cells, cleaved and noncleaved cells, immunoblasts), *minimally polymorphous* (plasma cells, lymphoid cells with plasmacytic features), and *monomorphous* (large noncleaved cells or small noncleaved cells) types.

Detection of Chimerism

Transplant pairs were pre-selected based on their differential reactivity to MHC class I antigens in NHPs using a specific antibody, H38 (One Lambda, Inc., Canoga Park, CA). Chimerism (percentage of donor leukocytes) was assessed in peripheral blood via flow cytometry twice weekly during the first week, and once every 2 weeks thereafter.

The following antibodies were used to assess chimerism: CD3-FITC (BD Pharmingen, , CD4-PerCP-Cy5.5 (BioLegend), CD8-APC, CD25-PE, FoxP3-AF647, CD11b-PE (BD Pharmingen, , CD20-PerCP-Cy5.5 (BioLegend, , CD154-APC, NKp80-PE, CD28-PE (Life Technologies), CD95-APC (BD Pharmingen), CD45RO-PE-Cy7 (BioLegend), mIgG3-FITC, mIgG1-PE, mIgG2b-PerCP-Cy5.5 rIgG2a-APC (___,___), H38-FITC. Samples were acquired on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Inc., Ashland, OR).

Mixed Lymphocyte Reaction Assay

The systemic immune status of recipient animals was assessed by proliferation dye based-mixed lymphocyte reaction (MLR) assays. Briefly, responder PBMCs (12×10^6 cells/mL) were obtained, washed and labeled with eFluor® 670 (eBioscience, San Diego, CA) before incubating at room temperature for 10 minutes. Stimulator PBMCs ($2x10^6$ cells/mL) were obtained from the donor, recipient, and third-party animals and irradiated. Responder and stimulator PBMCs were then co-cultured at 37^0C in triplicate in 96-well, V-bottom plates at 200 μL/well for 5 days before acquisition on flow cytometry. The dilution of cellular membranes was analyzed to investigate both proliferating and non-proliferating CD3+ lymphocyte populations.

RESULTS

Reconstitution of cells after BMT

Leucocytes:

The absolute number of leukocyte is an important data to monitor to evaluate the risk of bacterial infection after BMT. In our series of 6 NHPs, WBC dropped to less than 2 thousand/mm^3 for 24 days in average (*Figure 2*). At 2 weeks after BMT, M6514 was found to have respiratory distress and an infectious syndrome although fluoroquinolone-based antibiotic prophylaxis was given as per clinical practice (20) . Unfortunately we did not manage to cure him with adequate intensive care. Indeed corresponding WBC counts following conditioning were 1.5 → 1.5 → 2.0 → 0.6 mm^3/L. This was evidently inadequate coverage for NHPs**.** After further discussion with our transplant infectious disease experts at MGH, we revised our antibiotic prophylaxis regimen to IV vancomycin (20mg/kg) and IM cefepime (50mg/kg). The next 5 animals were under this antibiotherapy regimen and no infection occurred afterwards. We also maintained about 1 month of antibiotics, time for the animal to reach a WBC >2 mm^3/L.

Platelet:

The reconstitution occurred after 18 days post BMT (*Figure 3*). Similar outcomes was found in the literature (21). No bleeding reported.

Hemoglobin:

We have no reduction of the hemoglobin level after BMT except for 1 NHP (*Figure 4*). 1 of them was transfused 100ml of blood. Our indication to transfuse is when the hematocrit is less than 20% or Hb < 7g/dL.

Nonmyeloablative conditioning and BMT in a 2 month delayed fashion did not induce stable mixed chimerism in NHPs

Dose of bone marrow infused

Donor bone marrow cells were infused in 6 NHPs (*table 1*). The target dose is 3 x 10^8 mononuclear cells/kg (22). However it was not possible for certain NHPs. M6514 (Full MHC-

Mismatched Recipient) did not receive enough donor bone marrow (BM) cells (50 x 10^6 cells/kg due probably to the storage process.

No chimerism induced

M3815 remained rejection-free during the 2-month delay period and completed tolerance induction successfully with no adverse events. Following the weaning of immunosuppression, all medications were withdrawn for a total of 36 days before clinical rejection developed on POD 126 (*Figure 5*), which was confirmed as Banff II on histology. In view of the possibility of a waxing and waning course before tolerance was fully established, we decided to treat the rejection episode by reinstating FK506, and a steroid bolus and subsequent taper (as per our protocol for treating rejection). The VCA was salvaged and on a follow-up biopsy on POD 143, both clinical and histological resolution was achieved. We then withdrew once more immunosuppression; however rejection recurred on POD 172 (Banff II) only about 2 weeks after. In a same way, we treated with FK506 and steroids but on further biopsy on POD 194, rejection persisted at Banff II. After consulting with the veterinarian, we decided not to treat this animal further and to let the VCA reject completely before euthanizing on POD 224 (*Figure 5*).

In vitro analysis failed to detect any evidence of mixed chimerism (*Figure 6*). Corresponding MLR assays showed that following donor BM infusion, there was a reduction in anti-donor and anti-third party responses, which demonstrates the immunomodulatory effect of the donor's BM cells. However, in spite of such *in vitro* donor-specific unresponsiveness, clinical rejection still developed. Moreover, terminal samples of the VCA demonstrated on histology that the animal had developed chronic rejection with evidence of graft vasculopathy with both bony and muscular invasion and C4d deposition (manuscript in preparation). 5 others NHP did not show any sign of chimerism in the peripheral blood (figure).

Development of PTLD

M4415 developed around 2 weeks after BMT (Figure 7), an increase in size of the VCA itself. It was diagnosed after deterioration of his general condition. Other pertinent clinical findings were

scrotal and bilateral lower limb edema, poor appetite and melenic stools. Despite conservative treatment with loop diuretics and omeprazole, a rectal mass was palpated and identified on ultrasound examination under sedation. In consultation with the veterinarian, a clinical diagnosis of PTLD was made in view of our previous experiments and the animal had to be euthanized. The VCA remained rejection-free at the experimental end point of POD 76. Final histopathology was indeed PTLD with local invasion of the surrounding soft tissues and regional and distant metastases to the para-aortic and mesenteric lymph nodes. M4515 was completely off immunosuppression for 25 days before it was found curled up in his cage with bloody diarrhea. PTLD was diagnosed and led to euthanasia on POD 115 (*Figure 8*). Final histology on experimental end-point revealed Banff I rejection although there were no clinical signs of rejection. Same results for M3316 that was found lying down on his cage with anemic sign (pale, tachycardia). On necropsy we found a disseminated PTLD on the IVC, angle between kidney and IVC compressing ureter. In all cases, their VCA were hard and immobile at the mobilization. Tumors had homogenous gross appearances and histology revealing confluency of lymphocytes and polymorphic features (*Figure 9*). The histologic appearance evaluated by a transplant pathologist was diagnosed as PTLD.

Others

M3516 bit his VCA (*Figure 10*), and was taken back to the operating room for fixing. We performed the debridement of the dirty tissue and washed out using sterile normal saline + betadine ®. Despite operative intervention, the animal continued to attack its own VCA and had to be euthanized after consultation with the attending veterinarian. M3516 was withdrawn from all immunosuppression for 8 days when it started to autophage its VCA. Final histology revealed no evidence of rejection.

DISCUSSION

Chronic Rejection

Case of chronic rejection (5) started to be reported. Contrary to SOT, it is not conceivable to replace a VCA by another VCA. One of the most logical reasons is the time of rehabilitation. For instance the time for a face transplant to move properly is in average 4 years (23). In addition the immunologic (24) and the surgical technique parts constitute the limiting factor. The identification and development of chronic rejection in our NHP model is also timely with recent similar reports emerging from long-term follow-up of VCA patients (25) showing the first case of necrosis on the face .The aggressiveness and velocity of rejection starting from deeper tissues mirrors the clinical experience and lends further support to the need for successful tolerance induction strategies.

Failure of mixed chimerism induction

Our laboratory has previously shown that stable mixed chimerism is required for long-term tolerance of VCA in a swine model across single haplotype full MHC mismatch barriers (i.e. haploidentical recipients) (9). Using the same swine model, we have also shown that the alternative hypothesis of transient mixed chimerism does not allow tolerance of VCAs (26). Therefore, the failure to develop stable mixed chimerism with resulting rejection in our NHP study is not unexpected. The infusion of donor BM cells would technically be equivalent to transient mixed chimerism because we were able to detect the presence of these cells during the time of BM infusion. However, the immunomodulatory effect of donor BM cells was not long lasting and most likely, resulted in rejection once its effect had worn off. The simple fact to infuse donor cells does not seem to induce tolerance. No one NHPs develop any sign of chimerism. This was demonstrated in M3815, which did not respond to aggressive steroid rescue therapy and rejection progressed inexorably to frank necrosis, mirroring the clinical challenge of steroid-resistant rejection in upper extremity VCA (27).

No thymus in NHP were found on necropsy. It can be explained by 2 reasons:

- Too old NHP, 5-6 years in average (m6514 : 4/12/11, m3815 : 1/15/09, m4415 3/23/11, m3516 : 6/21/10, m3316 :1/21/10)

- Effect of the Thymic irradiation (thymic involution)

The failure to develop delayed mixed chimerism in the context of VCA was surprising. Perhaps this protocol based on *in vivo* upregulation of regulatory T cells is not as robust as previously reported(10). Alternatively, rejection episodes may have led to further proliferation of memory T cell populations that act in some way to prevent successful engraftment after donor BM cell infusion. We also note that there was barely any gross thymic tissue visible on necropsy. This suggests that age-related thymic involution may negate successful mixed chimerism-based tolerance induction strategies because of the failure to induce both central and peripheral tolerance.

Development of PTLD in VCA.

The high, overall incidence of PTLD (3/6, 50%) was reported in MHC-mismatched NHP recipients but it was not unexpected based on the literature (28). Analysis of a terminal tissue samples from M3316 demonstrated PTLD of recipient-origin (H38- by chimerism FACS), CD20+ B cells. Clinical risk factors for PTLD development have been previously reported in the literature and have, unsurprisingly, been similarly confirmed in the context of VCA in this study – prolonged immunosuppression (29), T-cell depletion (ATGAM), calcineurin inhibitor therapy (cyclosporine, tacrolimus). Previous studies (30) in the field have attributed the high incidence of PTLD in NHP studies of VCA to simian lymphocryptovirus (LCV), which is genomically equivalent to human EBV . Unfortunately, testing of LCV status pre-transplant is not widely available and would presumably add on further direct experimental costs.

The clinical management of post-transplant nonhuman primates that are receiving multiple immunosuppressive agents can be complicated with the risk of PTLD and other opportunistic infections. The 3 cases presented here serve as potential risk reminders in the setting of potent immunosuppressive therapies, and a guide for veterinary pathologists to consider when evaluating pharmacologically or virally immunosuppressed animals. Post-BMT, a bridging course of cyclosporine is administered to prevent early rejection of the VCA while allowing the HSCs to engraft and develop mixed chimerism. However, studies have since shown that in lymphodepleted recipients, cyclosporine prevents T cell activation and would inadvertently, allow

replication of B cells infected with LCV/EBV, which probably accounts for the high incidence of PTLD seen in this study. Future studies ought to consider the use of MMF, an antiproliferative agent, additional rituximab for B cell depletion, or to replace ATGAM (largely T cell depletion) with alemtuzumab (depletes both T and B cells) (31). The usage of alemtuzumab for induction instead of ATGAM might be preferable as it is associated with a 71% increased risk of subsequent development of PTLD (32). Implementation of this change would however, require switching from Mauritian to Indonesian strain NHPs due to the propensity of Mauritian strain macaques to develop hemorrhage following alemtuzumab administration (33).In our protocol we inject ganciclovir as prophylaxia even if it actively blocks lytic EBV replication *in vitro* through inhibition of the late phase lytic replication. However, neither agent have any effect on EBV in its latent state or on the proliferation of EBV-transformed B cells. The average volume of 7,59 x 106 bone marrow cells from the mandible was 30 times that of fibular segments (2,8 x 106). When calculated in transplanted bone marrow cells per kilogram, composite facial allografts contained 2 x 108 cells/kg compared with 0.08 x 10^8 cells/kg in a model of composite fibular allografts (34). NHP models of organ transplantation are invaluable in the support of strategies for tolerance induction. They allow researchers to study the immune response to transplanted organs, and enable tests of new therapeutic agents before testing on human patients (Haustein et al., 2008). The clinical management of post-transplant non-human primates that are receiving multiple immunosuppressive agents can be complicated by the risk of PTLD and other opportunist infections. Cynomolgus Macaques develop PTLD in other transplant group. We cannot use the rituximab (induce nausea, anorexia…).

SUMMARY

Delayed Tolerance induction protocol has shown to be successful in NHP regarding the lungs and kidney. Transient mixed chimerism seems sufficient to induce tolerance for kidney except for the lungs which require stable mixed chimerism for long term survival. We reported, for the first time, the outcome about the delayed tolerance induction protocol on VCA in NHP.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

FIGURE LEGENDS

Figure 1. Delayed tolerance induction protocol. The sequence and timing of the protocol interventions are depicted relative to the timing of VCA and BMT. The VCA were maintained with conventional triple-drug immunosuppression for 2 months, followed by BMT using a nonmyeloablative conditioning regimen. After BMT, the recipients were treated with short courses of anti- CD154 mAb and anti-IL-6 receptor mAb, a single dose of anti-CD8 mAb, along with a 28-day course of CsA. After this, no further immunosuppression was given.

Figure 2. Chart showing the WBC in function of the time after BMT. The recovering period is around day 24 post BMT.

Figure 3. Chart showing the Platelet in function of the time after BMT. The recovering period is around day 18 post BMT. No bleeding occurred.

Figure 4. Chart showing the Hemoglobin in function of the time after BMT.

Figure 5. Clinical course for M3815 (haplo MHC-mismatch): A) first episode of AR occurring POD 126 Banff II B) recurrence up to POD 172 (Banff II) C) third episode of AR occurring POD 195 Banff II. D) Necrosis aspect POD 224

Figure 6. No evidence of chimerism

Figure 7. M4415 developed PTLD around 2 weeks after BMT. A) an increase in size of the VCA itself, the tumor can be visualized macroscopically (typical aspect) B) Posterior compression of the vena cava by the tumor.

Figure 8. M4515 developed PTLD around 5 weeks after BMT. Appearance of the tumor extra A) and intra B) luminal.

Figure 9. Histological aspect of PTLD A) B cells CD20+ staining B) hematoxylin and eosin showing an infiltration of mononucleal cells.

Figure 10. M3516 , aspect of its VCA after bite.

TABLE LEGENDS

Table 1. Study outcomes on subsequent cohort of animals on 2-month maintenance immunosuppression.

Recipient	VCA	MHC-Mismatch	Complications	Donor bone marrow cells (% of target dose)	Survival (day)	Off immunosuppression	Reason for Euthanasia
M6514	Face	Full	Banff I (POD 36) Banff II (POD 46)	16	74	No	Severe Sepsis
M3815	Face	Haplo-identical	Banff II (POD 126, 172 , 179)	96	224	36	Rejection
M4415	Face	Full	Banff II (POD 14)	100	76	No	PTLD
M4515	Face	Full	Banff I (POD 15, 119)	67	119	25	PTLD
M3516	Face	Haplo-identical	Banff I (POD 12)	63	100	10	Self mutilation
M3316	Face	Haplo-identical	Banff I (POD 27)	76	93	3	PTLD

MHC = major histocompatibility complex, POD = post-operative day, PTLD = post-transplant lymphoproliferative disorder, VCA = vascularized composite allograft.

SUPPORTING INFORMATION

None

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Delayed Induction of Tolerance to Vascularized Composite Allografts in Non-Human Primates: I, Analysis of Acute Skin Rejection While On Maintenance Immunosuppression

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Running Title: Acute Rejection of VCAs in Non-Human Primates

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Abbreviations:

ATG	anti-thymocyte globulin (equine)
FK506	tacrolimus
GvHD	graft versus host disease
IM	intra-muscular
IV	intra-venous
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
NHP	non-human primate
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
POD	post-operative day
PTLD	post-transplant lymphoproliferative disorder
SBT	steroid bolus and taper
SOT	solid organ transplantation
VCA	vascularized composite allograft

ABSTRACT

Reconstruction of extremity amputations and devastating craniofacial injuries can now be achieved through hand and face transplantation (i.e. vascularized composite allografts, VCAs). However, in contrast to solid organ transplantation, the incidence of acute rejection episodes targeted at the skin of VCAs approaches almost 90% in the first year after surgery and requires additional treatment to maintain viability of the allograft. Clinically, various induction, treatment and maintenance immunosuppressive protocols have been described with mixed results. Here, we demonstrate in a clinically relevant, non-human primate VCA model that acute rejection develops in a predictable manner following induction with anti-thymocyte globulin (ATG), tacrolimus, mycophenolate mofetil (MMF) and methylprednisolone prior to allograft perfusion. Post-operative maintenance immunosuppression consisted of tacrolimus, MMF and methylprednisolone. Prompt diagnosis and treatment of rejection episodes with steroid bolus and gradual taper is sufficient in preventing both allo-antibody formation and the early recurrence of rejection. However, prolonged maintenance immunosuppression can result in the development of lethal complications such as post-transplant lymphoproliferative disorder. Haploidentical recipients can remain rejection-free when maintained adequately on the same immunosuppressive regimen, which suggests that MHC sharing may lead to predictable outcomes on acute rejection in VCA.

INTRODUCTION

The transplantation of upper extremity and facial vascularized composite allografts (VCAs) is now an option at select centers for the reconstruction of complex trauma and soft tissue loss not amenable to conventional, autologous methods [1, 2]. Various immunosuppressive protocols have been reported but at the majority of VCA centers, induction therapy with T cell depletion is followed by post-operative maintenance on a triple drug combination of tacrolimus (FK506), mycophenolate mofetil (MMF) and steroids [3, 4]. Nevertheless, despite the use of such established immunosuppressive regimens, up to 85% of both hand and face transplant patients still develop at least one episode of acute rejection targeted at the skin component of the VCA within the first year, and at least 60% experience multiple such episodes [5, 6]. This is in stark contrast to solid organ transplantation (SOT), where the incidence of acute rejection in kidney allografts is less than 10% following transplantation under similar immunologic protocols [7].

Regardless, the development of acute rejection requires prompt recognition and treatment to prevent further progression, allo-sensitization, dysfunction and even loss of the VCA. Multiple treatment strategies have been described including high dose intravenous steroid boluses-only, increasing maintenance immunosuppression dosages, additional topical steroid and/or FK506 ointment (due to direct access to the skin component of the VCA), and further lymphocyte depletion with monoclonal or polyclonal antibodies (e.g. Campath-1, bortezomib, eculizumab) [3–6]. To date, VCA loss from acute rejection has not been reported in patients compliant with treatment. The requirement for additional immunosuppression to treat rejection episodes though, represents further morbidity to a VCA patient who is already chronically immunosuppressed. In turn, the overall burden of immunosuppression and risk of developing systemic complications is increased. Indeed, reno-vascular, infective, metabolic, and neoplastic disorders amongst others, have all been reported in VCA patients [3, 4].

Yet, despite almost 20 years of clinical VCA experience, the development and treatment of acute rejection episodes remains equivocal and poorly understood. Recent studies have demonstrated that human skin contains almost twice the number of leukocytes circulating in peripheral blood [8] and, in a series of clinical face transplant patients, acute rejection episodes were postulated to be accounted for by the persistence of donor-origin lymphocytes from the VCA itself [9]. The analysis of acute rejection in VCA may however, be confounded by variation in the induction and immunosuppressive protocol(s) utilized at different VCA centers [10].

Our laboratory has previously demonstrated the successful attainment of stable mixed chimerism and thus tolerance of VCAs in haploidentical [11] but not full MHC-mismatch recipient swine (unpublished data). Hence, the current study sought to investigate if the same single haplotype MHC mismatch may similarly lead to predictable outcomes on the development of acute rejection of VCAs.

MATERIALS AND METHODS

Animals

Mauritian strain, male cynomolgus monkeys (*Macaca fascicularis*) weighing approximately 5–10 kg were used (Charles River Primates, Wilmington, MA) as donor-recipient (1:1) transplant pairs based on ABO matching and MHC-mismatching [12, 13].

All surgical procedures and postoperative care of animals were performed in strict accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC) and the Animal Care and Use Review Office (ACURO) of the US Army Medical Research and Materiel Command (USAMRMC).

VCA Techniques

Upper extremity VCA procurement and transplantation were performed in non-human primates (NHPs) as previously described [14]. Facial VCAs were similarly performed as described by Barth et al [15].

Experimental Design and Immunosuppression Regimen

Ten cynomolgus monkeys received upper extremity or facial VCAs from MHC-mismatched donors (4 haploidentical recipients, all face VCAs) following induction with equine ATG (ATGAM; Pfizer, New York, NY; IV 50 mg/kg x 3 days on post-operative day (POD) 0, 1 and 2). Maintenance immunosuppression (up to POD 120) consisted of FK506 (IM 0.1 mg/kg BID on POD 0 and 1, then adjusted to keep plasma levels between 20-30 ng/mL), MMF (CellCept; Genetech, San Francisco, CA; IV 300 mg on POD 0, then given parenterally QD mixed into the animal's daily feed provided *ad libitum* with reduction to 100-200 mg QD by POD 14 and maintained to the end of experiment), and methylprednisolone (Solu-Medrol; Pfizer, New York, NY; IV 40 mg on POD 0 and 1, followed by gradual taper over 14 days to IM 1g QD maintenance to the end of experiment) (Figure 1).

Rejection Monitoring and Histopathological Examination

All VCAs were monitored twice daily for the first 72 hours and once daily subsequently. Protocol skin biopsies of the VCA were performed using a standard 6 mm punch biopsy kit at approximately 30-day intervals. Clinical diagnosis of rejection (i.e. increased erythema, swelling, ulceration etc.) required biopsies of the affected area(s) for histopathological confirmation and treatment with a steroid bolus and gradual taper (SBT, similar to our maintenance regimen – starting from 40 mg before reduction to 1 mg/day over the ensuing 14 days; Figure 1).

All skin biopsy samples were fixed in formalin and stained with hematoxylin and eosin (H&E), Histopathological examinations were performed by transplant pathologists (I.A.R and R.B.C) blinded to the study. The severity of acute rejection was graded according to the 2007 Banff Working Classification of Skin-containing Composite Tissue Allograft Pathology [16].

Detection of Chimerism

Transplant pairs were pre-selected based on their differential reactivity to MHC class I antigens in NHPs using a specific antibody, H38 (One Lambda, Inc., Canoga Park, CA). Chimerism (percentage of donor leukocytes) was assessed in peripheral blood via flow cytometry twice weekly during the first week, and once every fortnight thereafter.

The following antibodies were used to assess chimerism: CD3-FITC (BD Pharmingen, cat. 556611), CD4-PerCP-Cy5.5 (BioLegend, cat. 317428), CD8-APC (___,___), CD25-PE (___,___), FoxP3-AF647 (___,___), CD11b-PE (BD Pharmingen, cat. 557397), CD20-PerCP-Cy5.5 (BioLegend, cat. 302326), CD154-APC (___, ___), NKp80-PE (___,___), CD28-PE (Life Technologies, cat. CD2804), CD95-APC (BD Pharmingen, cat. 558814), CD45RO-PE-Cy7 (BioLegend, cat. 304230), mIgG3-FITC (____,____), mIgG1-PE (___,___), mIgG2b-PerCP-Cy5.5 (___,___) rIgG2a-APC (___,___), H38-FITC (___,___). Samples were acquired on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Inc., Ashland, OR).

Mixed Lymphocyte Reaction Assay

The systemic immune status of recipient animals was assessed by proliferation dye based-mized lymphocyte reaction (MLR) assays. Responder PBMCs from recipient animals were labeled with cell proliferation dye eFluor670 (eBioscience). Under vortex agitation, an eFluor670 solution was added to responder PBMCs and incubated at 37^0C for 10 minutes. The responder cells were subsequently washed, counted and re-suspended at $12x10^6$ cells/mL (total 2 mL). Stimulator PBMCs from donor animals ($2x10^6$ cells/mL; total 2 mL) were irradiated at 2500 cGy. Labeled responder and stimulator cells were plated together in 96 well plates (Costar Corning; Lowell, MA) and incubated for 5 days at 370C in 5% carbon dioxide and 100% humidity. Following this, cell proliferation of responder cells was analyzed with flow cytometry.

Measurement of Donor-Reactive Antibodies

This was performed by flow cytometric analysis as described previously [17].

Isolation and Characterization of Skin Resident Leukocytes

Punch biopsies taken from the VCA and host skin were placed overnight in digestion buffer (500 mL RPMI with L-Glutamine, 30 mL of Fetal Bovine Serum, 5.4 mL of 1M Hepes Buffer, 5 mL of DNASE I, 5 mL of Pen/Strep and 5 mL primacin) and 10% dispase. The following day, the epidermis was separated from the dermis using a pair of fine forceps.

Epidermis samples were placed in HBSS with trypsin. Samples were agitated on a heat shaker for 30 minutes. Digestion buffer was subsequently added to dilute the trypsin. Epidermis samples were then centrifuged and re-suspended in FACS media. The dermis was added to digestion buffer with collagenase and placed in an incubator at 37^0C. The dermis samples were filtered, centrifuged and re-suspended in FACS media. The cells from the epidermis and dermis were stained with the following antibodies: CD3-FITC (BD Pharmingen, cat. 556611), CD4-PE-Cy5 (___,___), CD8-AF700 (BD Pharmingen, cat. 557945), CD25-BV605 (___,___), CD45RO-PE-Cy7 (BioLegend, cat. 304230), CD45RA-APC (___,___), CD207-PE (BioLegend, cat. 352203) and H38-FITC (___, ___), mIgG3 –FITC (____, ____) and analyzed with flow cytometry [18].

RESULTS

Maintenance Immunosuppression for 4 Months

Results for upper extremity VCA (in M1413 and M4213) were previously reported [14]. Briefly, M1413 developed Banff II rejection on POD 97 (preceding FK506 level on POD 93 was 22.8 ng/mL) and resolved with SBT; M4213 developd Banff I rejection on POD 30 (FK506 level was 5.7 ng/mL on POD 27 and 27.3 ng/mL on POD 30), failed to respond to SBT, progressed to Banff IV rejection and necrosis of the VCA by POD 51 and had to be removed from study (Figure 2).

Using the same regimen in facial VCA recipients, M6014 (full MHC-mismatch) developed rejection by POD 19 but M6714 (haploidentical) remained rejection-free up to POD 79. However, both subjects had to be removed from study due to the development of post-transplant lymphoproliferative disorder (PTLD) on POD 107 and 79 (Table 1).

However, due to PTLD developing beyond two months on triple maintenance immunosuppression, we modified our study end point to POD 60. All remaining VCAs were also performed with the face model to simplify the logistics of animal care (e.g. repeated cast changes in upper extremity VCA recipients) and ensure consistency of results.

Maintenance Immunosuppression for 2 Months

Six recipients of facial VCA were studied (Table 2). Similar to M4213 (upper extremity VCA, full MHC-mismatch), M6514 (full MHC-mismatch) developed rejection on POD 36 (Banff I; corresponding FK506 level was 20.8). The episode was treated with SBT but recurred on POD 46 (Banff II; corresponding FK506 level was 19.8) and required another round of SBT before resolving. M3815, which was also a haploidentical recipient (similar to M6714), did not develop rejection clinically or on histology up to POD 60.

A review of our cases thus far led to the observation that rejection appeared to develop within three to four weeks after VCA in full MHC-mismatch recipients, but not in haploidentical animals. We therefore decided to incorporate an additional surveillance VCA biopsy at approximately two weeks after transplantation in our subsequent animals to determine if sub-clinical rejection could be diagnosed early on histology, especially when clinical presentation was equivocal.

In M4415 and M4515 (full MHC-mismatch recipients), the additional week 2 biopsy returned as Banff II and I respectively and the corresponding clinical appearance of the VCAs were not conclusive for rejection despite adequate levels of systemic FK506 (26.2 ng/mL and 17.8 ng/mL

respectively on POD 13 for both animals). However, prompt SBT at this time point was adequate in preventing recurrence of rejection (both clinically and on histology) up to POD 60 (Figure 4). In the last two recipients (haploidentical), subclinical rejection was diagnosed in M3516 on POD 12 biopsy (Banff I; corresponding FK506 level was 15.1); clinical rejection also developed in M3316 on POD 27 (Banff I; corresponding FK506 level was 23.9).

Leukocyte Infiltration of VCAs

Flow cytometric analysis of VCA skin biopsy samples showed that in the initial cohort of recipients, there was near-complete (>90%) turnover of skin leukocytes (CD4+, CD8+) in the VCA dermis from donor- to recipient-origin cells by the first protocol biopsy on POD 30. However, the kinetics of this turnover was slower in the epidermis where a variable amount (75-93%) of CD207+ T cells (Langerhans cell phenotype) were donor-origin at the same time point (i.e. POD 30) (Figure 5). When additional surveillance biopsies were introduced at week 2 in the second cohort of animals, similar numbers for cellular turnover were observed in VCA skin (Table 3). No quantitative differences in cellular kinetics were observed between the upper extremity and facial VCA recipients, or between full MHC-mismatch and haploidentical recipients (data not shown).

Mixed Chimerism, Systemic Immune Function and Sensitization

After transplantation, no evidence of mixed chimerism (either transient or persistent) was detected peripherally despite the incorporation of vascularized bone marrow as part of the VCA (data not shown). By MLR, the *in vitro* allo-response to both donor and third-party lymphocytes were comparable while the animals (both full MHC-mismatch and haploidentical recipients) were maintained on triple immunosuppression (Figure 6). Despite rejection episodes ranging from 1 to 3 in this study, no evidence of allo-antibody formation was detected in any of the study subjects (Figure 7).

DISCUSSION

Since the first clinical hand [19] and face [20] transplants, much effort has been invested towards understanding the mechanism(s) behind the development and treatment of acute rejection episodes in VCA. Mechanistic studies in small animal models have hinted at possible pathways involving the MHC but are considered less robust due partly to differences in cellular composition of the transplanted skin [21]. NHPs though, share close homology to humans and respond similarly to immunologic agents in clinical use [22]. While the extent of HLA mismatch reported in VCA patients has varied from 0/6 to 6/6 due to other considerations that take priority such as the matching of allograft size and skin color to optimize functional and aesthetic outcomes, there has not been a clear association or correlation with the incidence and subsequent development of acute rejection [3, 4]. In contrast, HLA matching has, until recently, been an absolute requirement in hematopoietic stem cell transplantation (i.e. bone marrow or peripheral blood stem cells) due to predictable outcomes such as the risk of developing graft versus host disease (GvHD) [23]. In this study, we utilize clinically relevant NHP models to study acute rejection of VCAs following transplantation under the most common regimen currently in use – T cell depletion followed by standard triple maintenance therapy – across both full and single haplotype MHC-mismatch barriers.

Related NHP studies in VCA have varied in their induction and maintenance immunosuppression regimens, as well as the extent of MHC mismatch (Table 4). Cendales et al. previously reported median rejection-free survival of 17 days (range, 5 – 76) with triple immunosuppression in MHC non-identical NHPs that had undergone upper extremity VCA without T cell depletion. The immunosuppression dosages used were, in comparison, lower than in this study. Of note, 6 of 7 animals in the study developed allo-antibody formation [24]. Silverman et al. introduced T cell depletion with ATG and dual maintenance immunosuppression with FK506 and rapamycin but only one recipient animal survived (to POD 129) long enough to develop clinical rejection episodes on POD 45 and 70 (at time points similar to the current study) that were treated with steroid bolus-only [25]. Barth et al. then attempted induction with high dose FK506 alone (target trough 30-50 ng/mL) before tapering to lower maintenance doses (target trough of 10-20 ng/mL)

from POD 28 onwards. This regimen was, encouragingly, able to achieve a mean clinical rejection-free survival of 116 days (range, 60 – 177) but all five recipient NHPs developed PTLD [26], similar to our initial cohort of animals. In the other three NHPs of the same study by Barth et al. [26], FK506 was converted to rapamycin after 28 days but rejection developed at POD 28, 49 and 70, again similar to rejection time points in our study. In a subsequent study, Barth et al. added MMF to the previous regimen but this led to mixed results in four NHPs: rejection developed on POD 44 and 163 (while on immunosuppression) in two NHPs, but did not occur till POD 205 and 322 in the remaining two animals while maintained on immunosuppression [27]. No MHC mismatch data was provided for the last three studies although the authors reported that transplant pairings were selected based on the *least* degree of proliferation by MLR, which suggests that perhaps, the immunologic challenges of a full MHC-mismatch barrier may not have been fully explored.

In our study, the first clinical signs of rejection developed around POD 19 to 36 in full MHC-mismatch recipients of both upper extremity and face VCA. Besides M4213 (upper extremity), all other animals developed rejection regardless of the preceding and corresponding systemic levels of FK506. Notably, by the time rejection was clinically apparent, corresponding flow cytometric analyses demonstrate near-complete turnover of leukocyte populations within VCA dermis from donor- to recipient-origin. The numbers of these recipient-origin cells then remained at approximately the same levels (>90%) up to experimental end-point, which suggests that these cells may be skin-resident in nature. Despite SBT when rejection was diagnosed clinically (in full MHC-mismatch recipients), the episode either progressed or recurred over the next two to three weeks. This was demonstrated in M4213, which developed steroid-resistant rejection (similar to clinical upper extremity [28]) that resulted in VCA loss, and in M6514 which had progression/recurrence of rejection from Banff I to II.

Interestingly, we also observed varying presentations of acute rejection in our face transplant recipient animals. In M6014, rejection was diagnosed when it developed maculopapular rashes of

different intensities (Figure 3); M6514 had a more focal presentation with involvement of the overlying epidermis (not shown); and in M4415 and M4515, clinical examination was equivocal (Figure 4). These diagnostic challenges have also been reflected in the clinical VCA experience [6]. While mucosal biopsies have been suggested to play a role in aiding the diagnosis of rejection [29, 30], this was not possible in our study due to the heterotopic location of the transplant where the medial aspect of the mandible and inner mucosal lining are located within the deepest aspect of the anastomosis site. By the same token, deeper biopsies of the masseter muscle and mandible were not possible with a punch biopsy and hence, were not evaluated in this study to determine if rejection of deeper tissues preceded that of the skin or vice versa.

Ultimately, a histological diagnosis of VCA rejection remains the current gold standard but in our study, a strong clinical suspicion would trigger presumptive SBT. Disconnect between examination and histological findings (e.g. in M6014, M4415, M4515, M3516) are not new and have been reported in clinical VCA [31]. However, this study has shown that the timing of SBT can successfully negate the early recurrence of rejection episodes, at least within an experimental setting.

Unfortunately, the low cellular yield from our skin assays (2-3 day process) precluded further mechanistic studies. We hypothesize that antigen presentation by donor epidermal Langerhans cells leads to the rapid infiltration of recipient-origin skin leukocytes. Related studies have shown though, that steroids can promote the upregulation of regulatory T cells (T_{regs}) through epidermal Langerhans cells [32]. Coupled with the finding of T_{regs} in skin biopsies with long-term follow-up of VCA patients at >5 years after surgery [33], our results suggest that induction with even higher dosages of steroids and/or maintenance at larger doses may potentially avert the early onset of acute rejection. Such findings also call into question the validity of steroid-sparing protocols that are currently in use at certain VCA centers, especially when these patients have subsequently required reinstatement or repeated bolus dosing of steroids due to multiple rejection episodes [34, 35]. Moreover, there is increasing recognition of the deleterious role of memory T cells, which

are capable of migrating to and remaining skin-resident, especially in the context of VCA [9, 36]. Such memory T cells are also more resistant to conventional immunosuppression [37], as is used in this study, and clinically, and may therefore account for the development of rejection episodes despite adequate systemic FK506 levels in full MHC-mismatch recipient animals.

Of note, despite essentially similar observations on MLR and flow cytometric analysis of skin leukocytes, haploidentical recipients (M6714, M3815) did not develop rejection clinically or on histology when immunosuppression was maintained within range and did not require SBT. Compared to SOT in which MHC class II sharing improves outcomes [38], our results suggest that the additional sharing of MHC class I antigens confers additional protective mechanism(s) in the context of VCA. Conversely, when systemic levels of FK506 were sub-therapeutic (e.g. in M3516 and M3316), the protective effect of MHC sharing did not enable the reduction of maintenance immunosuppression dosages. These observations warrant further consideration of HLA matching as a clinical criterion in future VCA. Applicability is confounded however by the extreme variability of clinical HLA. We do note though that our institution's first hand transplant patient [39] (HLA mismatch 4/6) has remained-rejection free at 4 years follow-up, which lends further support to the need for further investigation into HLA matching in VCA.

SUMMARY

Acute rejection of VCAs develops in a predictable, temporal sequence and histological changes are likely to precede clinical presentation. Although SBT is usually sufficient in aborting the rejection episode, the slower diagnosis on clinical presentation predisposes the recipient to early recurrence or even subsequent progression of rejection when fully developed. Therefore, there is a role for surveillance biopsies in the early post-transplantation period to guide and inform treatment. We have also shown, for the first time, that MHC sharing may confer additional protection against the development of acute rejection in VCA.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

FIGURE LEGENDS

Figure 1. Dosing schedule for methylprednisolone. Same regimen is used in the event of clinical acute rejection.

Figure 2. Clinical course for M4213 (full MHC-mismatch, upper extremity VCA): (A) Hint of rejection just distal to the suture line on POD 29, (B) rapid progression with increased erythema by POD 34; (C) biopsy showing mild perivascular inflammation without epidermal involvement (Banff Grade I), (D) rejection episode was steroid-resistant, worsened and the animal started to autophage its allograft hand on POD 39, (E) non-viable VCA with terminalization of digits on POD 41, (F) epidermal and dermal necrosis (Banff Grade IV) with thrombosis on POD 51 when the animal was euthanized due to a non-viable VCA. MHC = major histocompatibility complex, POD = post-operative day, VCA = vascularized composite allograft.

Figure 3. Clinical course for M6014 and M6714 (facial VCA): (A, B, C) Different intensities of a maculopapular rash on POD 28, 48 and 78 respectively for M6014 (full MHC-mismatch) and no histologic evidence of rejection; (D) biopsy on POD 48 showing no evidence of rejection; (E, F, G) corresponding pictures for M6714 (haploidentical recipient) on POD 28, 52 and 79 with no clinical evidence of rejection, (H) nor evidence of rejection on histology (on POD 79). MHC = major histocompatibility complex, POD = post-operative day, VCA = vascularized composite allograft.

Figure 4. Clinical course for M4415 (full MHC-mismatch): (A, B, C, D) Equivocal appearance of VCA on POD 14, complete resolution after steroid bolus and taper by POD 25, and no evidence of recurrence up to POD 60; (E, F, G) corresponding histology shows focal moderate to severe deep perivascular inflammation (Banff Grade II) with endothelialitis (inset) on POD 14, and no evidence of rejection on POD 32 and 60. MHC = major histocompatibility complex, POD = post-operative day, VCA = vascularized composite allograft.

Figure 5. *In vitro* analysis of donor and recipient skin cell contributions to VCA at POD 30; representative data from a full MHC-mismatch (H38-) recipient animal. Epidermis: (*left four panels*) isotype controls; (*right four panels*) 45% of CD3+ lymphocytes in the epidermis were donor-origin (H38+) and of these, 75% were CD207+ Langerhans cells. Dermis: (*left four panels*) isotype controls; (*right four panels*) 27% and 51% of CD3+ lymphocytes were CD8+ and CD4+ respectively, and 7.4% (of CD8+) and 17.2% (of CD4+) were of donor-origin (H38+), demonstrating rapid infiltration of VCA dermis by recipient-origin skin leukocytes that stay resident.

Figure 6. *In vitro* evidence suggests that the presence of vascularized bone marrow as part of a VCA did not provide additional immunomodulation based on mixed lymphocyte reaction assays, where the anti-donor and anti-third party responses were comparable (representative data shown; no difference observed between full MHC-mismatch and haploidentical recipients).

Figure 7. No *in vitro* evidence of allo-antibody (anti IgG or anti IgM) formation across serial dilutions from 1:10, 1:100 to 1:1000 despite the development of multiple acute rejection episodes. Representative antibody FACS analysis shown. FACS = flow activated cell sorting.

TABLE LEGENDS

Table 1. Study outcomes on initial cohort of animals on 4-month maintenance immunosuppression.

Recipient	VCA	MHC-Mismatch	Complications	Average FK506	Allo-Antibody	Survival	Creatinine Glucose	Reason for Euthanasia
M1413	Hand	Full	Banff II (POD 97)	28.8 ng/mL (16.7 – 40.2)	No	> POD 120	0.6 – 0.9 53 – 157	–
M4213	Hand	Full	Banff I (POD 30)	25.7 ng/mL (5.7 – 48.7)	No	POD 51	1.1 – 1.4 47 – 175	VCA loss (Banff IV)
M6014	Face	Full	Banff 0 (POD 19, 48, 78)	23.3 ng/mL (9.0 – 38.9)	No	POD 107	0.6 – 1.1 74 - 157	PTLD
M6714	Face	Haplo-identical	–	18.7 ng/mL (8.7 – 47.5)	No	POD 79	0.8 123	PTLD

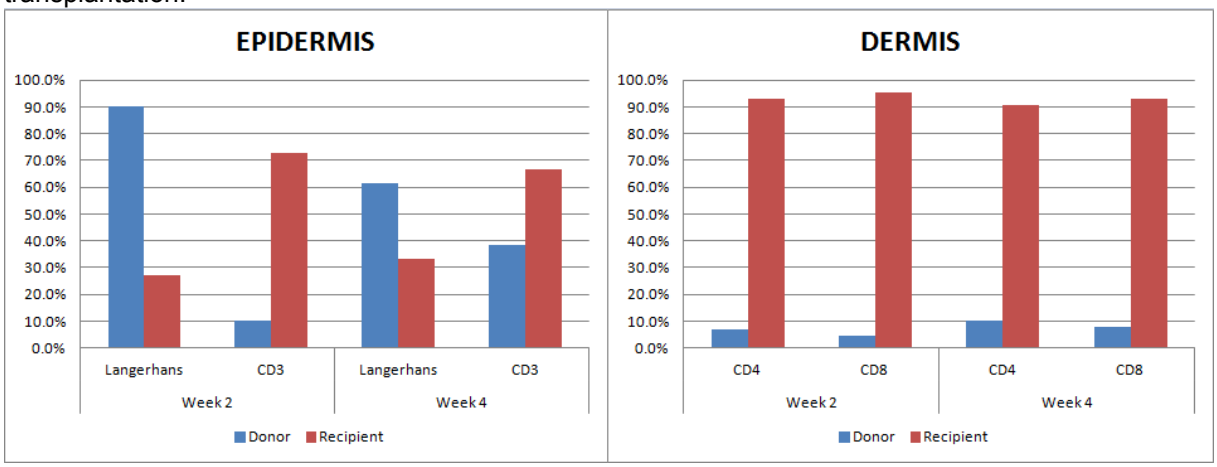
MHC = major histocompatibility complex, POD = post-operative day, PTLD = post-transplant lymphoproliferative disorder, VCA = vascularized composite allograft.

Table 2. Study outcomes on subsequent cohort of animals on 2-month maintenance immunosuppression.

Recipient	VCA	MHC-Mismatch	Complications	Average FK506	Allo-Antibody	Survival	Creatinine Glucose	Reason for Euthanasia
M6514	Face	Full	Banff I (POD 36) Banff II (POD 46)	21.2 ng/mL (9.6 – 33.0)	No	> POD 60	0.6 – 0.8 59 – 151	–
M4415	Face	Full	Banff II (POD 14)	20.5 ng/mL (9.0 – 29.8)	No	> POD 60	0.6 – 0.8 94 – 131	–
M4515	Face	Full	Banff I (POD 15)	23.4 ng/mL (10.2 – 34.8)	No	> POD 60	0.7 – 0.9 73 – 140	–
M3815	Face	Haplo-identical	–	29.1 ng/mL (17.0 – 40.6)	No	> POD 60	0.7 – 0.9 73 – 115	–
M3516	Face	Haplo-identical	Banff I (POD 12)	21.2 ng/mL (10.2 – 33.7)	No	> POD 60	1.0 – 1.1 111 – 131	–
M3316	Face	Haplo-identical	Banff I (POD 27)	23.1 ng/mL (11.0 – 34.3)	No	> POD 60	0.7 – 0.7 114 – 119	–

MHC = major histocompatibility complex, POD = post-operative day, PTLD = post-transplant lymphoproliferative disorder, VCA = vascularized composite allograft.

Table 3. *In vitro* analysis of leukocytes from VCA skin biopsies at 2 and 4 weeks after transplantation.



Donor-origin Langerhans cell populations are near 90% at week 2 in the epidermis, with corresponding CD4+ and CD8+ populations > 90% recipient-origin by this time in the dermis. By week 4, the donor-origin Langerhans cell population decreases to about 60% but the CD4+ and CD8+ dermal populations remain at > 90% recipient-origin, suggesting tissue residence. No difference was observed between full MHC-mismatch and haploidentical recipients (not shown). VCA = vascularized composite allograft.

Table 4. Related NHP studies investigating various immunologic protocols and outcomes on acute rejection

Author	N =	VCA	MHC-Mismatch	Induction	Maintenance	Rejection	Allo-Antibody
Cendales et al. [24]	7	Hand	"Non-identical"	No	FK506, MMF, Steroid	Median POD 17 (range, 5 – 76)	Yes (6/7)
Silverman et al. [25]	3	Face	?	ATG	FK506	POD 45, 70	Not checked
Barth et al. [26]	5	Face	?	High dose FK506	FK506	No (but developed PTLD from POD 60 to 177)	No
	3	Face	?	High dose FK506	Rapamycin (after 28 days)	POD 28, 49, 70	No
Barth et al. [27]	4	Face	?	High dose FK506	FK506, MMF	POD 44, 163	No

ATG = anti-thymocyte globulin, FK506 = tacrolimus, MHC = major histocompatibility complex, MMF = mycophenolate mofetil, POD = post-operative day, PTLD = post-transplant lymphoproliferative disorder, VCA = vascularized composite allograft.

SUPPORTING INFORMATION

None

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