Cutting Edge of Transplantation Optimizing Long-Term Transplant Survival: Therapeutics, Targets & Technologies

December 21, 2015 – December 21, 2016

This activity is jointly provided by Global Education Group and the American Society of Transplantation.

This activity is supported by an educational grant from Astellas.

Target Audience

The educational design of this activity addresses the needs of healthcare professionals (physicians, surgeons, pharmacists, nurses, and others) and researchers working in the field of transplantation medicine.

Statement of Need/Program Overview

Healthcare professionals and researchers who have dedicated their careers to transplant medicine continue to be challenged by a number of key questions pertaining to optimization of long-term transplant survival. Specifically:

- Why have we failed to advance long-term survival, is it due to a lack of technology, targets or therapeutics?
- Is testing for early injury a more important target than tolerance?
- Have we avoided the organ as a target as we uncovered recipient immune responses?
- Did we select targets based on therapeutics that we had? Should we now reassess what therapeutics we should be developing?

This manuscript summarizes the highlights presented at the 2015 Cutting Edge of Transplantation annual meeting jointly provided by American Society of Transplantation and Global Education Group.

Educational Objectives

Overarching objectives identified for this meeting are listed below. After completing this activity, participants should be better able to:

1. Recommend the most up to date therapeutics for optimizing long-term graft survival, based on the latest evidence
2. Describe recent developments in identifying novel targets for future therapeutic advances in long-term graft survival
3. Describe the current state-of-the-art technologies and the potential for optimizing long-term transplant survival

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Manuscript compilation completed by:
Colleen Manitt, PhD

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Summary
This manuscript is based on data presented at the 2015 *Cutting Edge of Transplantation* (CEOT) conference by the American Society of Transplantation (February 5-7 2015, Chandler, Arizona).

Organ transplantation is often the best therapeutic option for end stage organ failure. However, transplant medicine continues to struggle with several key issues. Graft rejection, which occurs when a recipient's immune system mounts a response against donor-specific alloantigens, is a common problem, and controlling this immune response remains an enormous challenge. Immunosuppression is the only available therapy for preventing graft rejection. However, advances in our understanding of the mechanisms involved in the regulation of immune responses have provided hope for new therapies. The paucity of available organs has been a major problem. Xenotransplantation has been one possible solution to overcoming this obstacle. Recent advances in identifying and targeting xenoantigens responsible for causing xenograft rejection have provided promising results. An overwhelming number (ex.: 85% of donated lungs) of donated organs are never used because they are deemed unsuitable for transplant. Another area of research that has aimed to address the shortage of available organs has been to design *ex vivo* organ repair systems that will preserve organs for longer periods of time *ex vivo*, permitting more opportunity for organ transport, evaluation, and repair. These systems also reduce ischemia-reperfusion injury. A syndrome known as Primary Graft Dysfunction (PGD) is believed to result from ischemia-reperfusion injury, and is associated with significant morbidity and mortality following transplant. Finally, exciting advances have been made in stem cell biology and tissue engineering. These innovations provide new hope for organ repair and regeneration, and even organogenesis.

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I- Rejection: Controlling immune responses in the host

A. Regulatory T-cells and their uses in cell therapy and transplantation.

The immune response to transplantation is complex. A dialogue is initiated between the recipient's innate immunity and the graft immediately after it is transplanted, leading to activation of adaptive T- and B-cell responses. This dialogue is ongoing, and controlling this continuing immune response remains an enormous challenge, and for many patients this is not achieved successfully over the long-term.

Alloantigens are released from the graft into the host immune system for the lifespan of the transplant. Current approaches to avoid rejection involve suppressing immune function. However, these approaches prevent the immune system from engaging with transplant alloantigens and compromise its ability to bring regulatory networks into play. As such, the host immune system can never learn to become tolerant to the graft and its alloantigens.[1]

The immune system has multiple control mechanisms that it can use to regulate itself in different circumstances.[2] In this way, it can respond to an antigen in an appropriate manner, which includes mounting an immune response that is not excessive or that continues indefinitely. Indeed, there are many regulatory mechanisms that can be brought into play to shut down a host immune response to a graft, or to create an environment that will allow tolerance to the donor alloantigens to emerge. This includes the contribution of cells that are initially involved in the immune response to the transplant.[3]

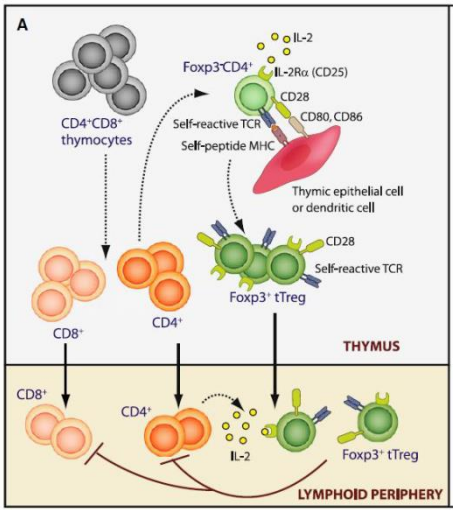

Figure 1. Regulatory T cell lineage

The regulatory T cell

The regulatory T-cell is not a homogeneous cell subtype - there are various subsets of regulatory T-cells that can work together to control immune responses *in vivo*. One population of regulatory T-cell emerges through selection in the thymus alongside the conventional CD-4+ and CD-8+ T-cell populations that can mount an effective rejection response against the allograft. Along with those thymus-derived cells is a separate population of regulatory T-cell that can develop in the periphery. These are referred to as P-, or peripheral, Treg-cells that are induced to become regulatory and express Fox P3, an important transcription factor, when they encounter an antigen. Fox-P3 is a transcription factor involved in controlling immune responses. Studies of humans carrying Fox-P3 functional gene mutations show that loss-of-function of this transcription factor leads to dysregulated immune responses and autoimmunity. So, when these cells are present in sufficient numbers and in the right place, they can really be very effective at controlling unwanted immune responses (Figure 1).[4]

Indeed, these regulatory T-cells can be identified with intracellular as well as cell surface markers. In this way, Treg-cells can be identified from a heterogeneous group of cells, and these markers can also be used to purify/isolate them out of a heterogeneous cell suspensions to use them for cell therapy. Treg-cells from the CD-4 subset express high levels of CD-25, sustained Fox-P3 expression, as well as other markers. CD-25 is the alpha chain of the IL-2 receptor, the hallmark of a regulatory T-cell population within a CD-4 compartment.

The conditions that are present when these cells encounter antigen allows them to acquire a regulatory function that can control the activity of unwanted populations of T-cells and other cell types. In response to alloantigen *in vivo*, they change their intracellular signaling machinery. It has been shown in mouse that these cells transiently spike γ-interferon expression in this microenvironment, which triggers a molecular cascade that allows these cells to control aggressive CD-4 and CD-8 T-cells that would otherwise reject an allograft. It will also allow regulatory T-cells to control alloantibody production by B-lymphocytes and affect other innate populations as well. A number of factors mediate these Treg-cell processes, including soluble molecules (interleukin-10, TGF-β), cell-cell contact events, and CTLA 4 which exerts inhibitory effects on effector T-cells. Once present in the transplant microenvironment, Treg-cells are very effective at controlling unwanted immune responses. The combination of both the thymus-derived and the peripheral Treg- cells contribute to the overall control of the immune response after transplantation.[5]

These cells either exert their function within the transplant itself or within the draining lymphoid tissues associated with the graft. The majority of the experimental data suggest that these cells are actually much more efficient when they are resident in a tissue.[6]

Drug therapies to enhance regulatory immune cell function

It may be possible to modify standard immunosuppressive regimens to allow these cells to emerge as a more predominant population, allowing them exert a greater effect on the post-transplant immune response.[7] Indeed, leukocyte depletion strategies either using alemtuzumab or anti-thymocyte globulin have generated data in clinical studies to suggest that Treg-cells do emerge as a population in those patients that are depleted at the time of transplantation.[8, 9, 10]

Regulatory T-cells as a cellular therapy

Regulatory T-cells can be isolated and grown *in vitro,* and a number of recent advancements have permitted enrichment of specific T-cell populations. Current efforts are aimed at using Tregs as a cellular therapy; the goal is to augment the level of control these cells can exert over the immune system by increasing cell number.[7]

A few studies have begun to explore Treg-cell therapy in the treatment of graft versus host disease. These are generally phase 1-2-A safety studies that have reported limited adverse events in bone marrow transplantation. One study used either adult-derived or umbilical-cord-derived regulatory T-cell populations. The study reported limited adverse events in this immunosuppressed patient population.[11] A study performed in Italy showed that an infusion of donor-derived non-expanded Treg-cells resulted in the prevention of graft vs host disease and the enhancement of immune reconstitution in these bone marrow transplant recipients.[12] These studies indicate that regulatory T-cells can be a benefit in controlling the unwanted immune response in graft vs host disease.

<u>Cell therapies – ongoing clinical trials</u>

In kidney transplantation, a European phase 2-1-safety study - the *One study*[13] - is evaluating the safety of cell therapies, including a number of regulatory T-cell subtypes, tolerogenic dendritic cells and regulatory macrophages in the setting of living donor kidney transplantation. A randomized, open label study at the University of California, San Francisco (UCSF) aims to use regulatory T-cells to reduce inflammation detected in biopsies six months after kidney-transplantation. In liver transplantation, there are also phase 1-2-A studies in progress at UCSF[14] and at King's College London (KCL)[15]. These studies are examining an immunosuppressive regimen designed to be Treg-cell supportive – to promote persistence and functionality of the regulatory T-cells. Tregs will be infused 11- 13 weeks post-transplant, and

escalating doses will be assessed. The Treg infusions will be expanded *ex vivo.* The expansion protocols used in the studies will differ based on the regulatory standards between the countries: polyclonal expansion (KCL) versus expansion in the presence of cells expressing the HLA of the organ donor (UCSF). See Appendix 1 for more detailed descriptions of these clinical trials.[7]

These studies represent the recent progress in both isolating and manufacturing regulatory T-cells for clinical use, which has made it possible to move these studies to clinical safety trials for kidney and liver solid organ transplantation.

Future Directions[7]

Cell enrichment: The One study will generate data comparing different isolates of regulatory T-cells, the CD-25+ CD-4 population being the one that can be isolated using magnetic bead-technology. However, these cells can be further enriched by eliminating other contaminating populations by including an additional marker, CD127.[16] Low levels of this marker, the alpha chain of interleukin 7 receptor, enrich for regulatory functionality.

Polyclonal vs donor-alloantigen reactive Treg-cells: As expected, data show that donor alloantigen reactive Treg-cells are much more efficacious on a per cell basis than the polyclonally-expanded populations.[17, 18, 19] Safety data comparing polyclonal and donor alloantigen reactive regulatory T-cell populations will also emerge from these ongoing clinical trials. This will be very useful because a more enriched population would require fewer cells to achieve the same efficacy.

Cell number: The dose-escalation studies will provide some insight into the optimal cell number that is required. However, dosing parameters are within the range that regulatory authorities have allowed, which may not include the optimal dose.

Cell viability and distribution: A number of centers are using tracking technology, such as deuterium-labeled glucose or gadolinium, to understand where infused Treg-cells go. Developing these approaches is crucial because in order for these cells to be useful in the long-term, it is necessary to determine their viability and where they go.

Important questions

Many factors remain to be determined in the clinical indication for using Treg-cells as a cell therapy.

When to infuse: Will it be more effective to infuse early to induce natural regulatory mechanisms, or will it be more effective to intervene when there is evidence of inflammation or rejection?

What is an optimal permissive/supportive environment: Should the cells be infused into a leuko-depleted environment because the cells will be more likely to niche and therefore function more effectively, or should they be competing with the existing cell populations? There is evidence that supports a competition approach. However, the leuko-depleted environment appears to have the most supporting data.[7]

If Treg-cells with donor-alloantigen reactivity could be enriched *in vivo* by creating an environment that would be more permissive to the cells that are infused*,* this would promote the cells' persistence, expansion, and regulatory function. Studies in the liver will determine whether an everolimus or a sirolimus-based regimens are permissive in that context. Leukocyte-depletion has been suggested as a

way of minimizing competition for the cells that one infuses and clearly the liver protocols will also assess whether using lymphocyte-globulin as an agent is effective.

The interleukin-2 conundrum: Regulatory T-cells require interleukin-2 (IL-2) to both expand and survive.[20] This poses a conundrum because too much IL-2 stimulates effector populations. However, data now coming from both experimental studies and some recent clinical data indicate that regulatory T-cells require very low levels of IL-2 to expand and function *in vivo*. If the amount of IL-2 present in vivo could be titrated, it may be possible to maximize Treg-cell survival and function.

Two studies in patients exhibiting graft vs host disease and a recent study from a type 1 diabetes safety study have reported that low-dose IL-2 therapy creates an environment that is permissive for regulatory T-cells to expand, stabilize, and perform their function. One transplant study explored whether IL-2 at optimal dose would allow conversion of peripheral Treg-cells and augment the overall pool of available Tregs.[21] A significant benefit of the low-dose IL-2 therapy on the severity of the chronic graft vs host legions was also reported. The same group also carried out a number of functional analyses that reported evidence of 1) low-dose IL-2 therapy selectively increasing stat-5 phosphorylation-positive signaling in the regulatory T-cell pool, 2) increased proliferation, and 3) decreased apoptosis.[22] The study conducted in patients with type-1 diabetes was a dose escalation study. The highest dose used in that study induced a 5% increase in the number of detectable regulatory T-cells.[23]

B. Immune monitoring and therapeutic targets - borrowing from other fields

In transplant rejection, the host mounts an immune response against donor tissue. Some principles to control this unwanted immune response can be borrowed from research into autoimmune diseases, which is when the immune system fails to discriminate the “self” from the “non-self” and responds to one of its own tissues as foreign.

There are two types of IBD: ulcerative colitis and Crohn's disease. There is a significant overlap between the genes implicated in the two diseases (GWAS).[24] Ulcerative colitis is typically associated with the colon only whereas Crohn's disease can affect any part of the digestive tract. The incidence of IBD is increasing, and is currently about 1% in North America.[25] It is most prevalent in economically-developed countries.

Similar to the challenges in transplant medicine, the goal of therapy is to help regulate the patient’s immune system and keep it from recognizing the digestive tract as “non-self” and mounting an immune response against it. The mechanisms involved in immune regulation in the gut is complex, particularly due to the fact that the digestive tract is continuously exposed to dynamic changes in dietary antigens, microbial antigens and metabolytes.[26]

About IBD

In the gastrointestinal tract, immune effector cells and trillions of microbes live in close proximity.[27] Regulatory mechanisms and a sophisticated barrier maintain vital host-microbial interactions and tissue homeostasis. The pathogenesis of inflammatory bowel disease includes an abnormal immunological response to disturbed intestinal microflora, inflammation, and disruptions in barrier function. [28]

The micrographs in Figure 2 show the distribution of IGA (green), IGM (blue), and IGg (red) antibodies in the normal bowel (top panel) versus in IBD (bottom panel). In IBD, substantial disruption is observed – Changes in B cells occur during mucosal inflammation: IgM and IgG antibodies are increased, and these antibodies are autoreactive. These events are seen in gingivitis, gastritis, celiac disease, and IBD. *(Figure 3 provided by P. Brandtzaeg*.)

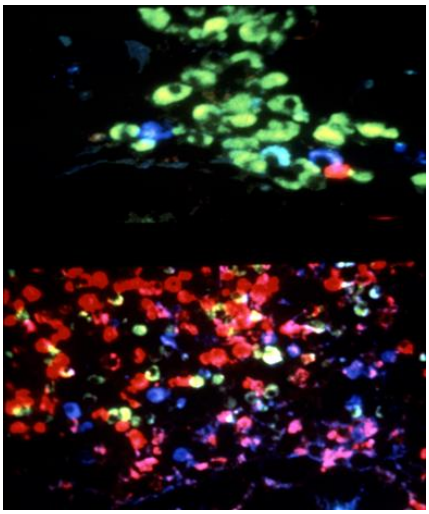

Figure 2. Changes in B cells during mucosal inflammation

Inflammatory processes and autoimmunity versus tissue type in defining pathogenicity

Most therapeutics agents used to treat IBD were borrowed from other fields such as rheumatoid arthritis (RA). This suggests that autoimmunity and inflammatory processes are more relevant than the tissue itself, as it seems to work across many diseases. Available therapeutic drugs include steroids and purine synthesis inhibitors such as methotrexate, sulphasalazine, and azathioprine.[29] The field also relies heavily on TNFα neutralization, which blocks many TNFα-dependent processes in Crohn's disease and ulcerative colitis. TNFα neutralizing drugs include infliximab, adalimumab, and golimumab, and most work for multiple diseases.[29] TNFα-neutralization is believed to produce therapeutic benefits by influencing signaling pathways involved in pro-inflammatory cell clearance, and by changing the phenotype of antigen-presenting cells to a less inflammatory M2 phenotype.

While most drugs work in multiple disease settings, some drugs such as etanercept do not work in IBD.[30] This might be due to the fact that different doses are required in IBD, suggesting that tissue type may be important as well.[26]

Table 1. Biologic therapies for IBD

Biologic Therapies for IBD: What are the Choices?

Ulcerative Colitis	Crohn's Disease
•Anti-TNF agents	•Anti-TNF agents
– Infliximab	– Infliximab
– Adalimumab	– Adalimumab
– Golimumab	– Certolizumab pegol
– Biosimilars on the way..	– Biosimilars on the way..
•Anti-integrin agents	•Anti-integrin agents
– Vedolizumab	– Vedolizumab -selective
	– Natalizumab – non-selective
	•Anti-IL-12/23 agents
	– Ustekinumab

Current Therapeutic strategies (Table 1)[26]

The anti-TNFs

Although biologic agents directed against TNFα are effective in many patients with IBD, 30 and 45% of patients are either refractory to anti-TNF therapies or they discontinue treatment due to a loss of response, or adverse events.[31, 32]

The integrin agents

Alternative pathways involved in the inflammatory process need to be targeted for therapeutic interventions. Increased interactions between lymphocytes and endothelial cells in IBD perpetuate the inflammatory response. As such, the adhesion molecules involved in these interactions may provide potential targets for new therapeutic interventions. The humanized monoclonal antibody against human α4 integrin, natalizumab, was the first integrin agent effective in Crohn's disease. However, it has been associated with a risk of progressive multifocal leukoencephalopathy. The second-generation of anti-integrin agents inhibits interactions between α4β7-integrin and mucosal addressin cellular adhesion molecule 1(MadCAM-1).[32, 33]

The interleukin agents

These include agents that target IL-12/23, and the classic pro-inflammatory cytokine IL-6. IL-23 plays a key role in the adaptive immune responses in IBD. Ustekinumab, a human monoclonal antibody that targets the p40 subunit of both IL-12 and IL-23, has been shown to be effective in phase 2 trials in both Crohn's disease and ulcerative colitis. Antibodies targeting IL-6 are also being studied in Crohn's disease.[32]

C. What can we learn from cancer research and tumor rejection?

Studying how tumors escape the host's immune system provides insight into what pathways are important in immune tolerance, and can provide targets for trying to achieve tolerance in transplantation medicine.[34]

Prior to any immuno-therapeutic intervention, patients can already be categorized based on the degree to which a natural autoimmune response against the tumor has already occurred (Fig. 3).[35] There is a major subset of patients that shows T-cell inflammation in the tumor micro-environment at baseline. These patients already exhibit CD8+ T-cell infiltration into the tumor, and these tumors have a particular chemokine profile that is likely responsible for attracting T-cells into the tumor micro-environment.[36] These tumors also have a type 1 interferon (IFN) signature. In contrast to this, patients who do not exhibit a natural autoimmune response lack these signatures; they do not exhibit the same chemokine profile, they do not have a type 1 IFN signature, and they do not exhibit CD8+ T cell infiltration.

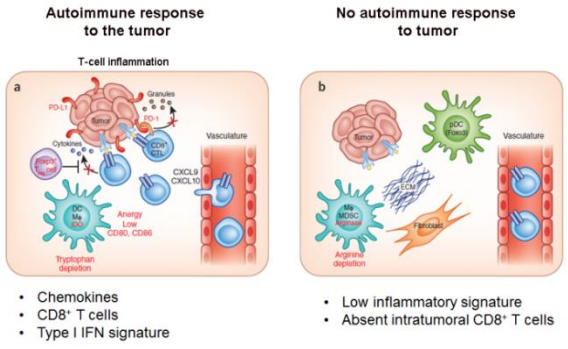

Figure 3. Immunobiology of T cell-inflamed versus non-T cell-inflamed tumors

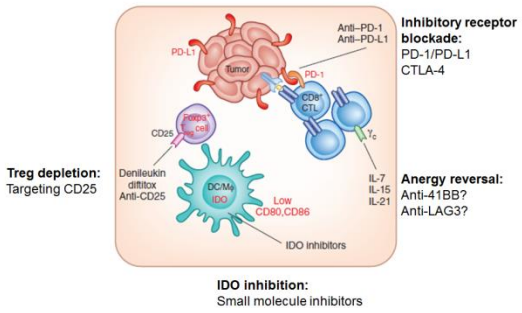

Figure 4. Possible interventions to target negative regulatory mechanisms in T cell-infiltrated melanomas

At least four mechanism seem to contribute to the dysfunction of the T-cells that have successfully infiltrated the tumor, but have been ineffective in eradicating it (Fig. 4).[35] These provide specific therapeutic targets, and all of these pathways have been validated in pre-clinical models. *Possible Interventions*: 1) Depletion of regulatory T-cells by targeting CD25, 2) Blocking receptors in the inhibitory pathway, with antibodies targeting PD-1/PD-L1, 3) Blocking indoleamine-2,3-dioxygenase (IDO) function with small molecule inhibitors, and 4) Blocking the recently identified cell surface markers - 4-1 BB and Lag3, using monoclonal antibodies. An additional intervention would be to reverse T-cell dysfunction- anergic cells.

Indeed, clinical trials with anti-PD1 antibodies in melanoma have led to two FDA-approved antibodies.[34] Notably, a predominant proportion of responsive patients are those who have exhibited an autoimmune response with T-cell infiltrate in their tumor.[37] In response to anti-PD1 treatment, T-cells in the tumor become Ki67-positive, a cellular marker of proliferation. Consistent with this, an increase in the number of T-cells invading the tumor is observed. They attack the tumor from within in those patients, inducing tumor regression. PD1 is active in at least 7 other cancers. Anti-PD1 is anticipated to become FDA approved in this broad spectrum of cancers.[34]

Many other pathways are involved in inhibiting T-cell responses in those tumors. Studies in pre-clinical models have shown that targeting two pathways simultaneously has additive effects (CTLA4 + PDL1, CTLA4 + IDOi, PDL1 + IDOi).[38] Thus it is possible to block two pathways simultaneously and get potent tumor regression in mice, in the absence any other conventional cancer therapies. Examination of T-cells in the tumor revealed that successful therapies aimed at restoring immune function most strongly correlate with the re-emergence of IL-2 expression by T-cells and an ability to proliferate directly within the tumor site.[39] This was demonstrated pharmacologic using FTY 7-20, which blocks the exit of any additional T-cells from the lymph nodes.[40] Thus, restoration of immune function is happening by inducing changes in the existing tumor environment.

These combinations are currently being tested in human clinical trials. One study has reported initial data showing more than 50% tumor shrinkage with very deep responses using anti-CTLA-4 and anti-PD1.[41] Preliminary from a study that used an IDO-inhibitor and anti-CTLA-4 foreign antibody indicate that about half of the patients responded to the therapies.[42] Other permutations are also being tested based on these pre-clinical studies.

The anti-PD1 field is currently dominating cancer immunotherapy. However, blocking LAG-3 in combination with 4-1BB, proteins on the surface of dysfunctional T-cells, produced potent effects as well (Horton B and Gajewski T, *manuscript in preparation*). [34] Thus, it may be possible to target other molecular targets in addition to PD1 and CTLA4, with comparable clinical efficacy.

What approaches can be developed to gain therapeutic efficacy in non-T cell-inflamed tumors?
The next challenge in the field will be to develop therapies for the remaining patient population that does not exhibit a natural autoimmune response. This profile applies to the majority of cancer patients. In order to develop therapies for these patients, it is necessary to understand the mechanisms underlying this response.

Type 1 interferon is key. In a recent study, type 1 interferon was found to be upstream of T-cell priming.[43] This is consistent with the fact that type 1 interferon is found to be expressed in tumors with T-cell infiltration, but not in tumors without the innate immune response. Type 1 interferons are made very early after tumor implantation. In mice that lack the receptor, the T-cell response does not happen. To promote T-cell infiltration Interferon must act directly on a very specific subset of dendritic cells - the Batf3 lineage dendritic cells which activate T-cells against tumor antigens.[43]

The sting pathway is nature's response to tumors. [44] Tumor-derived DNA can be found in the cytosol of tumor-infiltrating dendritic cells. This DNA activates the sting pathway, and the sting pathway is driving type 1 interferon production and it also drives complete activation of the Batf3 dendritic cells. Consistent with this, STING knockout mice cannot generate this type 1 interferon production and they cannot mount an anti-tumor immune response. Conclusion: Tumors induce dendritic cells to make Type 1 IFN.[44]

STING is polymorphic in the human. Compounds designed to target all the known polymorphic variants are extremely potent molecules – In one study, one or two doses into tumors completely eradicated these tumors in the entire mouse experimental group. When these mice are re-challenged, they're resistant. This has now been observed in in five different tumor models.[45]

Radiation induces Type 1 IFN through the STING pathway. Tumor irradiation also leads to DNA transfer to the host antigen-presenting cells and this activates the STING pathway.[46] The therapeutic effect of

radiation of these mouse models is lost in STING knockout mice. Activation of the STING pathway is required to maximize the effects of radiation.

Translational bioinformatics - Identification of new molecular pathways to induce an immune response within the tumor. This addresses inter-patient heterogeneity.

The T-cell inflamed phenotype has a distinct genetic signature. One subset of tumors expresses a panel of chemokines, T-cell markers, and type 1 IFN genes that defines that phenotype. A second major subset of tumors is completely negative for all of those genes, and a third group contains intermediate tumors. Bioinformatics were obtained for the two extremes (snip analysis, tumor exome sequencing, etc.).

A series of studies were conducted to profile the oncogene pathways that might be on or off in T-cell inflamed and non-inflamed tumors. [47] **48% percent of non-T-cell inflamed tumors showed evidence of active β-catenin signaling.** Very few of the T-cell inflamed tumors do. In fact, approximately 70% of these non-T-cell inflamed tumors have functional mutations in β-catenin, about 23% have loss-of-function of mutations in *inhibitors* of the β-catenin pathway. The remainder have over expression in either ligands, receptors, or beta-catenin itself. In a mouse tumor model, expression of β-catenin in tumor cells is sufficient to exclude T-cells out of the tumor micro-environment[48], confirming that β-catenin function is causally related to the absence of an immune response.

The mechanism explained[34]

When β-catenin is on in the tumor cells, it extinguishes expression of key chemokines. The chemokine CCL4 is necessary for recruitment of that small population of the Batf3 lineage dendritic cells into the tumor. Therefore, tissues, in this case the tumor cells, help the immuno-response normally. The tumor cells make a chemokine that brings dendritic cells into the tumor that in turn activate T-cells. When β-catenin is on however, those chemokines are not released. Absence of β-catenin results in recruitment of dendritic cells, T-cell activation, and the T-cell inflamed phenotype. Thus, in tumors that lack β-catenin in a mouse model (T-cell inflamed group) targeting CTLA4-PD1 produces a therapeutic effect. However, tumors that express β-catenin are completely resistant to checkpoint blockade. Further, there are no T-cells at baseline in those tumors, and no augmentation of T-cell infiltration or activation after treatment.

These data suggest that β-catenin expression within the tumor could be a primary mechanism of resistance to immunotherapies, which means this is one molecular pathway that should be targeted and tested therapeutically.[34]

Microbiota

The intestinal microbiome has a profound impact on anti-tumor immune responses to tumors that are distal from the intestine. One study examined the rate of subcutaneous tumor growth in mice that are genetically similar but have two different gut microbiome compositions due to the facility where they were bred. Tumors grew faster in one mouse (Taconic) than the other (Jackson Labs), and this difference was abolished when the mice were house together prior to the experiment.

Examination of T-cell infiltration at the end of the experiment correlated with this; the mice with the faster growing tumors did not exhibit any T-cell infiltration. In a separate study, anti-PD-1 treatment in conjunction with exposure to fecal matter of the mice with the more efficient anti-tumor response (Jackson) produced substantial tumor control in most of the *Taconic* mice, and about half of the mice rejected the tumor completely. In the *Jackson* mice - with the already more efficient anti-tumor microbiota, anti-PD-1 alone works, but this is not the case in the *Taconic* mice. The clinical implications of

these studies are very exciting. The specific bacterium responsible has been isolated, and a clinical trial testing anti-PD1 therapy in conjunction with this probiotic are being planned (Sivan A and Gajewski T, *manuscript in preparation*).[34]

Conclusions

- The degree of T-cell infiltration in the tumor microenvironment may serve as a predictive bio-marker for current immunotherapies.
- The STRING pathway is required to produce a T-cell inflamed micro-environment driven by DNA transfer to host antigen presenting cells. STING agonists could be one therapeutic approach to promote immune responses in the non T-cell inflamed tumors
- Specific somatic alterations, intestinal microbiome, and germline snips are important

II- Xenotransplantation

The shortage of organs is one of the biggest problems in transplant medicine. More than 120,000 people in the United States alone are waiting for organs. The first obstacle to successful xenotransplantation is the need to produce a donor animal with fewer antigens that will elicit an antibody-mediated rejection of the donor organ. Work performed in Joseph Tector's lab (Indiana School of Medicine) illustrates how the field of xenotransplantation evolved in tandem with advances in genome modification approaches.

Genes have been identified that play an important role in xenograft cross-reactivity. Two are genes that humans have deleted during the course of evolution, and pigs have not: α Gal (GGTA1 gene) and HD antigen (CMAH gene). A third gene is β1,4 N-Acetylgalactosaminyl Transferase.[49]

Using homologous recombination, Tecter and colleagues made a homozygous αGal-knockout pig. However, when they transplanted kidneys from these pigs, using FDA-approvable immunosuppression, the results were disappointing.[50]

The age of DNA nuclease

Advances in genome modification techniques have permitted more rapid and sophisticated knockout studies aimed at identifying xenoantigens responsible for graft rejection.[50] The schematic in Figure 5 illustrates how genetic modification in somatic cells is achieved by zinc finger nucleases (ZFN) versus the CRISPR/Cas9 system.

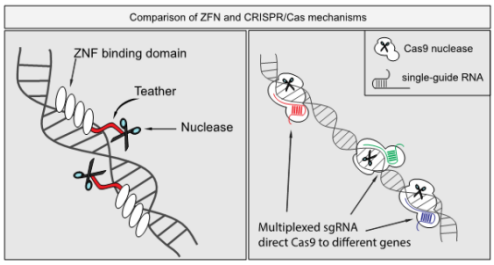

Figure 5. Zinc finger nuclease (ZFN)-mediated genetic modification

Zinc Finger Nuclease (ZFN)

Because studies in the previous αGal knock-out had not yielded promising results, a double knock-out pig was engineered using zinc finger nucleases. CMAH was deleted first, followed by αGal.[51] The double- knockout pig was found to be a better crossmatch to humans than chimpanzees. Indeed, results showed that 4 of the 5 human samples tested exhibited less antibody-binding for IGM and IGG in the double knockout than in the chimpanzee.

CRISPR/Cas9 system

In the CRISPR/Cas9 system, a Cas-9 nuclease is used with a single-guide RNA. The advantage of this system is that the nuclease is untethered - it can knock-out more than one gene in a single reaction. In this way, multiple genes can be targeted by using their corresponding guide RNAs that are in order like PCR primers.

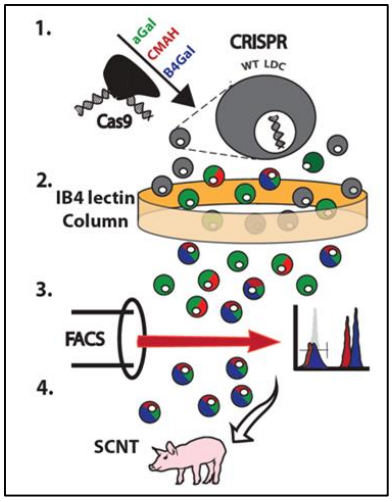

Figure 6. βGal/CMAH/B4GALNT2 triple knockout engineered using the CRISPR/Cas9 system.

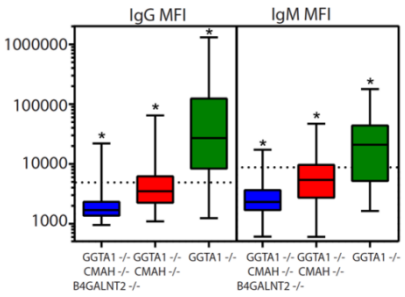

Figure 7. Degree of reactive antibody binding according to Knockout.

A triple knockout pig was engineered (Fig 6).[52] The following genes were targeted: αGal, CMAH, and B4GALNT2. The Cas 9 plasmid was introduced along with the guide-RNAs to these 3 genes. The cells were passed through a lectin column to isolate gal knockout cells, and then FAC sorted for CMAH and B4GALNT2. Nuclear transfer was then performed.

Serum from 40 people was tested with peripheral blood mononuclear cells (PBMCs) from the different knockouts. The amount of binding decreased linearly with addition of gene knockout (Fig 7).

Panel Reactive Antibody (PRA) is an immunological laboratory test routinely performed on the blood of people awaiting organ transplantation. A proportion of patients awaiting transplantation are considered sensitized – they have exceptionally high antibody levels that react to foreign tissue.

This diagram in Figure 8 illustrates the cross match between the three knockout pigs and individuals with across sensitized categories. Highly sensitized (80-100 PRA, red), moderately sensitized (20-79% PRA, orange), and non-sensitized (1-19% PRA, Yellow). There is less binding across PRA score groups, including highly sensitized individuals, in the triple knockout compared to other knockouts. The data points outside the grey box in the graphs on the right indicate that there are still outliers, which suggests that some individuals are exhibiting cross-reactivity to additional xenoantigens. Previous studies had suggested that HLA antibodies cross-react with swine leukocyte antigens (SLA) in sensitized patients (high PRA score) but not in non-sensitized patients. [53]

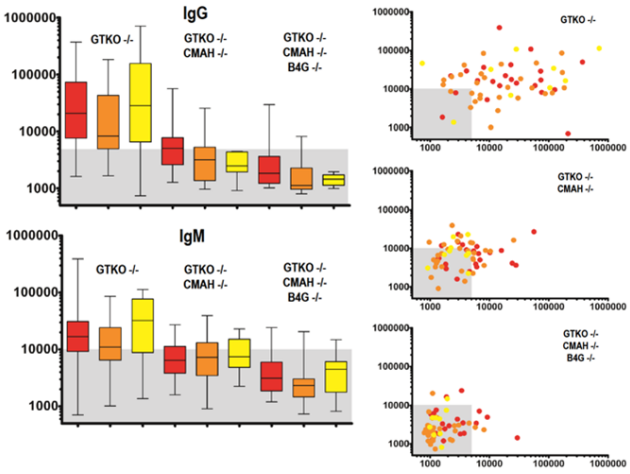

Figure 8. Cross match across PRA score groups and the three knockout pigs.

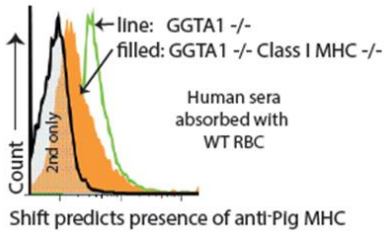

Figure 9. The highly sensitized profile is almost entirely attributable to HLA antibodies

Comparing whether the gal + class 1 MHC (SLA-1) knockout was a better cross match then the gal knockout alone (Fig 9). The shift of the double knockout peek toward the human indicated that class 1 MHC is the xenoantigen for this person. Indeed, further analysis indicated that the highly sensitized profile is almost entirely attributable to HLA antibodies. Consistent with this, when pre-adsorption almost completely abolishes cross-reactivity.

Conclusion

Genome editing to delete xenoantigens is effective in reducing rejection risk. The triple antigen knockout pig has a crossmatch with human serum that is good enough too not expect early antibody mediated rejection in a pig to human renal xenograft for most patients. Deletion of class 1 swine leukocyte antigen may make it possible to cross match approximately 95% of waitlisted patients. These pigs are healthy and they grow normally.[50]

III- Ex vivo organ repair

A major challenge associated with transplant medicine, in addition to organ rejection, is the paucity of donor organs. However, approximately 85% of the lungs that are offered for transplantation are never used, and this is similar for other organs as well.[54] Despite this high threshold for judging an organ suitable for transplantation (best 15%), severe primary graft dysfunction occurs in about 13-35% of cases.[55]

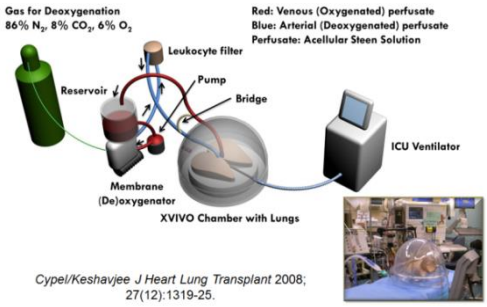

Figure 10. The Toronto Ex Vivo lung perfusion (EVLP) system.

A number of factors could improve the number of organs that can be used from the available pool. First, approaches that would increase organ viability *ex vivo* would provide more time to assess the condition of the organ outside the body at the time of transplantation. The ability to maintain the organ outside the body for a longer period of time would also provide an opportunity to rescue or salvage damaged organs.[54]

An Ex Vivo Lung Perfusion (EVLP) system has been developed to keep the lung alive outside the body (Figure 10).[56 ,57] The system consists of a dome where the lung is kept, which is ventilated by a standard ICU ventilator, and perfused with a centrifugal pump at appropriate pressures - it removes oxygen, adds carbon dioxide. In this way, the lung can be ventilated in the system and it is possible to assess the ability of the lung to do what it is supposed to do – add oxygen, remove carbon dioxide – and monitor its physiology.

The first experiment showed that keeping normal pig lungs in the EVLP system at 37 degrees for 12 hours did not produce any deterioration in function. Addition of cellular preservation solutions improved results further. Transplantation of these lungs was successful and exhibited excellent organ function.[58]

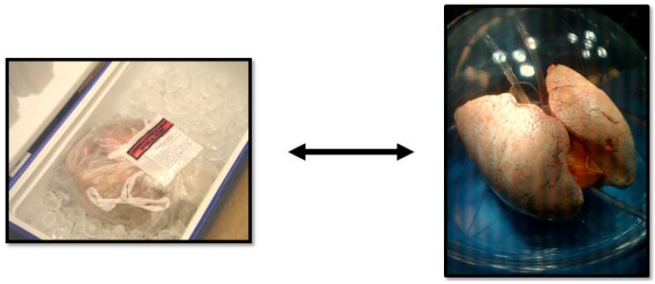

Figure 11. Cold static preservation versus normothermic EVLP

An extension of the preservation period was attempted to allow for long-distance organ transport. An experiment was designed where all lungs were first stored with cold static preservation, followed by either 12 hours of continued cold static preservation or 12 hours in the *ex vivo* lung perfusion system at normothermic temperatures. The lungs that had the 12 hours in the EVLP circuit recovered and had improved function after transplantation (Figure 11).[59] This protocol has become standard practice at the Toronto General Hospital (172 transplants have been performed) – lungs are being transplanted 22-23 hours after removal. These approaches have contributed to optimizing lung preservation, and provide the opportunity to accurately assess, diagnose, treat, and repair the organ and confirm treatment results.

A clinical trial that compared standard transplants to transplants using marginal lungs that were perfused in the *ex vivo* circuit reported comparable success between groups.[60] Further, recent data suggest that the EVLP patients may actually be doing better in the long-term. This may have relevance for organ rejection issues. Acute injury in the donor organ may have a significant impact on the recipient's immune response.[54]

In 2012 the EVLP was approved by Health Canada and also by the Ontario Health Technology Assessment committee, which is a committee that assesses new technologies independently.[54] The committee investigates and then advises the government about whether this should be funded or not, and they advised the ministry of health to fund EVLP. So *ex vivo* lung profusion has gone from inception, animal experiments, to clinical trial, to a fully funded and reimbursed procedure in Canada in a very short time, because regulatory authorities understood its importance and the unmet need - that 20-30% of people on the lung transplant list die because they do not get a lung in time.[54]

Developing approaches to treat lungs *ex vivo*

One powerful advantage of the EVLP system is the ability to diagnose, and possibly treat, the lung before transplantation. The common reasons for rejecting a lung for transplant, in many instances, are problems that may be possible to reverse - pulmonary edema, brain death associated inflammation, infection, pneumonia, aspiration or unrecognized aspiration, pulmonary embolism, and finally, every organ has ischemia-reperfusion injury.

Available therapeutics: The *ex vivo* system provides more time to intervene, manage, and treat the lungs. Some of the very expensive biologic drugs, like recombinant drugs that are $10,000/dose and have a 20-minute half-life *in vivo*, have a 32-hour half-life in the *ex vivo* system. Therefore, it becomes possible to use many of the available therapeutics and optimize a way to apply these.[54]

It is common to find pulmonary emboli in a donor lung. While it does cause inflammatory injury, the lungs are usable. The concern is whether that donor patient actually has a thrombotic disease or chronic pulmonary hypertension, which would make that lung dangerous to transplant. A lung with emboli was put into the *ex vivo* circuit, treated with alteplase, and monitored as pulmonary-vascular resistance decreased over time in the circuit. D-dimers were measured for evidence of clot lysis, and the function of the lungs over time improved. A biopsy was also taken and sent for analysis and there were no signs of chronic pulmonary arterial disease, and the lungs were deemed safe to transplant. The patient was returned to ICU with PO2 over 500, extubated in 12 hours, normal CT scan, and normal pulmonary artery pressure.[54]

Gene transfer: The possibility of using gene transfer approaches to induce expression of interleuken-10 (IL-10) because of its key regulatory role in transplant immunobiology - both in the early inflammatory responses and in alloimmune response – has also been explored. Putting adenoviral vector encoding human IL-10 into rats 12 hours prior to lung removal, followed by 24 hours of storage and then transplantation resulted in significantly better function of the lung. [61] These results have been replicated in pigs. [62] These studies show that it is possible to genetically treat the donor lung in the donor and have it work better after transplantation.

The standard of organ-preservation is flush cooling. Organ preservation at 4 degrees slows metabolic processes down to 5% of normal. The processes involved with organ deterioration after removal are also slowed down and that is what has made transplantation possible. However, all metabolic processes are slowed, including healing or regenerative processes, and gene therapy would slow down too. Thus, the

use of normothermic EVLP organ preservation was assessed in IL-10 gene therapy. Lungs were treated *ex vivo* for 12 hours in the *ex vivo* circuit under normothermic conditions. IL-10 gene upregulation was observed, similar to the effects produced with the *in vivo* treatment in the donor, and the lungs exhibited superior function.[63] Thus, the process was moved from the donor to the organ. This protocol also allows for longer transport times - 12 hours of cold storage, 12 hours to move the organ, diagnose it, upregulate the gene, and transplant it.

These studies have been carried out in the human.[64] Donor lungs from brain-dead donors that were rejected because of unacceptable functional parameters were brought into the lab. The lungs were treated with IL-10 gene therapy. As a result, oxygenation of the lungs was increased and pulmonary vascular resistance was improved, bringing these lungs to transplantable quality *ex vivo*. This approach reversed the inflammatory cytokine profile found in the brain-dead multi-organ donors; IL-1β, IL-8, TNFα, IL-12 and IL-6 were all down-regulated. These damaged human lungs that were not used for transplant, were brought to transplantable parameters with *ex vivo* IL-10 gene therapy.

IL-10 gene therapy during *ex vivo* lung perfusion in lung transplantation is in a phase 1 clinical trial. Using a bronchoscope IL-10 gene therapy will be delivered into the lung, and then the lung will be monitored for improvements in function. This is a phase 1 trial – the primary endpoint is whether the target IL-10 expression can be achieved safely. The target level of expression is based on the dosage at which positive effects were seen in rodents and pigs.[54] Secondary endpoints will include the incidence of primary severe grade 3 primary graft dysfunction. Dose and function will be monitored and titrated over time, and inflammatory markers will be assessed for indications that inflammatory injury has been reversed in these lungs.

How to diagnose the lung

A number of key genes have been identified that predict transplant outcome.[65] However, to obtain these answers in diagnosis and treatment of an organ pre-transplant, conventional methods like PCR are too time consuming. Shana Kelly, a nano-biology engineer from the U. of Toronto has invented a nano-chip. This chip has little hairs on it that have the sequence of the gene of interest.[66] So instead of PCR annealing, a gene is annealed to this nano-hair, and the read-out is an electrochemical signal. In this way, one can go from sample to readout in less than 20 minutes.

An organ repair center

The ORA team at Toronto Gen. hospital is the first organ repair laboratory in the world. A lung from Wisconsin was brought to Toronto, repaired at Toronto Gen. Hosp., and transported to Chicago where it was successfully transplanted – an organ repair center in operation.[54]

IV- Engineering organ repair

A. Making Hepatocytes in the lab

The hepatocyte is the most abundant cell in the liver, and it performs most of the liver's functions. Producing hepatocytes in the lab has the potential to provide many therapeutic benefits, including cell therapy. Successful infusion of hepatocytes into the liver has already been reported, and in that landmark paper therapeutic efficacy in the patient as well as safety of the procedure were clearly established. [67]

However, hepatocyte transplantation into the liver remains an experimental procedure, yet to be used in the clinical setting. A number of challenges in the field are likely at the root of this, including the fact that it has been difficult to obtain high-quality hepatocytes. Further, the human liver contains about 200 billion hepatocytes, but only about 5 billion can be safely infused. This falls short of the 20% repopulation that needs to be achieved in order to produce a therapeutic effect. So there is a need for cell expansion post-transplantation and there is currently a lack of capacity of the produced cells to react to regeneration stimulus in vivo in order to achieve this repopulation. Another, challenge is to prevent rejection. An ideal source would therefore be to produce hepatocytes from the recipient's own cells. [68]

Potential approaches

Make induced pluripotent stem cells (iPSCs, Fig 12). A suitable cell type is taken from the patient, such as a fibroblast, and the cell is reprogrammed toward pluripotency using the Yamanaka transcription factors.[69] In the case of the liver, directed differentiation then drives the cells toward a hepatocyte fate. Given that the cells are derived from the patient, it is expected that they will not be rejected following transplantation. Insight into the factors required for the directed differentiation of hepatocytes, or induction of pluripotent stem cells into hepatocytes, is obtained from developmental studies.

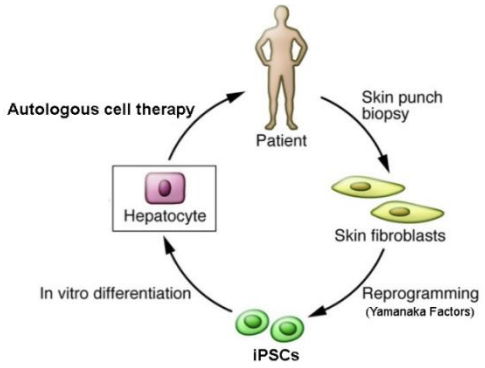

Figure 12. Engineering pluripotent stem cells (PSC)

AFP and albumin are markers of immature liver progenitor cells. The hepatocytes derived from induced pluripotent cells (IPS) highly express AFP, and also exhibit robust expression of albumen. In comparison to primary Adult Hepatocytes (AhePs), iPSC hepatocytes express similar levels of albumen. However, markers of metabolic function (CYP expression), are dramatically lower in iPSC hepatocytes in comparison to AhePs. The implication of these data for cell viability and function after transplantation are not clear.

There are other ways to transform a fibroblast into a hepatocyte, and these have advantages and disadvantages.

Partially reprogram the cells (Fig 13). In this protocol, the same Yamanaka factors are used when reprogramming toward pluripotency. However, instead of allowing the cells to become pluripotent, the cells are directly exposed to cues that will drive them toward the desired cell type, in this case, endoderm. Thus, endoderm progenitors are produced by this partial reprogramming approach.[70] Consistent with this, the partially reprogrammed endoderm cells begin to express FOXA2, a marker of endoderm 18 days into the procedure, and they do not express markers of pluripotency, like NANOG. Pluripotent cells can become teratoma. This has been a major concern in transplant settings for many types of tissues. Partially reprogrammed cells address this.[68]

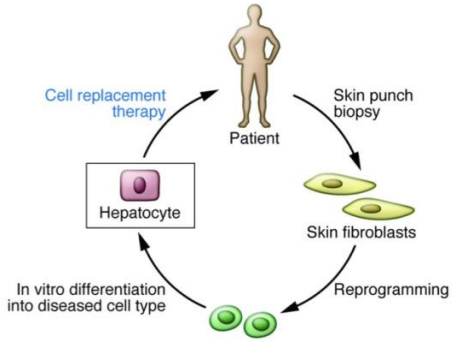

Figure 13. Engineering partially reprogrammed multi-potent progenitor cells (iMPC)

These cells first become endoderm progenitor cells, and therefore go through the whole differentiation process they would undergo *in utero*. As a result, these cells have the ability of endoderm progenitor

cells to proliferate quite extensively. Thus, it is conceivable that one clone could produce the billions of cells needed for a human transplant – an important prerequisite for clinical translation. In addition, these cells acquire more hepatic properties as they expand in the experimental conditions used, such as HNF4 expression, which is a hepatocyte-specific transcription vector in the liver.[70]

The gene expression pattern of the two induced hepatocytes are very different from primary hepatocytes. It may be debatable whether these cell can be labeled as hepatocytes at all.[69] However, when transplanted into Fah-deficient mice, these cells repopulated the liver similar to primary human hepatocytes; they started to expand and secrete albumen.[69] This proliferative capacity was maintained over time, with these cells exhibiting expression of the proliferation marker Ki-67. These cells were later recovered from these mice, and the global gene expression profile was again analyzed. Cells that were transplanted *in vivo* for months had a gene expression profile that was indistinguishable from primary hepatocytes (Fig 14). Thus, *in vivo*, these cells mature, and assume a genetic profile that is indistinguishable from normal hepatocytes. Consistent with this the drug metabolizing capacity of these cells was analyzed and found to be similar to primary human hepatocytes.[71]

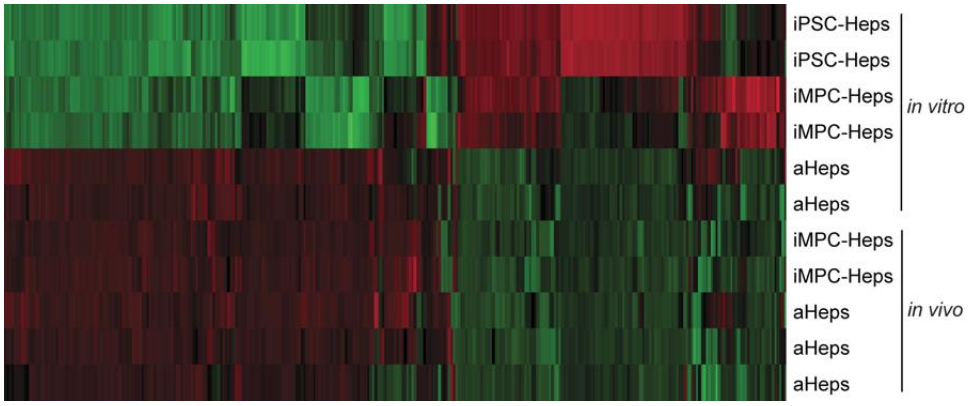

Figure 14. Gene expression profile of primary hepatocyates (aHeps) in comparison to iPSC-Heps and iMPC-Heps *in vitro* versus *in vivo*

More recently an alternative approach has emerged that involves driving the fibroblast to become a hepatocyte without reverting towards a progenitor fate in between. You can actually force the fate of a fibroblast, for example, into a hepatocyte by just delivering the key fate determining transcription factors. And so they use slightly different transcription factors. Two studies have published promising results with this approach. One used six transcriptions factors[72], and the other used three.[73] However, both protocols required making cell-cycle alterations using oncogenes or by knocking down tumor suppressors. If these cells are to be considered for transplantation, it becomes necessary to consider ways to circumvent the use of oncogenes and tumor suppressors. Ongoing studies are investigating this.[68]

B. Stem Cells and Organ Scaffolds

The focus of this work is to combine cells with scaffolds using principals of developmental biology to develop 3-dimensional tissues. These 3-dimensional tissues that are developed in the laboratory can be used for pharmacokinetics, disease modeling, and down the road, eventually, for cell-laden scaffold transplantation or lab-made organs in the future.[74]

1- Engineering the LIVER

In Figure 15, the left panel shows a micrograph of a normal liver, where the hepatocytes can be seen. A decellularized liver in which all the cells are gone is shown in the panel on the right. These scaffolds are put in custom-made bio-reactors that are connected to sensors, in order to monitor different key parameters in the growing organ.[75, 76]

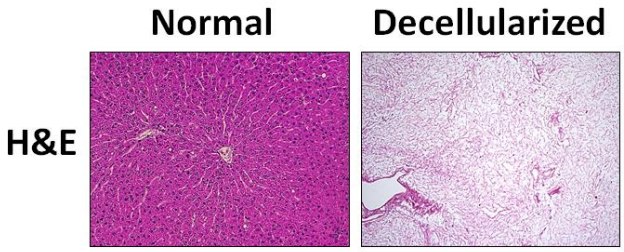

Figure 15. Cross-sections through a normal liver (left panel) and a decellularized liver.

AIMS

The long term goal is to develop a protocol that would make it possible to take a skin biopsy from a patient heading towards transplantation (moderate MELD score), make IPS cells, differentiate those cells into billions of hepatocytes (or kidney cells), and infuse those cells into a decellularized liver in a bio-reactor, through a vein canula.[74] Figures 16 depict IPS-derived hepatocytes that are growing within a liver scaffold over 14 days.

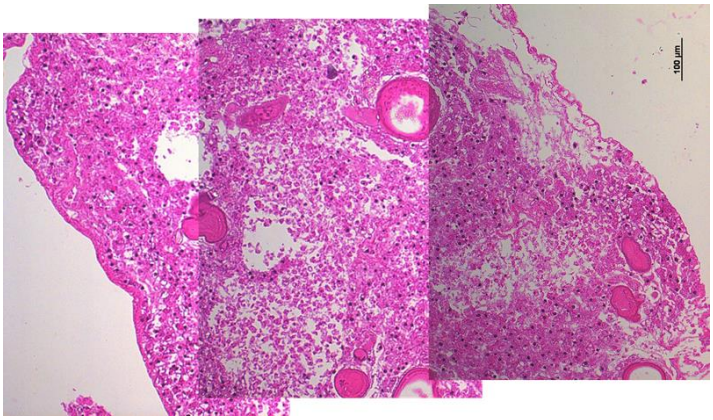

Figure 16. iPSC-derived Hepatocytes after 14 days in bioreactor

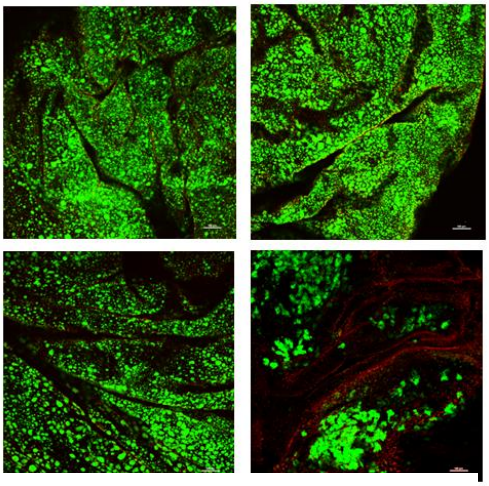

Figure 17. Live-dead staining of iPSC-derived hepatocytes after 14 days in bioreactor.

Live-dead staining indicates that a substantial proportion of cells are alive in the scaffold after 14 days in a bioreactor (Fig 17). A cross-section through that growing liver tissue shows that the distribution of the hepatocytes is heterogeneous within the parenchyma; sometimes they are a little bit more heavily packed, sometimes less densely packed. However, albumin expression emerges during this time and increases steadily. Because the organ is perfused in the bio-reactor, it is possible to take samples from the circulating medium and run different assays and tests for bio-markers.[76]

IPS-derived hepatocytes tend to remain very immature *in vitro*. To assess the functional properties of the IPS-derived hepatocytes in the scaffold system, an assay system was devised, since it is not feasible to test culture conditions in these larger scaffolding systems. The system involved designing smaller sections, of a decellularized liver in which it would be possible to observe how cells grow in three dimensional space on small sections of decellularized liver.

The IPS-derived hepatocytes were plated in two types of scaffold, either an ECM scaffold - the decellularized liver, or the PLLA scaffold, and cell viability at day 14 was assessed (Fig 18). All the cells were alive (green labeling).[74]

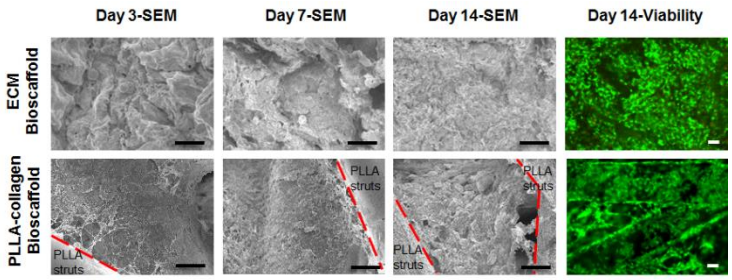

Figure 18. Cell growth on the PLLA versus ECM scaffold

The functional characteristics of these IPS-derived cells within these scaffolds were examined. These cells do not proliferate very much. However, when looking at gene expression, CYP-1A2, 2C9, 3A4, and HMGCR expression was increased in IPS derived cells grown either on the ECM scaffold or on the PLLA scaffold. However, the ECM scaffold seemed more effective. CYP 3A7 is expressed during fetal development as well as AFP protein. The expressions of these genes decreased by about 50% in the ECM scaffold. However, in a 2D printed scaffold, they did not change at all. So there is still something specific about these more heterogeneous ECM scaffolds that may suggest that these cells mature more within these ECM scaffolds.[74]

However, the most important question is how similar are these cells to primary human hepatocytes. CYP2C9 activity in IPS derived hepatocytes goes from essentially undetectable at day 3 to high by day 7 on the ECM scaffold. 3A4 also increases significantly in this system.

2- Engineering Urological Structures

There are three essential components of tissue engineering[77]:
Cell source: A number of different cells can be used: embryonic stem cells, progenitor cells, adult cells.
Scaffolding materials: metals, ceramics, synthetic polymers, natural polymers.
Signals that would guide tissue formation: growth factors, drugs, mechanical forces.

Current challenges for translation of tissue engineering.[77]

1. Engineering tissue with the right anatomy is an important first hurdle. However, the ultimate goal is to engineer tissue that can perform the same functions as the organ/tissue it is modeled upon.
2. Must be able to move beyond simple tissues: Flat 2D tissue - to 3D hollow tissue - to solid 3D tissue.
3. This work has to be successfully moved into clinical trials that would show the safety, feasibility, and eventually efficacy of these types of approaches.

Simple tissue engineering (flat-2D): Urethral stricture

A small biopsy was retrieved from patients. Urothelial and smooth muscle cells were expanded, seeded on a tubular scaffold made with polyglycolic acid – a polymer, and then re-implanted. All five patients in the study exhibited patent urethras at a five year follow-up.[78]

The Hollow organ – Bladder

In the clinical studies, a small biopsy was taken from patients and the urothelial and smooth muscle cells were isolated. Patient also underwent a CT scan to determine bladder shape and size. These parameters were used to design the scaffold, which was then seeded with the isolated cells. The seeded scaffolds were incubated in the laboratory from 3-7 days, and then implanted. The follow-up extended up to several years. In terms of functionality there was a 3-fold increase in the volume of the bladder, which exhibited normal pressures.[79]

The above studies summarize work in two organs: the urethra, which is a simple, tubular structure that has now been used in patients for several years, and the bladder, which is a hollow organ. For the bladder, proof of principle studies have lent support the efficacy of this approach in the clinical setting. The current, more ambitious, goal in tissue engineering is to make a complex solid organ, such as the kidney.

The KIDNEY

Rat kidneys can be decellularized using different strategies. Special bio-reactors for these 3-D scaffolds have been developed for the kidney.[76, 80] It will be possible to monitor certain indices, such as pressure and O2, as with the liver. And recirculate the media over time and sample a look at different bio-markers as cells proliferate within a scaffold.

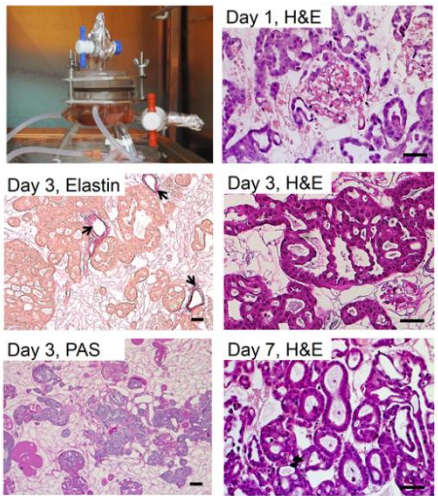

Figure 19. Development of tubules *in vitro*

Importance of sequence and location of implantation. Wertheim and colleagues conducted one study that compared whether putting cells into the scaffolds through the ureter would be more successful than putting them through the arterial system.[74] Introducing these cells through the arterial system was found to yield a better recellularized scaffold. By 7 days post infusion, some cells had coalesced into structures that resemble tubules (Fig 19). They appeared to be in the tubule compartment because, although the cells were infused in the arterial system, they were not observed in the small arterioles. It is not clear how cells migrate within the system.

Orientation.

Wertheim and colleagues have observed that even though these cells are injected in through the artery, they end up in the correct orientation.[74] Over a period of 7 days, there is an emergence in E-cadherin expression, which is a marker of tubular cells. Further, these cells become polarized (Fig 20). Thus, while the cells are injected at random through the arterial system, they somehow assume the correct orientation within the tubules, suggesting the existence of an early mechanism of polarization. E-cadhering staining is only observed around areas that are believed to contain tubules within the scaffold. This suggests that the extracellular matrix may be directing some of the cells to either differentiate within a specific pattern, or function differently in the case of the IPS derived cells. Expression of genes involved in cell-cell interactions also emerges during this time frame, including tight junction 1 and tight junction 2, and claudens.

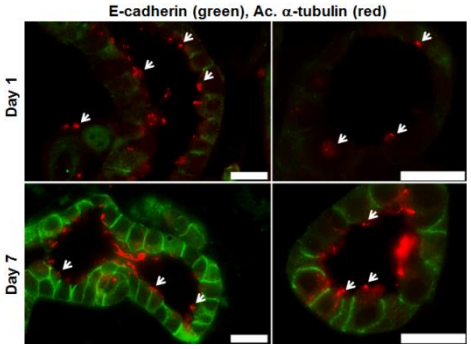

Figure 20. Proper tubule polarization

Enhanced cell maturity is obtained from these cells when early immature cells are cultured on extracellular matrix. The mechanisms that underlie this are currently under study. What are the extracellular matrix cues that instruct cells? Do they promote cellular functionality as well?[74]

C. Mechanisms of kidney development

The mechanisms of kidney development are instrumental to devising strategies for kidney biogenesis.[81] Nephron development initiates about 10 days into embryogenesis in the mouse, and at about 5 weeks in the human. Mesenchyme cells grow out of the ureteric bud. That population of mesenchyme cells has been pre-specified to generate the kidney and it also provides the signals that induces the outgrowth of that bud.[82]

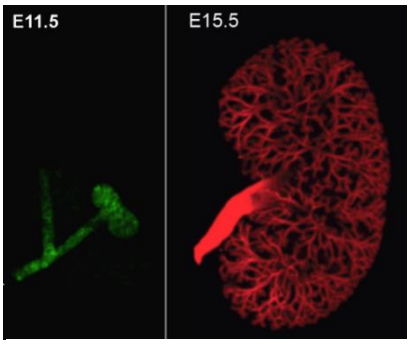

Figure 21. Branching morphogenesis of the ureteric bud

As the bud develops, it undergoes extensive branching (Fig 21). This establishes the arborized network of the ureteric epithelium collecting duct network that will form the drainage system for the kidney to which the nephrons attach. The signals involved in this branching process and the generation of the arborized network have been identified.[83] This includes signals that prevent ectopic branching, ensuring that branching occurs at the appropriate place. These signals are produced locally, by stem cell progenitors in the adjacent mesenchyme in the tips of the bud. These stem progenitor cells are uncommitted, unspecialized cells, which, when they move out from that tip domain, generate the more specialized cells of that ureteric collecting duct network.

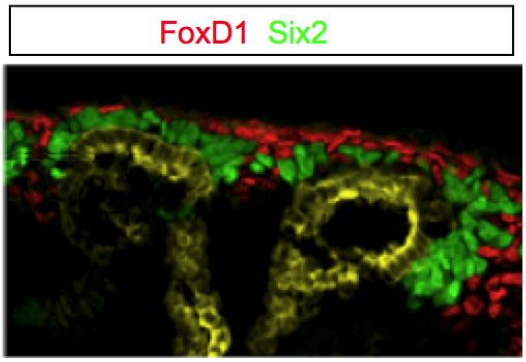

Figure 22. Fate of FoxD1+ and Six2+ cell populations

The epithelial network of the main body of the nephron is generated by mesenchyme or progenitor stem cells that lie adjacent to the tips of that collecting duct network. In Figure 22, there are green cells that surround that branching tip, and red cells that lie just outside that layer of cells. These two distinct populations are labeled with two transcriptional regulators: Six2 (green) and FoxD1 (red). The fate of those cell population during kidney development was traced. The descendants of those FoxD1-positive cells generate the entire interstitial cell network of the developing kidney, whereas the descendants of the Six2-positive population generate the main body of the nephron.[84, 85]

Six2 in this process is critical for kidney genesis (Fig 23). It's essential within the progenitor cells for maintaining the progenitor cell state. In the absence of Six2 function, those progenitor cells prematurely generate epithelial structures that presages formation of nephrons. On the left side are wild-type cells where the progenitors that lie above the tips are still mesenchymal, and on the right side Six2 mutant cells form little epithelial balls, the renal vesicles that are the precursors of the nephron. And the premature formation of those structures means that instead of generating a mouse kidney with 14,000 nephrons, only a few nephrons are formed.[86]

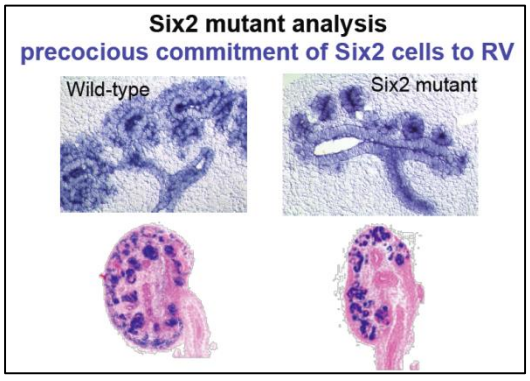

Figure 23. Six2 mutant analysis

More recently, other groups have been exploring processes that are going on in these two populations, the branching population and the nephron-inducing population, by introducing new technologies like

single-cell profiling of the progenitor population to see whether there are subsets of cells that may identify new properties that we can't see when looking at the population level.[81]

The mouse kidney contains approximately 14,000 nephrons. In the human it is closer to a million. The structure of the human kidney is more complex; it has a highly globular organization, and the organization of its primary branching events is different from the mouse. In the human kidney there is an arcading of nephrons, where nephrons attach to other nephrons. In addition, there is outgrowth of the ureteric epithelium, not just by dichotomous branching, but by growing out almost like a missile through cortical space.

While there are many similarities between mouse and human in terms of the distribution of genes critical for the nephron development, interesting differences can also be found in the two species as well. For example, Six2 is a critical regulator of progenitors in the mouse, and it has a closely related gene called Six1 in human. In the mouse, Six2 is expressed from the earlier stages of kidney development all the way to the end of nephrogenesis. However, Six1 has only a very transient period of expression, less than 24 hours right at the outset of kidney development.

How mechanisms of kidney development can guide research into kidney organogenesis and repair
Endogenous cell therapy: Progenitors are no longer present after development. Therefore, what is the nature of endogenous repair processes that occur later in life? How could these be manipulated for therapeutic purposes? Is there any way those silent developmental programs can be reactivated to any therapeutic effect?

Regenerative medicine and the kidney
Nephron progenitors can be isolated, but their properties are rapidly lost in culture. Thus, better approaches have to be developed to mimic the environments in the normal kidney, to be able to expand, manipulate and handle those cells in culture. These cells only differentiate in very complex cultures. Therefore, they are currently not well-suited for understanding this process or for isolating those nephrons.

With respect to renal-assist devices, cell transplantation, or recellularized scaffolds. A better understanding of the molecular characteristics of the cells is required. New approaches are needed to generate the cells, track them, and establish better structure function correlates.

For endogenous repair programs approaches to track the cells that are involved in repair. A greater understanding of how repair mechanisms relate to development processes and how they are distinct is needed. Evidence so far suggests that they are actually fairly distinct.

Appendix 1
Clinical studies in Treg Cell therapy

The One study - kidney[87]
EU network of contributing researchers across Europe led by Ed Geizler out of Regensburg. The Network was subsequent joined by Jeff Bluestone as UCSF and Larry Tucker and Jim Markmann at Massachusetts General Hospital. The phase 2-1-A safety study will evaluate the safety of cell therapies, including regulatory T-cells, but also tolerogenic-dendridic cells and regulatory macrophages in the setting of living donor kidney transplantation. In terms of regulatory T-cells then there will be a number of different types of regulatory T-cells compared within the study: polyclonally-active naturally occurring T-regs, donor alloantigen reactive regulatory T-cells (those that are expanded in the presence of donor alloantigen), and the interleukin-10- and TGFbeta-secreting type 1 regulatory (TR1) T cell – that suppress antigen-specific T-cell responses via cytokine-dependent mechanisms - pioneered by the Milan group and Maria Grazia Roncarolo. The study is ongoing.

There is a comprehensive immune monitoring program alongside the One study assays, such as flow cytometry. These assays have been developed by the European council, a team that have been working now together for a number of years, funded by the European commission. And the assays that are being investigated have been both developed and validated prior to the initiation of the One study. I should particularly highlight here Bergit Sevitsky from Berlin who has really developed the flow cytometry assays beautifully with Beckman Coulter that have been used throughout the study.

So that's the One study. Phase 1-2-A safety, hopefully will generate some interesting data allowing us not only to understand whether the cell therapy is safe but also giving us some insights as to which cells we should take forward for future analyses.

Flavio Vincenti and colleagues at UCSF - kidney[88]
What they're doing in this randomized, open label study is to use regulatory T-cells to try and reduce the severity of inflammation detected in biopsies detected six months post-kidney-transplantation. The presence of inflammatory infiltrates in renal allograft biopsy is associated with progressive renal dysfunction. The hypothesis of this study is that infusion of regulatory T-cells when these cellular infiltrates are detected will either prevent or inhibit the decline in renal function in the patient's that receive the cell infusions.

At the six months protocol biopsy stage, patients who are exhibiting this sub-clinical inflammation on the biopsy will be randomized to receive either standard of care, polyclonally expanded regulatory T-cells of recipient origin, or donor alloantigen reactive T-cells - T-cells that have been expanded in the presence of HLA-matched stimulating populations that will allow an enrichment of donor alloantigen reactivity.

University of California, San Francisco (UCSF) - liver[89]
In liver transplantation, there are also phase 1-2-A studies in progress both at UCSF and in London. Slides from Sandy Feng at UCSF and Alberto Sanchez-Fueyo in London have designed what they hope will be a T-reg supportive immunosuppressive regimen. T-regs will be infused later (week 11 to 13), post-transplant. And the idea is that the T-reg infusions will be based on T-reg that have been expanded ex vivo in the presence of cells expressing the HLA of the organ donor. So blood will be taken at the time of enrollment into the study. Blood from the donor will also be banked to generate these cell lines that are obviously of donor origin, and these will be used in the expansion strategy to enrich the regulatory T-cells as they expand for donor alloantigen reactivity. The cells that are expanded will be infused later in

the protocol once this conversion to what is hoped to be a permissive regimen for regulatory T-cell persistence and functionality has been implemented. And obviously this is also a safety study which will have serial sampling of the patients to understand what's happening as a result of T-reg infusion and obviously monitoring very carefully for potential adverse events. It's a dose escalation study with three phases culminating in the maximum dose of 800 million T-reg infused.

King's College London (KCL) - liver[90]

The London study also in liver recipients is based along very similar lines. But in this case the regulatory T-cells will be expanded polyclonally and interestingly there are differences between what the regulatory authorities will allow in Europe at the moment and in the United States. So in Europe at present we don't have permission to introduce cell-sorters into the CGP facilities where the regulatory T-cells are expanded. We have to isolate the cells using magnetic Bead-technology and obviously CD-25 then is the marker of choice for enriching regulatory T-cells. So these T-cells will be expanded ex vivo but they won't have enrichment for donor alloantigen reactivity. Again, in Alberto study, the regimen will promote hopefully the persistence and functionality of the regulatory T-cells infused by introducing rapamycin as a drug of choice in the post-transplant course. And the cells, again, will be infused late after transplantation once this regimen has been in place. So again there'll be extensive monitoring of these patients to determine the saftey of the T-reg infusions, but the ultimate aim of this study if successful is to achieve tapering of immunosuppression in a larger population of patients than can be achieved in the absence of the regulatory T-cell infusion.

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