T cell-directed immunosuppressive therapy and regulatory T cells for heart transplant survival in non-human primates

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T CELL-DIRECTED IMMUNOSUPPRESSIVE THERAPY AND REGULATORY T CELLS FOR HEART TRANSPLANT SURVIVAL IN NON-HUMAN PRIMATES

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Heart transplant survival in non-human primates

*T cell-directed immunosuppressive therapy and regulatory T cells for promotion of heart transplant survival in non-human primates*

**Overleving na harttransplantatie**

*T cell-gerichte immunosuppressieve therapie en regulatoire T cellen ter verbetering van de overleving na harttransplantatie in primaten*

**Proefschrift**

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Introduction and Outline
GENERAL INTRODUCTION

Heart transplantation significantly enhances the life expectancy of adult patients suffering heart failure, and infants born with malformations of their heart. However, there are many hurdles such as rejection of the transplanted organ, or side effects of the immunosuppressive drugs, that are yet to be overcome. This dissertation focuses on two components that may promote heart transplant survival, and survival of a transplanted organ in general. This Introduction reviews all the important components that are discussed in the thesis; it provides a general overview of the anatomy and function of the heart and the medical relevance of heart transplantation, the importance of non-human primate (NHP) transplant models to carry out the studies for this thesis, and the background of each research topic in relation to its medical relevance.

THE HEART

It’s all about the heart
The heart has always captured the imagination more than any other organ. People used to believe that the heart was the center of a person’s emotions, and many expressions that we still use today lead back to that concept. We can lose our heart to someone, be broken hearted, or win this very person’s heart back after all. We can pour out our heart to a friend (who hopefully has his or her heart in the right place) or claim that someone has a heart of gold...or stone. In the eyes of a Medical Doctor, however, it is simply a hollowed muscle that pumps blood through the blood vessels by contracting rhythmically.

Historic view on heart and circulation
The word cardiac (cardiology), which means ‘related to the heart’, is derived from the Greek word καρδία, kardia, for ‘heart’. In ancient Greek times the function of the heart and blood circulation was explained by Claudius Galen (131-201). In this theory blood vessels originated in the liver, blood was continuously formed from food and was consumed by the organs. There was no circulation; blood did not return to either the heart or the liver. The blood that reached the heart was either directed to the lungs, or it passed through the septum and reached the left ventricle, where the ‘internal fire' transformed it into ‘spirit of life' (spiritus vitalis). This spirit made the heart expand, and during diastole the motion of the arteries sucked the blood out of the heart. After death, the blood collected in the veins, leaving the arteries empty. The arteries were therefore thought to contain air during life too, and were named after that; the word ‘artery' is derived from aèr, containing air.
Interestingly, when Leonardo da Vinci (1452 – 1519) made his famous sketches of the heart and its blood vessels, the drawings were anatomically correct (anatomical notebooks, year unknown) (Figure 1); however, its function was still not clear. It took until 1629 for William Harvey to describe the new theory of blood circulation, with the heart as the central pump (1, 2).

Anatomy and function
The human heart is about the size of a fist, and lays in the thoracic cavity behind the sternum. It consists of striated muscle and connective tissue, and is divided into four chambers: the left and right ventricles, that expel blood into the body and the lungs, and the two atria, where venous blood returns to the heart. The blood follows two circuits: (1) deoxygenated blood returns from the body into the right atrium, flows into the right ventricle and gets pumped into the lungs; (2) from the lungs, the oxygenated blood flows back into the left atrium, passes into the left ventricle, and gets pumped throughout the body (Figure 2).

The sinoatrial (SA) node is a region in the heart that functions as the pacemaker. It initiates the contraction of the heart without need to be connected to the nervous system. However, the heart is also innervated by the parasympathetic vagal nerve, which enables our heartbeat to respond to emotions.

HEART TRANSPLANTATION

Indications for transplantation
Heart transplantation is required when a patient’s own heart is not capable of pumping sufficient blood through the body. Two large groups of patients can be identified that potentially need a heart transplant; adults suffering from heart failure, and infants that are born with a malformation of the heart or the vessels near it (congenital heart defects, CHD).
Heart failure is the condition that develops after part of the heart tissue has been damaged, usually in patients with coronary artery disease who experience myocardial infarction. During the infarction, no blood can reach part of the muscular tissue of the heart, leaving dead muscle in place. This part of the heart muscle will never contract again, leaving more work for the remainder of the cells. Working harder, these cells may become hypertrophic and dilate due to the extra stress that is put upon them with every contraction. Finally, the heart will no longer be able to supply the body with sufficient blood; first only during exercise, later even in rest. This end-stage heart failure can be supported by a mechanical left-ventricular assist device (LVAD) or the failed heart can be replaced by a heart transplant. Coronary artery disease is the leading cause of death in the world (3).

Figure 2: Blood flow through the heart
The blood follows two circuits: (1) Oxygen-poor blood returns from the body through the vena cava and enters the heart in the right atrium. The muscle walls of the right atrium contract and push the blood through the tricuspid valve into the right ventricle. Once the right ventricle has filled with blood, the right ventricle contracts and pushes the blood through the pulmonary valve, into the pulmonary artery. This artery transports the blood throughout the lungs.

(2) The oxygen-rich blood is then returned from the lungs to the left side of the heart, into the left atrium. Contraction of the heart muscle of the left atrium pushes the blood past the mitral valve, and into the left ventricle. Once the left ventricle is full of blood, the muscle walls contract pushing the blood past the aortic valve, into the aorta, and out into the body.
A congenital heart defect (CHD) is a malformation of the heart that is present at birth. These malformations can involve an abnormal circuit of blood flow through the heart, hypoplasia of part of the heart, or obstruction of part of the blood flow. CHD are among the most common birth defects with an incidence of 9 in 1,000 people. Although some defects do not need treatment, the more complex malformations require medication, surgery, or a heart transplant. In severe cases, a transplant is often required at very young age. Congenital heart defects are the leading cause of infant death resulting from birth defects (3).

**Life expectancy without transplantation**

Currently, only patients with a life expectancy of less than 1 year because of their heart failure are eligible the waiting list for transplantation. Therefore, for all patients who require a heart transplant, life expectancy if they do not receive a transplant is rather poor.

Children born with moderate to severe CHD have a much lower immediate (<1 year) mortality nowadays compared to half a century ago: in the 1950s, only 20% survived the first year of life; by 2006 90% of these children reached at least 18 years, due to medical and surgical interventions (4). This group of patients (that includes those who need or have received a heart transplant) has a significantly shorter life expectancy with a mean age of 37 +/- 15 years despite the medical advances (5). Nevertheless, if these children do not receive a heart transplant, they would most likely not reach adulthood at all.

"What this patient needs is a new heart!" – Development of heart transplantation

Except for a few single cases, most developments in heart surgery were made in the period between 1938 and 1982. Proper anesthesia and the use of sterile techniques were introduced by the mid-19th century, and subsequently blood transfusions and techniques for suturing blood vessels together were developed by the end of the 19th century. After these developments were established, the development of the first primitive heart-lung machines (1935, John Gibbon) and anti-coagulating drugs enabled some of the greatest steps in surgery. The first milestone in heart surgery was 1938, when Robert Gross developed the technique to correct a patent ductus – a vessel close to the heart. The first successful open heart surgery, a correction of congenital heart disease, was carried out in 1952 by John Lewis and Walton Lillehei (6).

It was Norman Shumway who had made most progress in laboratory experiments leading up to the first heart transplant. He became involved in it by coincidence, when during experiments in dogs involving the heart-lung machine, he started taking the
heart out and suturing it back in, mainly to practice his surgical skills while waiting. Later, he found that the dogs would survive for a period of time even if he transplanted a different dog’s heart. Importantly, his group perfected the surgical technique, they showed that the heart does not need to be connected to the nervous system to function properly, and they showed that the transplanted animals survived for several years.

However, the first human-to-human heart transplant was carried out by Christiaan Barnard on 3 December, 1967 at the Groote Schuur Ziekenhuis, Cape Town, South Africa. His intentions to do this procedure are vividly described by my mentor Dr. David Cooper: “I can personally vouch that Barnard had heart transplantation in his mind as early as 1965, when I visited his unit in Cape Town. He invited me to join him on a ward round visiting his patients, and introduced me to one patient with severe heart failure. When we had moved away from the patient’s bedside, Barnard turned to me and said, “What that patient needs, of course, is a new heart.” I agreed but, so far in the future did I see this form of treatment, I thought the remark had been made almost as a joke.” (6) Two years later the first heart transplant was a fact, and it remains one of the most important medical breakthroughs in the 20th century (7).

“Another goddamn miracle!” – Heart transplant techniques today

There are currently two different surgical techniques for transplantation of the heart; most often used is the orthotopic technique which involves removal of the native heart; the donor heart is sewn in its place instead. Less often used is the heterotopic transplant, in which the native heart is left in place; the donor heart is sewn alongside it (Figure 3) (8). This technique was developed by Losman and Barnard for patients whose heart function was more likely to recover, and it has also been proven beneficial for patients with severe pulmonary hypertension for which it is still used (9). After transplantation, when the donor heart has been sutured in and blood circulation through the heart has been restored, it may need to be defibrillated or may even start up by itself. Several hours after its removal from the donor’s chest, it suddenly bursts into life again.

On the waiting list for a heart transplant

As with any organ, the major hurdle of heart transplantation is the availability of too few suitable donor organs, and a long time on the waiting list resulting from that. Data from the United Network for Organ Sharing (UNOS; www.unos.org) (10) shows that annually around 3,500 patients are on the waiting list for a new heart in the United States. These are only patients that have a life expectancy of less than 1 year. The mean waiting time for a donor heart depends on UNOS status (severity of the condition), age and blood type of the patient, and is currently 50 – 300 days depending on
these factors. Out of the total 3,390 patients that were on the list in 2011, 2,311 (68%) received a transplant; 332 (10%) of patients died while on the waiting list, and another 272 (8%) had become too sick to undergo transplant.

Transplantation of a donor heart to an infant with congenital malformations is a particularly striking problem, as these infants often require a transplant at a very young age. Because of their small size, only a few suitable donors are available, but the distribution of transplants that can be carried out is similar to that in adults: UNOS data shows that there were 510 infants on the waiting list in 2011, of which 371 (73%) received a transplant; 46 (9%) died and 42 (8%) were too sick to undergo the transplant.

Patient survival
The most important outcome after heart transplantation is of course patient survival. The 5-year survival after heart transplantation is currently around 70%; 68% for infants and 72.5% for adults (www.unos.org) Mortality after transplantation is caused by rejection of the donor organ due to insufficient immunosuppressive therapy on one hand, and side-effects of these strong immunosuppressive drugs, such as infection, on the other. Combined with the fact that only 70% of the candidates on the waiting list

Figure 3: Heterotopic heart transplant
In heterotopic transplants, the native heart is left in place; the donor heart is sewn alongside it, connecting the donor and recipient left atria, right atria, pulmonary artery (PA) and aorta. Using this technique, both hearts receive venous blood from the body and the lungs, and both hearts pump blood back into the circulation.
list actually received a transplant, this results in a total of about 50% of patients who are in need of a heart transplant who will accomplish a 5-year survival.

Other outcomes after transplantation are related to quality of life; although a heart is only transplanted in cases where the condition of the patient is very poor and not likely to improve, the donor heart generally gives a much improved condition, and the patient can quickly pick up normal life activities that were not possible before. On the other hand, the life-long use of drugs that are required daily to prevent rejection result in many side effects. The most important is infection, since the drugs suppress the patient’s whole immune system and not only the response to the donor organ. This translates to a much shorter life expectancy and recurrent health problems, even if a patient belongs to the group of lucky ones, the 50% of patients who do receive a transplant and have a survival of more than 5 years. Today, receiving a heart transplant is considered a swap of a fatal disease for a chronic condition. Clearly there is a good deal of improvement to gain in (1) donor organ availability, (2) suppression of the rejection, and (3) avoidance of complications.

THIS DISSERTATION:
PRECLINICAL MODELS USED TO STUDY HEART TRANSPLANTATION

Primates as study subjects
Studies in this dissertation were carried out in non-human primates (NHPs). Particularly in the field of transplantation, the use of NHPs is considered the last step towards a clinical trial, where a novel therapeutic drug or procedure would be tested in human patients. These studies are designed based on previous evidence from studies carried out in mice or on cells, which then need to be validated in a larger animal model. Non-human primates are the most adequate model to study human-like immunological responses. Often only very small study groups are used, partially because of the costs of each experiment, because these studies are used as a proof of principle, and most importantly for humane reasons.

For the studies involving transplantation in infants (chapter 2, 3, 4), we studied infant baboons (papio anubis) receiving an artery patch transplant from blood group-incompatible baboons or pigs. Transplantation of an organ from a different species (xenotransplantation) may be a realistic option in the future, initially to bridge the period until a suitable human donor is identified. Pig tissue expresses galactose-α1,3-galactose (Gal) (11), a carbohydrate similar to A and B blood group antigens. Like humans, Old World monkeys and baboons do not express Gal on their cells, and
consequently make natural anti-Gal antibodies. These preformed anti-Gal antibodies are responsible for hyperacute rejection that develops within minutes to hours when a pig organ is transplanted (12). In this model we studied the effect of T-cell and antibody development when immunosuppressive therapy and a transplant were given at a very young age. By using baboons for this study, we were able to evaluate both anti-blood group as well as anti-Gal antibody development, and translate results to what may be expected in humans.

For studies involving the lymphocyte-depleting immunosuppressive drug, alemtuzumab (chapter 5, 7, 8) cynomolgus monkeys (Macaca fascicularis) of Indonesian origin were used. This particular subspecies of primates are the only species of NHP so far identified that, like humans, do not express CD52, the target of alemtuzumab, on their red blood cells. In any other type of NHP, the administration of alemtuzumab would lead to lysis of all erythrocytes and cause death of these animals. Each primate for these studies was thoroughly selected to ensure no CD52 was expressed on the erythrocytes before including the animal in the study.

Surgical models: artery patch and heterotopic heart transplantation

Heart transplantation in humans is generally carried out as an orthotopic transplant: as soon as a donor is identified, the patient’s chest will be opened and prepared to take out the native heart. This involves connecting the patient to a heart-lung machine. Only after the donor heart has arrived in the operating room, the native heart is removed from the patient’s chest, and the donor heart is sutured into the exact same location to replace it. For the studies in this thesis, the main goal was not to keep a very sick patient alive with a new heart, but to study the immunological responses after transplantation. Therefore, two surgical approaches were used as a model to avoid this complicated surgery.

A heterotopic heart transplant was carried out in the cynomolgus monkeys (chapter 5,7,8). Unlike the technique used in humans, where the heart would be sutured onto the patient’s sick heart in the chest, in our monkeys the heart was transplanted to the abdomen. Briefly, an atrial septal defect is created after the donor heart is harvested, and the veins connecting to the atria are closed. The recipient’s abdomen is opened and the abdominal aorta and inferior vena cava (IVC) are isolated and cross-clamped. Then the donor’s aorta is connected to the recipient’s abdominal aorta, and the donor’s pulmonary artery is connected to the IVC, resulting in blood flow through all the compartments of the donor heart. Just like in human heart transplants, after removal of all clamps and restoration of blood flow through the heart, the donor heart would start beating. By using this technique the heart is connected to the circulation and
therefore all immunological responses of the recipient against this transplant will be comparable to those if the heart would be transplanted into the chest. However, it avoids surgical complications of opening a small monkey's chest, and monitoring of rejection was easily done by palpating the monkey's abdomen to feel the strength of the contractions of the donor heart. In some cases, the pulsations could even be seen through the abdominal wall.

In the studies in infant baboons (chapter 2,3,4) an even simpler model was used to study the immunological response: only a patch of donor artery was transplanted. Briefly, a piece of donor artery is harvested and cut into a piece of approximately 1 x 2.5 cm. The recipient's abdomen is opened, the abdominal aorta is identified and clamped. An incision of approximately 2.5 cm is made in the length of the aorta, and the artery patch is transplanted as a full-thickness graft into the wall of the aorta. By using this technique there is exposure of donor endothelium to the recipient's circulation, which triggers the immunological response. Monitoring of rejection could only be done by laboratory assays, focusing on antibody detection and cellular responses. However, this approach avoided major surgery and its related complications in infant baboons that were 3 months old and around 1 kg in weight at the time of transplant.

T CELL-DIRECTED IMMUNOSUPPRESSIVE THERAPY IN INFANTS

Immunological response in an immature recipient

Infants born with a severe malformation of the heart need a transplant at a very young age, often within the first year of life. The immature immunological response has been thoroughly studied in infant laboratory mice (reviewed in 13). However, much less is known of the development of the immune system of humans and NHPs. Natural antibodies are the pre-formed IgM antibodies which develop during infancy. An important finding is that in human and baboon infants, this development of natural antibodies does not begin until 3 months of age, and subsequently develop during the first year (14). In human infants, an ABO-incompatible heart transplant can be carried out before these natural antibodies have formed, leading to B-cell tolerance to the donor graft (15). Similar to anti-blood group antibodies, the development of anti-pig antibodies, mainly directed against the carbohydrate Gal that is expressed on pig tissue, does not start until 3 months of age (16). Importantly, hyperacute rejection does not occur after a pig organ is transplanted into infant baboons that receive no immunosuppressive therapy (17). We investigated the effect of a transplant from a blood-group incompatible baboon or a pig donor (xenograft), as well as the effect
of immunosuppressive therapy alone, on the development of natural antibodies in infant baboons (chapter 2).

Laboratory mice are born lymphopenic and show lymphopenia-induced proliferation of T cells (18). We sought to study the natural development of memory T cells in untreated infant baboons, as well as the lymphopenia-induced proliferation and recovery of memory T cells that was induced by the administration of T-cell directed immunosuppressive therapy (chapter 3).

**Side effects of immunosuppressive therapy**

The immunosuppressive therapy that is needed to prevent rejection can cause serious side effects and illness in patients who receive any type of transplant. Two types of complications were studied in detail. In chapter 4 we discuss the development of a collagenous colitis-like disease in four infant baboons. Although it is not commonly associated with an immunocompromised state, cases of colitis have been reported in patients with organ transplants who were receiving immunosuppressive therapy (19). Only one case of collagenous colitis has been reported in NHPs previously, but this was not associated with a transplant or immunosuppressive therapy (20).

Second, we studied several cases of sepsis associated with immunosuppressive therapy. Although it is a common side effect after transplantation and associated daily use of immunosuppressive drugs (21), the diagnosis of sepsis in the large animal model is more difficult than in the clinical setting. Associated studies using T-cell directed immunosuppressive therapy in a cohort of young monkeys and pigs was evaluated in an islet transplant setting (chapter 5). These diabetic animals developed severe hypoglycemia and acidosis after immunosuppressive therapy was initiated. We believe this report describes a hitherto unreported syndrome.

**ALEMTUZUMAB AND REGULATORY T CELLS FOR HEART TRANSPLANTATION IN NON-HUMAN PRIMATES**

Alemtuzumab

Alemtuzumab (Campath-1H) is a humanized anti-CD52 monoclonal IgG antibody, which profoundly depletes all cells expressing CD52 on their surface (22). In humans, CD52 is expressed by all lymphocytes, natural killer (NK) cells, dendritic cells and monocytes. Unlike humans, most NHP species express CD52 on both white and red blood cells. The Indonesian sub-species of cynomolgus monkey, however, does not express CD52 on its erythrocytes, and can be given alemtuzumab safely (23). After alemtuzumab has been administered, a rapid and profound depletion of 99%
of all circulating lymphocytes is seen in the blood of both humans and cynomolgus monkeys, and particularly CD4+ T cells will remain depleted for a long time. For this reason it has increasingly been used as induction therapy for transplantation (24). Additionally, the period of lymphopenia could be used as a window of opportunity for cell therapy, such as the administration of regulatory T cells. These cells would have much more effect if only a limited number of the recipient’s T effector cells remained.

**Regulatory T cells**

Regulatory T cells (Treg) offer potential for improving long-term outcomes in cell and organ transplantation. This specific subpopulation of CD4+FoxP3+ T cells is involved in the control of immunity (25, 26) and tolerance to auto- and alloantigens (27). The NHP model is a valuable resource that can be used to address issues concerning the transfer of Treg therapy to the clinic. The current knowledge on Treg in NHP is discussed in chapter 6.

**The effect of alemtuzumab on regulatory T cells**

Alemtuzumab is thought to mostly deplete lymphocytes in the blood and not in the bone marrow (28). Its half-life is approximately 8 days in both humans and cynomolgus monkeys; however, its depleting effect can last for up to 2 months. Long-term (>1 year) effects in human renal transplants include a high proportion of repopulating memory effector T cells, and a transient increase of Treg in the circulation (29). Very little is known about its effect on lymphocytes in lymph nodes, which play a central role in antigen presentation, and serve as a major reservoir and homing base for circulating lymphocytes. Therefore, the potency of alemtuzumab to deplete subsets of lymphocytes, including Treg, from monkey lymph nodes was studied (chapter 7).

As future aims are to infuse Treg in patients after they have received alemtuzumab, a major concern would be that the remaining alemtuzumab in the circulation could potentially deplete the cells that are to be administered. Naturally-occurring, circulating Treg display relatively high levels of CD52 compared with other lymphocyte subsets, and are susceptible to killing by alemtuzumab in vitro (30, 31). However, little is known about CD52 expression on Treg after their expansion. In chapter 8 we studied the effect of alemtuzumab on both freshly isolated Treg as well as Treg that were expanded ex vivo, that could potentially be administered to monkeys receiving a heart transplant.

**Induced regulatory T cells**

As naturally-occurring, thymus-derived Treg are relatively rare, alternative options to obtain sufficient cell numbers were assessed. Induced regulatory T cells (iTreg) can be generated from naïve T cells in peripheral lymphoid organs during the immunological
response to antigen (Ag) stimulation, or in vitro through stimulation of mouse or human T cells under specific conditions. Various groups have observed that iTreg can be generated easily by conversion of much more abundant naïve CD4+ T cells in vitro, allowing much higher numbers of Treg to be obtained (32, 33, 34). The mechanisms of conversion and the suppressive potential of these in vitro-generated cells are reviewed in chapter 9.

We studied the generation of these induced Treg as well as their function after conversion, using particles that release the necessary cytokines in a controlled fashion (chapter 10). These induced Treg offer a potential treatment option either alone or in combination with naturally occurring Treg.
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OUTLINE

Two related components of T-cell related transplant immunity were studied in this thesis, with the final goal to promote heart transplant survival. In all studies, non-human primates were used as a preclinical model to validate future studies in humans. Both T-cell directed immunosuppressive therapy, which significantly depletes circulating T cells pre-transplant, as well as the use of regulatory T cells, which potentially enhance suppression of the rejection, were evaluated. The studies were conducted either in an actual heart transplant setting in adult monkeys, or by using artery graft transplantation in infant baboons as a model for heart transplantation for congenital heart malformations.

First, the effect of T-cell directed immunosuppressive therapy (in the form of anti-CD154 monoclonal antibody) was studied when this potent T-cell depleting drug was administered to infant baboons at 3 months of age, before the development of natural antibodies occurred (chapter 2). Both naïve infant baboons as well as infants receiving an artery transplant were included in this study. We then evaluated the effect of this drug on their T-lymphocyte homeostasis and population of memory cells (chapter 3). Additionally, as some of the animals receiving T-cell directed immunosuppressive therapy developed drug-related side effects, two syndromes that occurred in several cases were studied in detail; a collagenous colitis-like condition that developed in some of the infant baboons receiving anti-CD154 monoclonal antibody (chapter 4) and a syndrome of severe hypoglycemia and acidosis in diabetic monkeys and pigs, that were used for related studies on islet transplantation (chapter 5).

Second, a different aspect of T-cell directed immunomodulation in the transplant setting is the use of regulatory T cells, a rare subpopulation of CD4+ T cells that have a suppressive function. These cells were first described in 1995, and although studies in the mouse model are very promising, only few studies have been conducted in non-human primates up to date. The current knowledge on regulatory T cells in non-human primates is discussed in chapter 6. The effect of alemtuzumab, known for its potent depletion of lymphocytes from the circulation, was studied for its depleting potency on subsets of lymphocytes, including regulatory T cells, in monkey lymph nodes (chapter 7) and on expanded monkey regulatory T cells that could potentially be administered to monkeys receiving a heart transplant (chapter 8). As naturally occurring regulatory T cells are relatively rare, alternative options to obtain sufficient cell numbers were assessed. The generation of regulatory T cells from a naïve T cell subpopulation (conversion) was reviewed in chapter 9; the conversion of T cells into Treg and their suppressive function were investigated using particles that release the necessary cytokines in a controlled fashion (chapter 10).
Section 1

T cell-directed immunosuppressive therapy in immature non-human primate recipients
T cell-based immunosuppressive therapy inhibits the development of natural anti-AB blood type and anti-pig antibodies in infant baboons


*EMD and CM contributed equally

Transplantation 2012; 93:769-776.
Background
We set out to determine whether B cell tolerance to A/B-incompatible allo-antigens and pig xeno-antigens could be achieved in infant baboons.

Methods
Artery patch grafts were implanted in the abdominal aorta in 3-month-old baboons using either A/B-incompatible allografts (AB-I) or wild-type pig xenografts (pig). Gp1 (controls, n=6) received no immunosuppressive therapy (IS) and no graft. Gp2 (n=2) received an AB-I or pig graft, but no IS. Gp3 received AB-I grafts+IS (Gp3A: n=2) or pig grafts+IS (Gp3B: n=2). IS consisted of ATG, anti-CD154mAb, and MMF until age 8-12m. Gp4 (n=2) received IS only, but no graft.

Results
In Gp1, anti-A/B and cytotoxic anti-pig IgM increased steadily during the first year. Gp2 became sensitized to donor-specific AB-I or pig antigens within 2w. Gp3 and Gp4 infants that received anti-CD154mAb made no or minimal anti-A/B and anti-pig antibodies while receiving IS.

Discussion
The production of natural anti-A/B and anti-pig antibodies was inhibited by IS with anti-CD154mAb, even in the absence of an allograft or xenograft, suggesting that natural antibodies may not be entirely T cell-independent. These data are in contrast to clinical experience with AB-I allotransplantation in infants, who cease producing only donor-specific antibodies.
INTRODUCTION

A major hurdle in transplantation (Tx) is the long waiting time to obtain a donor organ. This problem is particularly striking in infants with congenital heart defects, who often require heart Tx at a very young age (1). The pig could provide an alternative source of organs if the immunological barriers could be overcome (2). The initial barrier is related to the presence of natural (preformed) antibodies (Abs) in the recipient directed to antigens on the vascular endothelium of the pig organ (3, 4). Ab binding initiates activation of the complement cascade, resulting in hyperacute rejection (5, 6).

Natural IgM Abs develop during infancy, a process believed to be associated with colonization of the gastrointestinal tract with bacteria/viruses that express galactose-α1,3-galactose (Gal) antigens (7-9). This natural Ab production is considered to be T cell-independent (10), though there is some evidence that it may be T cell-dependent (11). The development of anti-Gal Abs is similar to that of other Abs directed to carbohydrate antigens, e.g., the A and B blood group antigens (12). Natural anti-A/B Abs are usually absent during the first 3 months of life in humans and baboons, but subsequently develop during the first year (9, 13). The relative absence of Abs during the first few months has provided a ‘window of opportunity’ during which AB-incompatible (AB-I) organ Tx can be carried out successfully (14). Infants who received an AB-I organ did not reject the graft, and subsequently developed donor-specific B cell tolerance, defined by the absence of donor-specific Abs in the presence of a functioning graft and normal development of non-specific Abs, which is confirmed by a negative anti-donor agglutination titer and/or ELISPOT (15, 16).

Anti-pig Abs are primarily directed against Gal antigens expressed on pig cells, which share their core structure with the ABO antigens (12). Like anti-A/B Abs, Abs to wild-type (WT) pig cells in human and baboon infants do not develop until approximately 3m of age (9). Early studies demonstrated that hyperacute rejection does not occur after WT pig heart Tx into untreated newborn baboons (17).

We hypothesized that if an infant received a pig organ graft before the development of natural anti-pig Abs, these Abs might never develop, and B cell tolerance to the pig graft would result. We have investigated this by carrying out WT pig or AB-I baboon artery patch Tx in baboons of 3m of age. We could not confirm that this hypothesis is correct; however, all baboons that received anti-CD154mAb-based immunosuppressive therapy (IS), irrespective of the presence or source of a graft, showed inhibited development of both anti-pig and anti-A/B Abs compared to their age-matched controls, suggesting that natural Abs are T cell-dependent.
MATERIALS AND METHODS

Animals

Infant baboons (Papio anubis, Division of Animal Resources, Oklahoma University Health Sciences Center [OUHSC], Oklahoma City, OK) were housed at the University of Pittsburgh (UPitt) or in the specific pathogen-free facility at OUHSC. Donor pigs (White/Landrace, Country View Farms, Schellsburg, PA) and donor baboons (OUHSC) were housed at UPitt. Baboon aorta or pig carotid artery was harvested on the day of Tx.

Selection of baboons

AB blood type was determined in the first month after birth by staining of buccal smears from 43 colony-raised infant baboons, and was confirmed at least once after 3m of age. To allow monitoring of development of Abs to non-self (AB-I) blood group antigens over time, only baboons of either A or B blood group were selected. Five A and 9 B baboons were selected for the study (Table 1).

<table>
<thead>
<tr>
<th>Table 1: Experimental Groups</th>
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*a Group 3 infants: immunosuppressive therapy: full regimen with ATG induction and maintenance anti-CD154 mAb + MMF.
*b Group 4 infant B209: CTLA4-Ig only.
*c Group 4 infant B309: anti-CD154 mAb only.
†Euthanized or died
Experimental Groups

Four groups of infants were studied (Table 1). Control baboons (Gp1: n=6) received no IS and no graft. Gp2 (n=2) received either an AB-I or WT pig graft, but no IS; both baboons were followed until 1m after Tx. Gp3 received AB-I grafts+IS (3A: n=2), or WT pig grafts+IS (3B: n=2). Gp4 (n=2) received IS only (either anti-CD154mAb or CTLA4-Ig only), but no graft.

Figure 1: Anti-A, -B, -Gal and -pig serum antibody levels in Group 1 (n=6) and anti-pig levels in Group 4 (n=2)

(A) IgM levels (mean±SD) of anti-self, anti-AB-I, and anti-Gal, showing undetectable levels of anti-AB-I and anti-Gal at 1m of age, comparable to anti-self (OD<0.2), and thereafter slowly increasing levels of anti-AB-I IgM and a more rapid increase in anti-Gal IgM with increasing age.

(B) IgG levels (mean±SD) of anti-self, anti-AB-I, and anti-Gal showing a relatively high level of anti-Gal IgG at 1m of age, with a slow decrease with advancing age, suggesting these were maternal IgG antibodies. AB-I IgG were undetectable throughout the period of study.

(C) The ELISA data were confirmed by flow cytometry analysis of infant baboon serum binding to WT pig PBMC. IgM levels were increasing by 3m of age, and continued to increase thereafter. Anti-WT pig IgG levels remained low (largely undetectable) for the entire period of follow-up. (*15 and 18 months: n=2).

(D) The single anti-CD154mAb-treated infant (Gp4, B309) showed low levels of binding to WT pig PBMC that remained low (but gradually rose) after discontinuation of IS. In contrast, the single CTLA4-Ig-treated infant (Gp4, B209) showed levels that increased slightly before discontinuation of IS, and rose more rapidly after discontinuation of IS. Colored area indicates time of IS in both baboons.
Artery Patch Transplantation

In Gps2+3 infants, a length of donor carotid artery (pig) or aorta (baboon) was transplanted as a full-thickness onlay graft (approximately 1.0x0.5cm, Supplementary Figure 1) into the wall of the abdominal aorta at 3m of age (Table 1). Donor grafts were obtained from adult AB-I baboons or from WT pigs of blood type O.

Immunosuppressive and Supportive Therapy

Immunosuppressive and supportive therapy is described in Table 2. Gp3 (n=4) received therapy from 3m of age (Table 1). Maintenance IS was discontinued at 8m, except in one baboon in Gp3A (B7507, discontinued at 12m). Gp4 received either anti-CD154mAb or CTLA4-Ig alone, which was discontinued at 8m.

Monitoring of Recipient Baboon

Follow-up in Gp1 was for 12-21m, Gp2 for 1m post-Tx, Gp3 for 5-18m post-Tx, and in Gp4 for 14m. Monitoring is detailed in Table 2.

RESULTS

Group 1: Naïve controls (n=6)

All naïve controls (Table 1) remained healthy and showed steady weight gain during the study.

All anti-self blood group OD values remained at <0.2, which we regarded as absent or undetectable Ab. All infants showed undetectable anti-AB-I (non-self) and anti-Gal IgM levels at 1m of age, followed by a gradual increase of AB-I IgM and a more rapid increase of anti-Gal IgM; both could be detected by 4m (Figure 1A). Initial high anti-Gal IgG (but not anti-AB-I IgG) levels were detected in 3 infants (at 1-3m), which was likely due to maternal IgG; at later ages, anti-Gal IgG steadily fell throughout the period of follow-up until undetectable after 15m, whereas anti-AB-I IgG remained undetectable (Figure 1B). No significant differences in Ab levels were noted between those housed in the OUHSC specific pathogen-free facility (n=4) and those at UPitt (n=2) (not shown).

Flow cytometry confirmed the ELISA data; all infants showed a gradual increase in IgM binding to pPBMC (Figure 1C) and pAEC (not shown). IgG levels were high in some infants after birth, but decreased over time (Figure 1C). Cellular responses of control and transplanted infants are discussed in Supplementary Results and are detailed elsewhere (Chapter 3).
Table 2: Immunosuppressive and Supportive Therapy and Monitoring

<table>
<thead>
<tr>
<th>Induction therapy</th>
<th>Dose</th>
<th>Duration</th>
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<tbody>
<tr>
<td>Thymoglobulin</td>
<td>2.0 - 2.5mg/kg i.v.</td>
<td>Day -3 and -1</td>
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<tr>
<td>Methylprednisolone</td>
<td>5mg/kg i.v.</td>
<td>Before each dose of ATG and on day 0. The dose was then reduced by 1mg/kg/d, and discontinued on day 5</td>
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<tr>
<th>Maintenance therapy</th>
<th>Dose</th>
<th>Duration</th>
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<tr>
<td>Anti-CD154 mAb*</td>
<td>20-25mg/kg i.v.</td>
<td>Days -1, 0, 4, 7, 10, 14, then every 5-7d</td>
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<tr>
<td>Mycophenolate mofetil (MMF)</td>
<td>20-150mg/kg/d p.o. divided in 2 doses</td>
<td>Begun on day -2 (to maintain a blood trough level of 3-6µg/ml)</td>
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<tr>
<th>Supportive Therapy</th>
<th>Dose</th>
<th>Duration</th>
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<tbody>
<tr>
<td>Cefazolin</td>
<td>25mg/kg bid i.v</td>
<td>For 3 days after surgery</td>
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<tr>
<td>Famotidine</td>
<td>0.25mg/kg bid i.v</td>
<td>From day -3 until 1 month post-Tx</td>
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<tr>
<td>Ganciclovir</td>
<td>5 mg/kg i.v</td>
<td>From day -4 until 1 month post-Tx</td>
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<tr>
<td>Ketorolac</td>
<td>0.5mg/kg i.v</td>
<td>Before every dose of anti-CD154mAb</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.01mg/kg bid i.v</td>
<td>For 3 days after surgery</td>
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<tr>
<th>Monitoring</th>
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<th>Frequency</th>
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<tbody>
<tr>
<td>Weight</td>
<td>Weekly. After discontinuation of IS: monthly</td>
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<tr>
<td>Blood cell count and chemistry</td>
<td>Weekly. After discontinuation of IS: monthly</td>
<td></td>
</tr>
<tr>
<td>Anti-CD154 mAb levels</td>
<td>Weekly, until discontinuation of anti-CD154</td>
<td></td>
</tr>
<tr>
<td>MMF levels</td>
<td>Weekly, until discontinuation of MMF</td>
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<tr>
<td>T and B cells counts</td>
<td>Pre-Tx, before and after each dose of thymoglobulin, weekly for one month, then monthly</td>
<td></td>
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<tr>
<td>Anti-A / -B / -Gal Abs (ELISA)</td>
<td>Pre-Tx, and at age 4, 6, 9, 12, 15 and 18 months</td>
<td></td>
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<tr>
<td>IgM and IgG binding to anti-WT pig PBMC and PAEC (FCM)</td>
<td>Pre-Tx, and at age 4, 6, 9, 12, 15 and 18 months (at 15 and 18 months, only PBMC were tested)</td>
<td></td>
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<tr>
<td>IgM binding to anti-GTKO pig PBMC (FCM)</td>
<td>Pre-Tx, and at age 6, 12, 15 and 18 months</td>
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<tr>
<td>Complement-dependent cytotoxicity assay</td>
<td>At age 1, 6, 12 and 18 months</td>
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<tr>
<td>Mixed leukocyte reaction</td>
<td>Pre-Tx, and at age 4, 6, 9, 12, 15 and 18 months</td>
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<tr>
<td>Histology (H&amp;E staining)</td>
<td>Transplanted artery patch and native aorta harvested at time of necropsy</td>
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<tr>
<td>Immunofluorescence (anti-A, -B, and -Gal staining)</td>
<td>Transplanted artery patch and native aorta harvested at time of necropsy</td>
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*Selected as, in our experience, it is the only immunosuppressive agent that effectively prevents a T cell-dependent elicited Ab response in baboons to pig antigens (22).

Group 3 infants (n=4) received the full regimen, including all of the above listed drugs, except for CTLA4-Ig.

Group 4 infants (n=2) received either weekly CTLA4-Ig (B209) or weekly anti-CD154 mAb (B309) only, plus weekly ketorolac to prevent thrombus formation.
**Group 2: Artery patch Tx without IS (n=2)**

One infant received an AB-I (B7707) and one a WT pig (B7607) graft at 3m, with no IS (Table 1). In B7707 (AB-I graft, A→B), on ELISA there was a strong increase in anti-A IgM and IgG, but not of anti-B or anti-Gal Abs (Figure 2). Flow cytometry showed no significant increase in IgM or IgG binding to WT pPBMC or pAEC post-Tx. In B7607 (pig graft), a strong increase in anti-Gal IgM and IgG, but not in anti-A or anti-B was detected by ELISA (Figure 2). On flow cytometry a strong increase in IgM and IgG binding to pPBMC and of IgG binding to pAEC after Tx was seen (not shown), indicating sensitization to the graft. Histological examination of the grafts showed extensive inflammatory cell infiltrates in both infants (not shown).

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**Figure 2:** IgM and IgG anti-AB-I and anti-Gal antibodies measured by ELISA in Group 2 baboons (n=2) and IgM levels in B5008 (Group 3).

In B7707 (AB-I graft, A→B), anti-A IgM (C) and IgG (D) increased rapidly post-Tx, whereas anti-self IgM (A) and IgG (B) and anti-Gal IgM (E) and IgG (F) remained low. In B7607 (blood group B, WT pig graft), anti-self IgM (A) and IgG (B) as well as anti-AB-I IgM (C) and IgG (D) remained low, while anti-Gal IgM (E) and IgG (F) strongly increased post-Tx. Donor-specific sensitization to the artery patch graft developed in these non-immunosuppressed infant baboons.

**G** In B5008, IS therapy was discontinued at 8m. A delayed increase in anti-Gal IgM Ab was observed (beginning at 12m, i.e., 4m after discontinuation of IS) whereas there was an absence of AB-I (anti-B) IgM Abs for >10m after discontinuation of IS, suggesting that a state of donor-specific B cell tolerance may have been achieved. There was no increase in IgG levels (H). The colored area indicates the period of time during which B5008 received IS.
Altogether, these data confirmed that sensitization specific to the donor type (AB-I or WT pig) had occurred.

Group 3: Artery patch Tx and IS (n=4)

Four baboons received an artery patch Tx and IS (Table 1). All baboons showed normal weight gain after Tx, and follow-up was uncomplicated for 4-9m. At that time, within the same period of 4w, all baboons developed features of a colitis (diarrhea, weight loss), which necessitated discontinuation of IS; one died and one was euthanized, and the other two recovered and remained in follow-up (Dons EM, et al, submitted).

Figure 3: T cell kinetics and antibody levels in Group 3 baboons (n=4) compared to Group1 (n=6).

(A) In the control Gp1, CD4⁺ T cell numbers remained steady. In Gp3, CD4⁺ T cell numbers were profoundly depleted by thymoglobulin induction therapy and maintained low throughout the course of IS. A slow recovery occurred after discontinuation of IS, but the number remained lower than in Gp1 for several months. (B) B cells were not affected by the immunosuppressive regimen: no difference was observed between Gp1 and Gp3. Mean anti-AB-I IgM (C) and anti-Gal IgM (E) levels in all Gp3 baboons remained lower than those in the Gp1 control baboons. The differences reached significance at two time-points (4m, p<0.05, and 12m, p<0.01). Both anti-AB-I and anti-Gal IgG (D and F) remained low at all time-points in Gp1 and Gp3, indicating that no sensitization occurred. Colored area indicates period of IS in 3 of the 4 Gp3 baboons. (B7507 received IS for 12m). (*15,18, 21 months: n=2 for both groups).
Chapter 2

T cell kinetics

Following thymoglobin induction, there was a marked reduction in T cell (CD4+ and CD8+) numbers in all baboons. The number of CD4+ T cells remained low throughout the course of IS, after which slow recovery began (Figure 3A). The immunosuppressive regimen or transplant did not have significant influence on B cell (CD20+) numbers (Figure 3B). Cellular proliferative responses are discussed in Supplementary Results and are detailed elsewhere (van der Windt DJ, et al, submitted).

Anti-A, -B, -Gal and –pig antibodies

By ELISA and flow cytometry, all baboons showed no or minimal increase in anti-AB-I (Figure 3C) or anti-Gal/pig (Figure 3E) IgM Abs while IS was being administered, after which there was a slow increase in levels. Besides the maternal IgG that was only detected pre-Tx, no anti-AB-I or anti-Gal/pig IgG Abs were detectable while IS was being administered. On ELISA, B5008 (AB-I graft, B$\rightarrow$A) showed an absence of anti-B IgM Abs for >12m after discontinuation of IS (Figure 2G), whereas there was a delayed increase in anti-Gal IgM Ab (beginning 4m after discontinuation of IS). Moreover, both anti-AB-I and anti-Gal IgG Abs remained undetectable long-term after discontinuation of IS (Figure 2H).

Histopathological examination of the aortic patch grafts

Microscopy of the Gp3 grafts showed that the two infants with AB-I grafts had developed some fibrosis in the connective tissue at the site of the graft, but there was no inflammatory cell infiltrate at the time of euthanasia (not shown). After pig xenografting, a minimal cell infiltrate was seen in B5508, which was euthanized shortly after discontinuation of IS, while the pig xenograft in B5708, that remained without IS for >13m, contained numerous tortuous vascular channels suggesting previous thrombosis and recanalization, and one dense focus of inflammatory cells (not shown).

Staining of sections of native aorta and graft for anti-A, -B and -Gal Abs showed that in all Gp3 infants, except for B5508 (pig graft), donor antigens could be identified on the vascular endothelium of the aorta and vasa vasorum (Supplementary Figure 2), confirming that the transplanted tissue still presented antigen to the recipient at time of euthanasia in 3 out of the 4 infants.

From these observations, we concluded that (i) donor-specific sensitization did not occur, (ii) natural Ab production to both A and pig antigens was inhibited, if not completely prevented, but (iii) B cell tolerance to pig antigens was not achieved. (Evidence for B cell tolerance to AB-I antigens was equivocal, and is discussed below).
Group 4: IS only (n=2)
As there was evidence that all Gp3 baboons showed an overall delayed production and lower level of Abs compared to their age-matched controls, which was not related to the type of graft, we added two infants to the study (Gp4). These received IS, but no graft. One received only anti-CD154mAb and the other only CTLA4-Ig (Table 2); no induction or other maintenance therapy was administered. As they did not have to be weaned or undergo Tx, the IS was begun at 1m of age, and discontinued at 8m.

Both ELISA and flow cytometry data indicated that in B309 (receiving anti-CD154mAb) both anti-A/B and anti-Gal/pig IgM remained undetectable at all time-points (Figure 1D). At 1m of age, this infant had high maternal anti-A, -B, and -Gal IgG that fell to almost undetectable levels by 6m of age, and remained undetectable. B209 (receiving CTLA4-Ig) initially showed low levels of IgM, but when IS was discontinued at 8m of age, the level was already comparable to that in the untreated Gp1 controls, and rose thereafter (Figure 1D).

These data indicate that, when compared with age-matched controls, anti-CD154mAb suppressed the development of Ab and the cellular response in an infant baboon even when given as monotherapy. In contrast, CTLA4-Ig as monotherapy did not have the same effect.

DISCUSSION
As demonstrated by West and her colleagues and others, the natural ‘plasticity’ of the immune system in infants can provide a window of opportunity to induce a state of B cell tolerance to an ABO-I allograft (1, 18). We investigated whether the encouraging results of ABO-I organ Tx in human infants in the absence of natural Abs to carbohydrate blood group antigens might be translated to similar results following pig tissue xenotransplantation.

A weakness of our study, as with many studies in non-human primates, is that it involved a very limited number of experiments, and more data are required to confirm our initial tentative conclusions. The IS selected might also be criticized as not being clinically-relevant, as anti-CD154mAb is unlikely to be approved for clinical application in view of its associated thrombotic complications (19, 20). However, it was chosen as, in our experience, it is the only agent that effectively prevents a T cell-dependent elicited Ab response in baboons to pig antigens (21, 22). Nonetheless, our findings are of relevance to clinical ABO-I organ Tx as well as to future clinical xenotransplantation.
In this study we provide further evidence of the development of natural anti-A/B and anti-Gal/pig Abs in infant baboons (Gp1). Anti-A Ab developed more quickly (in type B baboons) than did anti-B Ab (in type A baboons). We additionally confirm our previous observation (9) that anti-nonGal Abs remain minimal throughout the first year of life (Supplementary Results). We also demonstrate that, despite the young age of the recipients, a small artery patch graft from either an AB-I or pig donor is sufficient to elicit donor-specific sensitization in infant baboons not receiving IS (Gp2).

Infant baboons that received IS in the presence of a graft did not become sensitized to their graft while IS was continuing. There was no or minimal increase in IgM and IgG levels, and the cellular responses remained weak. After discontinuation of IS, there was a delayed increase in Ab production and in the cellular response on MLR, except in one case; B cell tolerance to AB-I blood group antigens may possibly have developed in B5008 but, as this was a B group graft into an A group baboon, this observation needs to be placed in the perspective of the very slow development of anti-B Abs in the control baboons of Gp1. Unfortunately it was not possible to investigate this possibility by ELISPOT, as this would have required an excessively large blood draw from the small baboon to obtain sufficient B cell numbers. In the other Gp3 baboon that was followed for 21m (B5708), anti-Gal Ab production did not increase until 4m after discontinuation of IS, but B cell tolerance was clearly not achieved.

A striking observation was the finding that all infants receiving anti-CD154mAb (n=5) showed a marked inhibition and delay in development of both anti-AB-I IgM and anti-pig/anti-Gal IgM Abs, which was irrespective of the presence or type of graft. This was not only seen in the infants receiving thymoglobulin induction and maintenance therapy (Gp3), but also in the single infant that received only anti-CD154mAb (with no graft or adjunctive IS) (Gp4). Despite the small number of animals, when comparing Gp3 (+/- B309 from Gp4) (total n=5) and Gp1 (n=6), there was a significant difference in anti-Gal IgM levels at 4m and 12m.

These observations question whether natural Ab production is T cell-independent. Ohdan et al showed that, following depletion of CD4+ and CD8+ T cells in GTKO mice, anti-Gal IgM increased significantly, indicating that the natural development of Abs was not inhibited by the absence of T cells, suggesting that the production of anti-Gal Ab is T cell-independent (10). In contrast, Cretin et al presented evidence indicating that natural anti-Gal Ab production is T cell-dependent (11). Our limited data, which to our knowledge are the first in nonhuman primates, suggest that the development of natural Abs to carbohydrate antigens is inhibited by the administration of T cell-directed IS, and therefore may not be entirely T cell-independent. In baboons,
anti-CD154mAb, when combined with MMF, is associated with a profound CD4+ T cell depletion, but not in B cell depletion (20, 21, 23-25). In contrast, costimulatory blockade with CTLA4-Ig did not appear to inhibit natural Ab production so effectively, although this was tested in only one baboon.

Our finding that anti-CD154mAb-based IS can delay natural Ab production in infants has implications for clinical organ Tx. An infant needing an organ transplant at birth could possibly be maintained on anti-CD154mAb-based IS to prevent natural Ab production while waiting for a donor organ. Although in our study in baboons this effect was only seen with anti-CD154mAb, further studies involving combinations of IS agents might possibly achieve the same result. In clinical studies of ABO-I heart Tx in infants, B-cell tolerance to donor-specific antigens was achieved, but no generalized reduction in Abs to non-specific antigens was observed (1, 15, 16). Of possible relevance is that 3 of the 4 Gp1 grafts (including B5008, to which no anti-B Abs developed) still expressed AB or Gal antigens some months after Tx; expression of antigen is important in the development of specific tolerance. Of possible relevance, there is evidence from rodent studies that both anti-CD154mAb (26) and CTLA4-Ig (27) may be associated with decreased autoantibody production. Variations in the effect of IS on Ab production may be related to differences between animals and humans, or to the IS administered, which in clinical cases has not included costimulatory blockade.

It is encouraging to believe that the absence of natural anti-pig Abs might allow the possibility of pig organ Tx, at least as a bridge to allogeneic Tx (28), avoiding problems related to Ab-mediated rejection or endothelial cell activation, which may be associated with the development of thrombotic microangiopathy and/or consumptive coagulopathy. If a xenograft were to be transplanted into a human, the immature immune system of infants would be advantageous, as it offers the possibility of modulating its immune response. Theoretically, if a pig organ was transplanted early in infancy, the child might never develop Abs to the graft, and conventional T cell-directed IS might be sufficient to maintain the graft.

This might be a particularly successful approach if the heart is taken from a genetically-modified pig (22, 29, 30), thus reducing the humoral and cellular immunological responses further (31-33). Pig hearts of suitable size for infants could readily be obtained. However, our experience is that the response to a pig organ in nonhuman primates is complex; as well as natural Abs and the adaptive cellular response, it involves an innate cellular response, coagulation dysregulation, and a marked inflammatory response (32). Nevertheless, particularly with regard to genetically-modified pig heart Tx, it might provide a bridge to allotransplantation.
ACKNOWLEDGEMENTS

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Abbreviations
Ab - antibody
CDC - complement-dependent cytotoxicity
Gal - Galα1,3Gal
GTKO - α1,3-galactosyltransferase gene-knockout
IS - immunosuppressive therapy
MLR - mixed leukocyte reaction
pAEC - porcine aortic endothelial cells
PBMC - peripheral blood mononuclear cells
Tx - transplantation
REFERENCES


CHAPTER 2:  
SUPPLEMENTARY MATERIALS

SUPPLEMENTARY METHODS

Infant baboons

All procedures were in conformance with the NIH Guide for Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 80-23, revised 1978) and approved by the OUHSC and UPitt Institutional Animal Care and Use Committees.

In Vitro Assays

Flow cytometry for monitoring baboon T and B cell counts

T and B cell counts were monitored as described (37). T and B cell numbers were calculated by measurement of their percentages of the total white blood cell (WBC) count.

ELISA for anti-A, anti-B, and anti-Gal Abs

Serum samples were titrated at 1:50, 1:250, 1:1250 and 1:6250 dilutions and measured in triplicate. Data from 1:50 dilutions gave clear detectable levels using a positive control (adult baboon) and are shown in the results; higher dilutions were used as controls. Ninety-six-well plates (Nunc MaxiSorp, eBioscience, San Diego, CA) were coated with 100µl of 5µg/ml of A, B, and Gal BSA trisaccharides (Cat# NGP6305, NGP6323 and NGP1334, respectively, all from V-Labs, Covington, LA). Serum IgM and IgG anti-A, -B and -Gal Abs were determined as described (38). The results are reported in optical density (OD). Relative OD levels were calculated by subtraction of the background (BSA coating only) for each individual sample.

Binding of Abs to WT and GTKO pig cells

Baboon sera were tested by flow cytometry for the presence of Abs that bound to WT or GTKO pig peripheral blood mononuclear cells (pPBMC, 5% dilution) or WT pig aortic endothelial cells (pAEC, 10% dilution), as described previously (39). It was not possible to carry out a similar assay for binding to baboon blood group A or B cells, as baboon erythrocytes and PBMC do not express blood group antigens. Relative MFI was calculated as follows: experimental MFI value / negative control MFI value. The negative control contained cells and secondary Ab, without baboon serum.
Mixed lymphocyte reaction (MLR)
As stimulator cells, (i) PBMC were isolated from 50ml of blood from Large White/Landrace/Duroc WT pigs or (ii) PBMC were isolated from 150ml of blood from an unrelated adult baboon and cryopreserved in aliquots to provide stimulator cells at $4 \times 10^6$ PBMC/ml for each experiment. Stimulator cells were irradiated with 2500cGy in a cesium$^{137}$ irradiator. As responder cells, $4 \times 10^6$ PBMC/ml were obtained from infant baboon heparinized blood (8-10ml), and were stimulated with irradiated adult baboon, WT, or GTKO PBMC at 1:1 ratio in 200µl AIM-V tissue culture medium (GIBCO BRL, Gaithersburg, MD) at 37°C and 5% CO$_2$ using 96-well round-bottom plates (Corning, Lowell, MA). All responder-stimulator combinations were set up in quadruplicate. After 4d, 10µl of 3H thymidine labeling medium (1uCi/well; New England Nuclear, Boston, MA) was added to the cells. At d5 (18h later), cells were harvested on glass-fiber filter mats with a cell harvester, and were analyzed by beta counting on a microplate scintillation and luminescence counter (Perkin Elmer, Waltham, MA). The results were expressed as counts per million (CPM) and stimulation index (SI, average counts in counts/min divided by anti-self response).

Hemagglutination
Unfortunately, anti-A/B hemagglutination titers, as used by Fan et al to monitor human infants (14), proved difficult to measure in baboons. This is most likely due to the necessity of using human RBC for this assay, as baboon RBC and PBMC do not express AB blood group antigens. The use of human RBC required adsorption of anti-human Abs by human O erythrocytes prior to agglutination assay; in most infant baboons, the titers were undetectable at all ages.

Histology and immunofluorescence staining
At time of necropsy, tissue samples were taken from the transplanted aortic patch and native aorta, and fixed in formalin or stored in OCT solution (-80°C) for histological and immunofluorescence examination. Sections of native aorta and transplanted artery patch were stained with hematoxylin and eosin (H&E), and underwent immunofluorescence examination for anti-A, -B, or -Gal Ab binding (42).

SUPPLEMENTARY RESULTS
Complement-dependent cytotoxicity assay (CDC)
In the CDC assay to pig cells (at 12.5% serum concentration), the sera of Gp1 infants showed an undetectable level of cytotoxicity to pPBMC at 1m of age (mean 3.3% lysis,
range 0–14.8%), that increased until 6m of age in all except one (mean 20% lysis, range 10–40%), after which it remained constant.

There was a modest reduction in CDC to WT pPBMC in B7707 post-Tx (Gp2, AB-I, A→B); by contrast, in B7607 (Gp2, pig graft), the CDC assay showed a strong increase from 3% pre-Tx to 36% post-Tx, confirming that baboons of this age can generate an Ab-mediated complement response capable of killing target cells.

There was no increase in lysis of pig cells during IS in any Gp3 infants, and lysis remained low after discontinuation of IS. However, 5008 and 5708 showed an increase in CDC at 18 months to the level of the age-matched controls. Similar to Gp1, both Gp4 infants showed low CDC pre-treatment at 1m. Similar to Gp3, the level remained low in B309 for 4 months after discontinuation of anti-CD154mAb, while B209 (receiving CTLA4-Ig) showed a 5-fold increase in level by 6m (while IS was continuing), reaching values similar to those in Gp1.

Antibody binding to GTKO pig cells
In addition to WT pig cells, IgM binding was measured against GTKO pig cells in two infants in each of Gp1 (4908, 5108), Gp3 (B5008, B5708), and Gp4 (B209, B309). During the first 12m, IgM binding to GTKO cells increased only minimally with increasing age in all groups, with no difference in the rate of development of anti-nonGal IgM between the groups. Apart from the first month when some maternal IgG was detected, anti-pig (WT or GTKO) IgG Abs remained undetectable at all time-points (Supplementary Figure 3).

Mixed leukocyte reaction (MLR)
All Gp1 infant baboons showed a weak cellular response to allogeneic baboon as well as pig stimulator PBMC as measured by MLR at 1–3m of age; this response did not vary significantly in strength with increasing age.

B7707 (Gp2, AB-I) showed a 3-fold increase to allogeneic PBMC at 1m post-Tx, but no increase to pig cells; by contrast, B7607 (Gp2, pig) showed a 4-fold increase in response to pig cells at 1m post-Tx. (The reaction to allo-PBMC could not be measured in B7607 pre-Tx due to a technical problem, but post-Tx the response was similar to that seen in the Gp1 infants.)

There was no increase in the cellular response to either allogeneic (baboon) or xenogeneic (pig) PBMC in any of the Gp3 infant baboons after Tx until IS was discontinued. At 18m of age, B5008 (AB-I graft, B→A) showed an increase in anti-pig response,
but no increase in anti-baboon response, following the trend of Ab production. This suggests that recovery of the cellular response to pig antigens was associated with increased anti-pig Ab production.

B309 (Gp4, anti-CD154mAb) showed a minimal response to both allogeneic and WT pig cells at all time-points (age 3-12m), while in B209 (Gp4, CTLA4-Ig) the responses were similar to those in Gp1 during IS therapy. No difference in response was noted at 12m (4m after discontinuing IS).
SUPPLEMENTARY FIGURES

Supplementary Figure 1: Photograph of segment of donor carotid artery placed as an onlay patch graft into the wall of the recipient infra-renal aorta.

The grafts measured approximately 1.0 x 0.5 cm (arrow) (see color figure on page: 229)
Supplementary Figure 2: Anti-A, -B and -Gal staining of artery patch grafts in Group 3 infant baboons.

(A) Positive B5008 (blood group A) AB-I graft stained for the B antigen, showing remaining B antigens on the graft endothelium (X40)

(B) Positive B7507 (blood group B) AB-I graft stained for the A antigen, showing remaining A antigens in the graft vasa vasorum (X40)

(C) Positive B5508 (blood group B) WT pig graft stained for Gal, showing no remaining Gal antigens in the graft endothelium or adventitia (X20)

(D) Positive B5708 (blood group B) WT pig graft stained for Gal, showing remaining Gal antigens in the graft endothelium and vasa vasorum (X10)

(E) Positive control for A staining; native aorta of blood group A infant baboon (X40) (similar to B staining in native blood group B aorta)

(F) Negative control for A staining; native aorta of blood group B infant baboon (X40) (similar to B staining in native blood group A aorta)

(G) Positive control for Gal staining; carotid artery of WT pig (X40)

(H) Negative control for Gal staining; native aorta of blood group B infant baboon (X40)

Arrows indicate positive staining; Solid arrows: anti-A or -B staining; Dotted arrows: anti-Gal staining; Red: Anti-A, -B or -Gal staining [FITC]; blue: cell nucleus [DAPI]; green: collagenous fibers [autofluorescent].

(see color figure on page: 230)
Supplementary Figure 3: Serum IgM binding levels to WT and GTKO pig cells.
IgM binding to WT cells was measured in all infants. IgM binding to GTKO cells were measured in Gp 1 (n=2, 4908, 5108) and infants receiving anti-CD154 mAb (n=3; B5008 [AB-I], 5708 [WT], 309 [IS only]). Mean IgM binding to GTKO cells showed no differences between Gp1 and anti-CD154 mAb-treated (Gps3+4) infants. During the first 12m, there was almost no increase in anti-nonGal IgM production in any baboon. However, compared to Gp1, IgM binding to WT pig cells was reduced in infants receiving anti-CD154 mAb, and only began increasing several months after discontinuation of IS. The colored area indicates the period of time during which the infants received IS.

Supplementary Figure 4: Mixed Leukocyte Reaction to allo- and WT pig cells.
The cellular response to allogeneic stimulator PBMC of Gp1 controls (n=6) and all Gp2+3 transplanted infants receiving either an AB-I (A) or pig (B) graft shows that only the Gp2 infant receiving an AB-I graft but no IS (B7707) has an increase in cellular response to allo-, but not to pig stimulator cells post-Tx (A).
T-lymphocyte homeostasis and function in infant baboons: implications for transplantation

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*DJvdW and EMD contributed equally

Introduction
Laboratory mice are born lymphopenic and demonstrate lymphopenia-induced proliferation (LIP) that generates memory T cells, yet they are prone to immunological tolerance.

Methods
Here we tested whether these fundamental immunologic observations apply to higher animals by studying the immune system of infant baboons using flow cytometry of peripheral blood cells.

Results
It was found that baboons are born relatively lymphopenic and subsequently expand their initially naïve T cell pool with increasing numbers of memory T cells. After transplantation of an artery patch allograft or xenograft, non-immunosuppressed recipients readily mounted an immune response against donor-type antigens, as evidenced by mixed lymphocyte reaction. Immunosuppression with anti-thymocyte globulin, anti-CD154 antibody and mycophenalate mofetil prevented T cell mediated rejection. After lymphocyte depletion, homeostatic T cell proliferation was observed. In conclusion, the baboon proved a suitable model to investigate the infant immune system.

Discussion
In the present study, neonatal lymphopenia and expansion of the memory T cell population were observed but, unlike mice, there were no indications that infant baboons are prone to T cell tolerance. The expansion of memory T cells during the neonatal period or after induction therapy may form an obstacle to tapering immunosuppressive therapy, or ultimately, achieving immunologic tolerance.
INTRODUCTION

Laboratory mice are born lymphopenic and demonstrate lymphopenia-induced proliferation (LIP) which generates memory T cells during the first few weeks of life (1). During the neonatal period, their T cell immune system exhibits remarkable plasticity and, although no longer believed to be immunodeficient, is prone to tolerance induction after transplantation (Tx) or adoptive transfer of splenocytes under certain conditions (reviewed in Adkins et al. [2]). Unlike rodent species, the limited knowledge available in humans indicates that human neonates are born with an almost entirely functional immune system, a difference believed to be influenced by the duration of gestation (3). However, also for humans there are several indications that the neonatal immune system still has immature features. For example, (i) the immune system of neonates is still antigen-inexperienced and is constituted by naïve T lymphocytes (4, 5) (ii) In the unfortunate event that an infant needs an organ transplant, the Tx of a heart across the ABO-blood group barrier is relatively uncomplicated (6-9), while in adults this may lead to graft loss from hyperacute rejection (10, 11). Moreover, when an ABO-incompatible (AB-I) donor organ is introduced before the development of anti-AB antibodies (Abs), B cell tolerance to the incompatible blood group antigen(s) can develop (12). (iii) Pediatric or adult recipients of umbilical cord blood cells experience less graft-versus-host disease than recipients of bone marrow Tx from an adult donor (13, 14).

To our knowledge, few studies have closely followed the development of the human infant immune system with advancing age (15), possibly due to ethical concerns of involving infants in research protocols. Alternatively, non-human primate (NHP) models can be of value for investigation of the developing immune system (16).

The aim of this study was to verify two fundamental immunologic principles observed in mice, i.e., neonatal LIP of T cells and the possibility of inducing T cell tolerance to transplant antigens, in a NHP model using infant baboons. We found that baboon T lymphocytes significantly increase in number after birth with transient appearance of memory T cells. After artery patch Tx at the age of 3 months, the T cell immune system appeared functional and, in the absence of immunosuppressive therapy, indications for the allowance of immunologic tolerance were not observed.

MATERIALS AND METHODS

Sources of Animals and Blood Samples
Healthy young infant baboons (Papio anubis) (Group 1, Table 1) were housed at the specific pathogen-free (SPF) facility of the Oklahoma University Health Sciences
Center (OUHSC) (17). From 6 baboons blood was drawn during the first week of life, and at 1, 2, 4, and 6 months of age, stored in tubes containing EDTA, and shipped at 4C to the University of Pittsburgh (UPitt) for analysis on the following day. The infants remained healthy and untreated throughout the time of study. Blood samples from healthy, untreated, young baboons aged 1 year (n=6), and 2-3 years (n=6) were drawn and shipped under the same conditions.

In addition, 5 infant baboons (Group 2) were housed at UPitt from the age of 2 months for immunologic and transplantation studies. Blood was drawn and analyzed before and at multiple time-points after transplantation of an artery patch graft (see below).

All animal care procedures were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985), and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

**Table 1: Experimental Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Experiment</th>
<th>Baboon #</th>
<th>Blood group</th>
<th>Graft type</th>
<th>Age at Tx (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No Graft, No IS</td>
<td>7707</td>
<td>B</td>
<td>A</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7607</td>
<td>B</td>
<td>Pig</td>
<td>102</td>
</tr>
<tr>
<td>2A</td>
<td>Graft, No IS</td>
<td>7507</td>
<td>B</td>
<td>A</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5008</td>
<td>A</td>
<td>B</td>
<td>107</td>
</tr>
<tr>
<td>2B</td>
<td>Graft, IS</td>
<td>5508</td>
<td>B</td>
<td>Pig</td>
<td>98</td>
</tr>
</tbody>
</table>

**Transplantation of baboon or pig artery graft**

At the age of 3 months, 5 baboons (Group 2) were transplanted with a length of donor carotid artery, as an immunologic model for pediatric heart Tx. Donor artery grafts were obtained either from adult baboons (OUHSC) that were AB-I with the recipient, or from wild-type (WT) pigs (Large White/Landrace) of blood type O (Country View Farms, Schellsburg, PA). Under full anesthesia, and after laparotomy and partial heparinization (100IU/kg), the recipient aorta was clamped distally to the renal arteries and at the bifurcation, incised longitudinally, and a 1.5×0.5cm patch of donor (baboon or pig) carotid artery was sutured into the aortic wall as a full-thickness onlay graft. The clamps were removed and, after assuring hemostasis, the abdomen was closed.
Two baboons (Group 2A) received either an AB-I or WT pig graft, but no IS; these baboons were followed for immunologic studies for 6 weeks until euthanization for pathologic studies. Three baboons (Group 2B) received an AB-I (n=2) or a WT pig graft (n=1), and were immunosuppressed throughout the follow-up after transplantation (Table 1). IS consisted of induction with anti-thymocyte globulin (ATG), and was maintained using an anti-CD154 monoclonal antibody (mAb) and mycophenolate mofetil (MMF) (Table 2). In the here reported studies, Group 2B baboons were followed for 15 weeks after transplantation, where after they remained in follow-up for studies of weaning of immunosuppression (IS) and B cell tolerance (these results are reported separately in Chapter 2).

### Table 2: Induction and Maintenance Immunosuppressive and Supportive Therapy in Group 2 baboons.

<table>
<thead>
<tr>
<th>Induction therapy</th>
<th>Dose</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymoglobulin</td>
<td>2.0-2.5mg/kg i.v.</td>
<td>Days -3 and -1</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>5mg/kg i.v.</td>
<td>Before each dose of ATG and on day 0. The dose was then be reduced by 1mg/kg/d, and discontinued on day 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maintenance therapy</th>
<th>Dose</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD154 mAb</td>
<td>20-25mg/kg i.v.</td>
<td>Days -1, 0, 4, 7, 10, 14, then every 5-7d</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>20-150mg/kg/d p.o. divided in 2 doses</td>
<td>Begun on day -2 (to maintain a blood trough level of 3-6µg/ml)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Supportive Therapy</th>
<th>Dose</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefazolin</td>
<td>25mg/kg bid i.v.</td>
<td>For 3 days after surgery</td>
</tr>
<tr>
<td>Famotidine</td>
<td>0.25mg/kg bid i.v.</td>
<td>From day -3</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>5mg/kg i.v.</td>
<td>From day -4 until 1 month post-Tx</td>
</tr>
<tr>
<td>Ketorolac</td>
<td>0.5mg/kg i.v.</td>
<td>Before every dose of anti-CD154mAb</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.01mg/kg bid i.v.</td>
<td>For 3 days after surgery</td>
</tr>
</tbody>
</table>

### Flow cytometry studies

The following fluorochrome-conjugated mAbs were used in flow cytometry experiments: anti-CD3 Pacific Blue (clone: SP34-2), anti-CD4 PE-Cy7 (SK3), anti-CD8 APC-Cy7 (RPA-T8), anti-CD20 FITC (2H7), anti-CD25 APC-Cy7 (M-A251), anti-CD45RA PE-Cy5 (5H9), anti-CD62L PE (SK11), anti-CD127 PE (hIL-7R-M21) (all from BD Biosciences, San Jose, CA), and anti-FoxP3 APC (PCH101) (eBioscience, San Diego, CA).

Whole blood was incubated with conjugated Abs or corresponding isotype controls, after which red blood cells were lysed using PharmLyse (BD). Intracellular staining for FoxP3 to identify regulatory T cells ($T_{reg}$) was performed according to the manufacturer’s protocol (eBioscience). Cells were analyzed with a LSRII multicolor flow cytometer (BD). Data were analyzed using FacsDiva 6.0 software (BD). Table 3 shows how different lym-
phocyte subsets were identified. Absolute cell numbers were calculated based on white blood cell counts obtained from our institution’s hematology laboratory.

**Table 3: Monoclonal antibodies used to define lymphocyte subsets by flow cytometric analysis**

<table>
<thead>
<tr>
<th>Lymphocyte subset</th>
<th>Pacific blue</th>
<th>PE-Cy7</th>
<th>APC-Cy7</th>
<th>FITC</th>
<th>PE-Cy5</th>
<th>PE</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>T and B cells</td>
<td>CD3⁺</td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>CD20⁻</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naïve T cells (Tₙa)</td>
<td>CD3⁺</td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>CD45RAhi CD62Lhi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total memory T cells (Tₜtot)</td>
<td>CD3⁺</td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>CD45RAlo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effector memory T cells (Tₑmf)</td>
<td>CD3⁺</td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>CD45RAlo CD62Llo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central memory T cells (Tₑcm)</td>
<td>CD3⁺</td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>CD45RAlo CD62Lhi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terminally differentiated effector memory T cells (TₑmrA)</td>
<td>CD3⁺</td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>CD45RAhi CD62Llo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulatory T cells (Tₑreg)</td>
<td>CD3⁺</td>
<td>CD4⁺</td>
<td>CD25hi</td>
<td>CD127⁻FoxP3⁻</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Mixed lymphocyte reaction (MLR)**

MLR measured by thymidine incorporation was carried out as previously described (18). Briefly, as stimulator cells, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of (i) 150ml of blood from an unrelated adult baboon (cryopreserved in aliquots to provide stimulator cells for each experiment) or (ii) from freshly obtained blood from Large White/Landrace WT pigs. As responder cells, PBMC obtained from buffy coats of infant baboon blood were isolated. In the MLR, responder cells (0.4x10⁶ cells/well) were stimulated with irradiated adult baboon or WT pig PBMC at a 1:1 ratio. All responder-stimulator combinations were set up in quadruplicate and were incubated for 5 days. Ten microliters of 3H thymidine labeling medium (1uCi/well; New England Nuclear, Boston, MA) were added to each well during the last 18h of incubation. Cells were harvested on glass-fiber filter mats with a cell harvester, and were analyzed by beta-scintillation counting on a liquid scintillation counter (PerkinElmer, Waltham, MA). The mean results of quadruplicate tests were expressed as counts per million (CPM) and stimulation index (SI, average counts of anti-baboon or anti-pig response divided by anti-self response).

**Graft histology**

Aortas including the graft fixed in 10% formalin and embedded in paraffin. Four micron (4μm) sections were stained with hematoxylin and eosin for light microscopy. Immunohistochemical staining for expression of A, B and Gal was performed.
Statistical analyses
Continuous variables are expressed as mean±SEM, and compared using the (paired) Student t-test, linear regression, and repeated measures ANOVA for changes over time, as appropriate. Repeated measures ANOVA was followed by pairwise comparisons of peak values with values at birth. P-values <0.05 were considered statistically significant. All analyses were performed with GraphPad Prism 4 for Macintosh (GraphPad Software, La Jolla, CA).

RESULTS

Early lymphocyte development in healthy infant baboons
In Group 1 baboons aged 0 to 30 months, a linear decrease in the proportion of CD4+ T lymphocytes (CD4+ cells) was observed (Figure 1A). This decrease was complemented by a steady increase in the proportion of CD8+ T lymphocytes (CD8+ cells) (Figure 1A). During the first months of life, an increase in the absolute number of lymphocytes occurred. Numbers of CD4+ cells were 2.8±0.8× higher at the age of 4

Figure 1: Changes in CD4+ and CD8+ T cells, and CD20+ B cells in healthy infant baboons (Group 1). Left panels show proportions of total lymphocytes (A, C), right panels show absolute numbers, which increased 3 to 7 fold during the first 4 months of life (B, D).
Figure 2: Lymphopenia-induced proliferation of memory T cells in healthy infant baboons (Group 1).

(A) Dot plot of CD4+ cells stained for CD45RA and CD62L to distinguish naïve and memory T cells. Proportions (B, E) and absolute numbers (C, F) of naïve and memory subpopulations among CD4+ and CD8+ cells. Absolute numbers expressed as a ratio of numbers measured <1wk after birth (D, G) show that expansion of T_{TotMem} was more significant than of T_{Naïve} indicating the occurrence of LIP. *P<0.05 vs. Naïve. (TotMem = total memory T cells; CM = central memory T cells; EM = effector memory T cells).
Memory T cells in infant baboons

Numbers of CD8\(^+\) cells were 6.6±1.4× higher at the age of 4 months compared with numbers at birth (repeated measures ANOVA, \(P<0.0001\)). Because of the changing proportions over time, the fold-increase in CD8\(^+\) cells was greater than of CD4\(^+\) cells (\(t\)-test, \(p=0.0344\)). After 4 months, a decline in CD4\(^+\) cells, and stabilization of the numbers of CD8\(^+\) cells was observed.

After observing the above trends in lymphocyte development early in life, we were interested in characterizing the phenotype of different T lymphocyte subpopulations. In the first week of life, the majority of lymphocytes (62.1±7.1% of CD4\(^+\), and 58.5±7.9% of CD8\(^-\)) possessed the phenotypic characteristics of naïve T cells (T\(_{N}\)), which stain positive for CD45RA and CD62L (Figures 2B, 2E). The absolute numbers of CD4\(^+\) and CD8\(^+\) T\(_{N}\) increased during the first 4 months of life (Figures 2C, 2F), indicating T cells leaving the thymus (new thymic emigrants). However, as a percentage of total CD4\(^+\) and CD8\(^+\) cells, a decline in T\(_{N}\) occurred during the first 2 months (repeated measures ANOVA, \(P=0.015\) for CD4\(^+\), \(P=0.003\) for CD8\(^+\), Figures 2B, 2E). Simultaneously, an increase in the percentages of effector memory T cells (T\(_{EM}\)) and central memory T cells (T\(_{CM}\)) was observed (in Figure 2 presented separately as well as combined as total memory T cells [T\(_{TtotMem}\)]). The changes were most pronounced in the CD8\(^+\) population. When compared for differences in kinetics, the appearance of T\(_{EM}\) occurred earlier than the appearance of T\(_{CM}\) (Figure 2E). Figures 2D and 2G show the development of T\(_{N}\) and T\(_{TtotMem}\) as a ratio of their respective baseline numbers at birth (set as 1.0), indicating that the expansion of T\(_{TtotMem}\) was more significant than of T\(_{N}\) (paired \(t\)-test).

**Figure 3:** Regulatory T cells in healthy infant baboons (Group 1)

(A) Identification of regulatory T cells among CD4\(^+\)CD25\(^{hi}\) T cells. Proportions among CD4\(^+\) (B) and CD4\(^+\)CD25\(^{hi}\) (C) cells, and absolute numbers (D). (Treg = regulatory T cells).
test, P<0.05 for most time-points). Beyond 2 months of age, the proportions of \( T_N \) and \( T_{TotMem} \) reversed back to proportions more comparable with those at birth. The percentages and numbers of terminally differentiated effector memory T cells (\( T_{EMRA} \)) remained relatively stable (not shown). No distinctive pattern in the development of CD20+ B lymphocytes (B cells) could be discerned (Figures 1C, 1D).

**Figure 4:** Stimulation index after mixed lymphocyte reaction (MLR) in control and transplanted (Group 2) baboons. Responses in healthy infant baboons (Control) are compared with responses in baboons after AB-I allo-Tx (A, B) and WT pig xeno-Tx (C, D) in the presence (IS) or absence (No IS) of immunosuppressive therapy. Data for B7607 after allo-stimulation are missing due to technical error (C). (E) Graft histology 6 weeks after Tx without IS (Group 2A) showed fibrosis and heavy lymphocytic infiltration. Normal vascular histology can no longer be recognized. (see color figure on page: 231)
The relative and absolute numbers of CD25<sup>hi</sup>FoxP3<sup>+</sup>CD127<sup>-</sup> T<sub>Reg</sub> peaked at the age of 4 months (repeated measures ANOVA, both P<0.0001, Figure 3), at the same time-point as the highest measured total numbers of CD4<sup>+</sup>. The dynamic rearrangement of T<sub>N</sub> and T<sub>TotMem</sub> did not seem to affect the functionality of the cellular immune system to mount an immune response, as investigated by MLR. The responses of infant baboon PBMC after stimulation with irradiated baboon or WT pig PBMC did not vary with age (P>0.05 for each), (Figure 4).

Changes in lymphocyte subpopulations after artery patch Tx

**Group 2A: Tx in absence of IS therapy**

After transplantation of an aortic patch graft in 2 untreated infant baboons, the gradual decline in percentage of CD4<sup>+</sup> and increase of CD8<sup>+</sup> cells did not appear different from
those in healthy untreated and untransplanted baboons of comparable age (Figure 5A). The presence of an AB-I or WT pig graft therefore did not seem to influence this evolution. Three weeks post-Tx, a slight and transient, and non-significant, shift to increased proportions of memory T cells (TTotMem, TEM, TCM) was seen for CD4+ as well as CD8+ cells (repeated measures ANOVA, P=0.374 for CD4+, Figure 6A, and P=0.243 for CD8+, Figure 6E).

After Tx without IS, no change in TReg as percentage of CD4+ was detected (Figure 7A). However, although we were not able to provide statistical evidence due to the small number of animals, within the CD4+CD25hi population, larger number of cells expressed FoxP3 (Figure 7B), resulting in a peak of absolute numbers of TReg 4 weeks after Tx (Figures 7C).

Although the changes in TN and TTotMem were small, during follow-up donor type-specific immunosensitization had readily occurred. This was evidenced by an increased response in the donor type-specific MLR (Figures 4A-D), the appearance of antibodies against graft antigens in recipient serum (Anti-A Abs in AB-I Tx, and anti-Gal Abs in WT xenoTx, respectively, Dons EM et al. manuscript in preparation), and fibrosis and lymphocyte infiltration of the graft on histological analysis (Figure 4E).

**Group 2B: Tx in presence of IS**

Induction therapy with ATG resulted in depletion of >95% of CD4+ cells, and >90% of CD8+ cells in 3 infant baboons (Figures 5B, 5D). CD4+ cells were maintained at very low levels with anti-CD154mAb and MMF, as has been previously described in older animals (19) (Figures 5A, 5B). CD8+ cells recovered during the 3.5 months follow-up to levels comparable with those measured at baseline (Figures 5C, 5D). The increased proportion of B cells within the lymphocyte gate (Figure 5E) was a consequence of depletion of T cells, as no significant change in absolute numbers of B cells was noted (Figure 5F).

During follow-up, pronounced changes in relative numbers of T_N, TTotMem, TEM, and TCM were seen. CD4+ T_N fell from 55.8±6.6% at baseline to 14.8±4.9% at 6 weeks post-Tx (repeated measures ANOVA, P=0.0133, Figure 6C). During this period, CD4+ TTotMem had increased from 42.0±6.6% to 82.2±6.7% (repeated measures ANOVA, P=0.0441). At the age of 4 months, a major shift from T_N to TTotMem as part of the natural development of the immune system would no longer be expected (compare with Figure 2B), and is therefore likely to be caused by lymphocyte depletion and repopulation phenomena. Although absolute numbers of CD4+ cells were still low, the altered proportions of T_N and TTotMem resulted in the earlier repopulation of TTotMem than
Figure 6: Naive and memory subpopulations among CD4+ and CD8+ T cells in infant baboons after Tx (Group 2).

(A, B, E, F) Group 2A – No Immunosuppression. (C, D, G, H) Group 2B – Immunosuppression. Shifts toward a memory phenotype (C, G) and significantly greater expansion of memory T cells (D) indicated homeostatic proliferation after induction therapy with anti-thymocyte globulin. *P<0.05 vs. Naive. (TotMem = total memory T cells; CM = central memory T cells; EM = effector memory T cells).
TN (Figure 6D). Starting at 2 months after Tx (at age 5 months), the relative numbers largely reversed to those measured pre-Tx (Figure 6C).

The changes in CD8 TN and T_{totMem} followed similar patterns to those in CD4 cells. CD8 TN levels fell from 38.9±6.3% at baseline to 21.1±3.6% at 3 weeks post-Tx (P=0.0028, Figure 6G). CD8 T_{totMem} increased from 58.2±5.3% to 71.3±3.2% (P=0.027). Six weeks post-Tx reversal to proportions comparable with baseline had occurred.

When changes in T_{Reg} after Tx without (Group 2A) and with (Group 2B) IS were compared, opposite phenomena were observed. While without IS the percentage of T_{Reg} among CD4+ did not change (Figure 7A), after T cell depletion and chronic IS a relative increase in T_{Reg} among CD4+ was seen (although non-significant, Figure 7D). However, this neither led to an increase in percentage among CD4+CD25hi, nor in an increase in absolute numbers (P>0.05, Figures 7E, 7F). This indicated that FoxP3 expression did not increase among CD4+CD25hi cells, and that the increase among CD4+ was more likely a result of lymphocyte depletion, which relatively spared T_{Reg}.

**Group 2A - No Immunosuppression**

- **Relative:**
  - CD4+ among FoxP3+CD127- Treg
  - CD4+ among FoxP3+CD127+ Treg
- **Absolute:**
  - CD4+ among FoxP3+CD127- Tn

**Group 2B - Immunosuppression**

- **Relative:**
  - CD4+ among FoxP3+CD127- Treg
  - CD4+ among FoxP3+CD127+ Treg
- **Absolute:**
  - CD4+ among FoxP3+CD127- Tn

**Figure 7:** Regulatory T cells in infant baboons after Tx (Group 2).

Proportions among CD4+ (A, D) and CD4+CD25hi (B, D) cells, and absolute numbers (C, F) in the absence (Group 2A - No Immunosuppression) or presence (Group 2B - Immunosuppression) of immunosuppressive therapy. (Treg = regulatory T cells).
The applied immunosuppressive protocol (ATG, anti-CD154 mAbs, MMF) adequately suppressed the response in the MLR with donor-type stimulation during the course of follow-up (Figure 4). The vigorous phenotypic changes therefore seem to have been overruled by the immunosuppressive regimen with respect to their in vitro functional potential. However, it should be acknowledged that at the time of the major $T_N \cdot T_{TotMem}$ phenotypic shifts, T cell numbers were too few to harvest in sufficient numbers to perform MLR. The intervals between MLR results are thus larger than the time required for phenotypic changes.

**DISCUSSION**

The aims of the studies presented here were to verify if infant NHP (i) undergo lymphopenia-induced proliferation (LIP) to expand their T cell numbers after birth, and (ii) are prone to the induction of immunologic tolerance of transplant antigens. Both phenomena have been previously documented in laboratory rodents, but it remained unknown if these fundamental immunologic principles are similar in NHP and humans. We observed that infant baboons were relatively lymphopenic at birth. The initial naïve immune status and subsequent LIP or 'homeostatic proliferation' of T cells which generated memory T cells did not affect the functionality of the immune system when challenged with AB-I baboon or WT pig antigens. IS including lymphocyte depletion was effective in the suppression of cellular rejection, but caused significant shifts in T cell phenotype.

During the first 4 months of life of baboons, the blood T lymphocyte pool expanded three to seven fold. Numbers of CD8$^+$ cells increased more significantly than the CD4$^+$ subpopulation (which relative numbers actually slightly declined, as previously reported in humans (5, 15)). Although early in life the thymus still contributes importantly to T cell homeostasis, in our studies evidenced by increasing $T_{N}$, a relatively greater expansion of memory T lymphocytes was seen. Two plausible mechanisms could have contributed to this observation, being (i) LIP, and (ii) exposure to antigens in the environment, likely as a result of intestinal colonization (20), while further antigen exposure was limited in the SPF environment. The observation that the shift toward increased $T_{TotMem}$ was transient and reversed after approximately 6 months of age suggests a dominant role for LIP. To our knowledge, the only available study in humans also showed an early increase in total number of CD4$^+$ and CD8$^+$ lymphocytes (15). However, memory T cells were not investigated.
Heart Tx can be the only life-saving therapy in certain congenital cardiomyopathies. In neonates, a heart can be successfully transplanted across the ABO-blood group barrier (6-9), likely because the natural Abs against non-self blood groups do not develop until later. Moreover, after Tx the development of Abs against the donor blood group never occurred, while Abs against non-donor, non-self readily appeared. This indicated that B cell tolerance was induced (12) (although it should be noted that recipients were thymectomized and remained fully immunosuppressed during follow-up). It is unknown if the T cell compartment contributed to this tolerant state, or whether it would be possible to reduce the (T cell directed) immunosuppressive therapy in infant Tx recipients. We therefore established the infant baboon artery patch Tx model to undertake immunologic studies.

Because heart Tx in infants is limited by the scarcity of size-matched donor hearts, we also investigated xenotransplantation, i.e., pig-to-human, which could provide at least a ‘bridge’ for the patient until a suitable human donor heart becomes available (21). The observation natural anti-pig Abs develop in a similar pattern as anti-A or B blood group Abs led us to hypothesize there might be an opportunity for tolerance induction to pig antigens (22). The outcomes regarding B cell tolerance will be separately reported (Chapter 2).

Regarding T cell immunity, no major phenotypic changes in the T_N and T_TotMem compartments occurred after Tx without IS during six weeks of follow-up. Donor-type specific sensitization was induced, as proven by an increased response in the MLR, the appearance of donor-type specific Abs in serum, and pathologic changes in the graft. These results indicate that the T cell immune system of baboon infants, although still antigen-naïve, can readily mount an immune response against transplant antigens.

The immunosuppressive protocol prevented the induction of anti-donor-type Abs, and reduced the response in the MLR against donor-type stimulators, as well as against third-party stimulators. Signs of selective tolerance induction were therefore not observed, as this is traditionally defined as specific unresponsiveness to a specific foreign (transplant) antigen while maintaining reactivity to other (third-party) antigens (23). Nevertheless, lymphocyte depletion with ATG caused significant shifts in naïve and memory T cells. After induction of lymphopenia, peripheral blood was mainly repopulated with memory T lymphocytes, a phenomenon known as homeostatic repopulation (24, 25).

Homeostatic repopulation of memory T cells after lymphocyte depletion has been observed in patients with organ transplants (26, 27), and can result in acute cellular rejection with T_TotMem predominating in peripheral blood and in graft histology (27).
Memory cells can be cross-reactive with graft antigens and cause rejection with reduced need for antigen presentation and co-stimulation, so called ‘heterologous immunity’ (23, 28). This represents a clinically-relevant problem, e.g., when weaning of immunosuppression is attempted. Moreover, memory cells have been found to represent a barrier to Tx tolerance (23, 29).

The ideal panel of conjugated mAbs used to identify lymphocyte subsets by their cell surface antigens remains a topic of debate. In our studies, we opted for differentiation of T_N and T_totMem by mAbs against CD45RA and CD62L, a strategy that is well-established in humans (28, 30) and NHP (31-34), and that we previously successfully applied in cynomolgus monkeys (35). However, Pitcher et al. postulated that memory T cells in rhesus monkeys can best be determined by anti-CD95 and CD28 staining (16). The different strategies, however, stained very significantly overlapping cell populations (16, 36), leading us to conclude that CD45RA and CD62L can be reliably used in baboon studies.

In conclusion, after birth, the immune system of infant baboons was relatively lymphopenic and naïve, and T lymphocytes significantly increased in number with a transient appearance of memory T cells. It was hereby confirmed that neonatal NHP undergo LIP to establish T lymphocyte homeostasis. After artery patch Tx at the age of 3 months, an age at which natural Abs against ABO-blood group and pig antigens are still practically undetectable (rood), the T cell immune system was able to readily and actively mount an immune response against transplanted antigens. On the basis of the assays performed, we did not identify a T cell ‘window of opportunity’ for tolerance induction. However, the PBMC MLR is a crude functional assay, and more subtle signs of immune tolerance, or opportunities to achieve so, may have been missed. More detailed investigations in this regard are warranted. Immunosuppression including lymphocyte depletion was associated with the expansion of memory T cells that may actually form an obstacle if immunologic tolerance is the goal.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Noriko Murase, MD, for help with the surgical procedures. EMD is the recipient of fellowships from the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences and the Stichting Professor Michael van Vloten Fund, The Netherlands. The presented studies were supported in part by NIH grants U01 AI068642 and R21 AI074844. The Division of Animal Resources at OUHSC is supported by NIH grants R24 RR016556 and P40 RR012317.
Abbreviations

AB-I  - ABO-incompatible
Abs, mAbs  - (monoclonal) Antibodies
ATG  - Anti-thymocyte globulin
IS  - Immunosuppression
LIP  - Lymphopenia-induced proliferation
MLR  - Mixed lymphocyte reaction
MMF  - Mycophenolate mofetil
NHP  - Non-human primate
PBMC  - Peripheral blood mononuclear cells
SPF  - Specific pathogen-free
T\textsubscript{CM}  - Central memory T cells
T\textsubscript{EM}  - Effector memory T cells
T\textsubscript{EMRA}  - Terminally differentiated effector memory T cells
T\textsubscript{N}  - Naive T cells
T\textsubscript{Reg}  - Regulatory T cells
T\textsubscript{TotMem}  - Total memory T cells
Tx  - Transplantation
WT  - Wild-type
REFERENCES


A collagenous colitis-like condition in immunosuppressed infant baboons

Eefje M. Dons, Gabriel J. Echeverri, Lora H. Rigatti, Edwin Klein, Claudia Montoya, Roman F. Wolf, Jan N.M. Ijzermans, David K.C. Cooper, Robert Wagner

Inflammatory Bowel Disease 2012; 18:1325-1332.
**Background:**
Collagenous colitis is a chronic inflammatory bowel disease of unknown etiology. It is fairly common in adult humans, but rare in infants, and has been associated with autoimmune disorders.

**Methods**
We report the diagnosis, treatment and follow-up of a collagenous colitis-like condition in four infant baboons (age 7-12 months) that had received an artery patch transplant at three months of age and subsequent immunosuppressive therapy for periods of 4-10 months.

**Results**
All presented identical symptoms within a period of four weeks, including weight loss associated with chronic watery diarrhea that was unresponsive to standard antimicrobial treatment. Clinical chemistry evaluations were within normal ranges, viral causes were ruled out, and fecal and blood cultures were repeatedly negative. At necropsy, two infant baboons were found to have a form of collagenous colitis. In the remaining two baboons that had identical clinical features, immunosuppressive therapy was discontinued and treatment with budesonide was initiated. Both baboons recovered and remained well on no medication until the end of follow-up (24 months).

**Conclusions**
Collagenous colitis has occasionally been reported in patients with organ transplants. It has been reported only once previously in baboons. The four cases reported here strongly suggest that (i) clinical features as well as histopathological findings of collagenous colitis in baboons are very similar to those in human patients; (ii) it was associated with the immunocompromised state of the baboons, as two non-immunosuppressed age-matched baboons in close proximity did not develop the condition, and (iii) it may have had an infectious origin as all four cases developed within a four week period of time.
INTRODUCTION

Collagenous colitis and lymphocytic colitis are two types of what is known as microscopic colitis (1). Both are chronic inflammatory bowel diseases, characterized by a normal macroscopic appearance of the mucosa of the colon, but distinct histopathological changes upon microscopic examination. Clinical symptoms are chronic non-bloody diarrhea, abdominal cramping, and weight loss. Biopsy is diagnostic and differentiates between collagenous and lymphocytic colitis. In humans, the incidence varies from 4-15 cases/100,000 population, and is higher in the elderly and in women (2, 3). The development of collagenous colitis in children is rare (4, 5). It is not commonly associated with an immunocompromised state, although cases of microscopic colitis have been reported in patients with organ transplants who were receiving immunosuppressive therapy (IS) (6).

There is almost no information on the occurrence of collagenous colitis in nonhuman primates. In baboons, only one case has been reported in a large cohort of 132 baboons presenting with chronic colitis (7, 8). We report four cases (two of which were confirmed histologically) which occurred in a group of six infant baboons in our animal facility. All four were receiving IS therapy and developed clinical features of colitis within the same period of four weeks.

CASE REPORTS

Study Population: six infant baboons

The six baboons in this study were obtained from the Oklahoma University Health Sciences Center, Oklahoma City, OK. All animal care procedures were in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). All protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Four infant baboons, ranging from 3-4 months of age and 1.3-2.3 kg in weight, received artery patch grafts into the wall of the infra-renal abdominal aorta (Table 1, described in detail in Dons EM, et al, submitted). The grafts were either from a blood group A/B-incompatible baboon (AB-I, n=2) or from a wild-type (i.e., genetically-unmodified) pig (pig, n=2). All four received IS therapy from the time of the transplant (Table 2). This consisted of induction with two doses of anti-human thymocyte globulin (ATG) and
maintenance with a combination of an anti-human CD154 monoclonal antibody (anti-CD154 mAb) and mycophenolate mofetil (MMF). Details of the IS regimen and of supportive medical therapy are provided in Table 2.

As the experiments were aimed towards documenting the development of anti-AB or anti-pig antibodies during infancy (9) in the presence of an AB-incompatible allograft or pig xenograft, two other baboons, matched for age and weight, were followed as controls (Table 1). These did not receive either an allograft or xenograft and did not receive any IS therapy or other drugs on a regular basis, but simply were maintained

Table 1: Clinical Details of 6 Infant Baboons

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Sex</th>
<th>Age at arrival in our facility (days)</th>
<th>Age at Tx (days)</th>
<th>Start of clinical features (age, days)</th>
<th>Period of IS (age, days)</th>
<th>Survival after Tx (days)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>B4908</td>
<td>M</td>
<td>70</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No Tx or IS No diarrhea Alive</td>
</tr>
<tr>
<td>B5108</td>
<td>M</td>
<td>64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No Tx or IS No diarrhea Alive</td>
</tr>
<tr>
<td>B7507</td>
<td>M</td>
<td>67</td>
<td>98</td>
<td>300</td>
<td>Anti-CD154 (317) MMF (307)</td>
<td>324 (Died)</td>
<td>Diarrhea Dehydration Collagenous colitis diagnosed at necropsy</td>
</tr>
<tr>
<td>B5508</td>
<td>M</td>
<td>57</td>
<td>98</td>
<td>125</td>
<td>Anti-CD154 (137) MMF (132)</td>
<td>146 (Euthanized)</td>
<td>Diarrhea Dehydration Collagenous colitis diagnosed at necropsy</td>
</tr>
<tr>
<td>B5008</td>
<td>M</td>
<td>65</td>
<td>107</td>
<td>130</td>
<td>Anti-CD154 (139) MMF (134)</td>
<td>&gt; (euthanized at 24m, end of follow-up)</td>
<td>Diarrhea Colon biopsy (non-diagnostic) Treatment with budesonide</td>
</tr>
<tr>
<td>B5708</td>
<td>F</td>
<td>43</td>
<td>87</td>
<td>127</td>
<td>Anti-CD154 (145) MMF (131)</td>
<td>&gt; (euthanized at 24m, end of follow-up)</td>
<td>Diarrhea Treatment with budesonide</td>
</tr>
</tbody>
</table>

Anti-CD154 mAb - anti-CD154 monoclonal antibody
IS - immunosuppressive therapy
MMF - mycophenolate mofetil
Tx - transplantation
in the same room, received the same diet and had blood drawn at the same time intervals as the four experimental baboons.

At the time of entry to our facility, all six baboons were between 43 and 70 days (median 64 days) or approximately 2 months of age. However, one of them had been born earlier than the others and was admitted to our facility and received a transplant and IS therapy approximately 5 months before the others.

The artery patch grafts were carried out when the four baboons were aged 87-107 days (median 98 days) or approximately 3 months of age. They received IS therapy continuously for periods ranging from as long as 10 months to as short as 5 months, at which time they all developed diarrhea and either died (B7507) or were euthanized (B5508) or had IS therapy discontinued (B5008 and B5708) (Table 1).

Clinical Features

All four immunosuppressed infant baboons developed severe unexplained watery diarrhea within the same time-period extending over four weeks (Figure 1). This was associated with either actual weight loss or discontinuation of the normal weight gain seen in infant baboons (Figure 2).

---

**Table 2: Summary of Drugs Administered to Experimental Baboons**

<table>
<thead>
<tr>
<th>Induction Therapy</th>
<th>Dose</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymoglobulin</td>
<td>2.0 - 2.5 mg/kg i.v.</td>
<td>Day -3 and -1</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>5 mg/kg i.v.</td>
<td>Before each dose of ATG and on day 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The dose was then reduced by 1 mg/kg/day, and discontinued on day 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maintenance Therapy</th>
<th>Dose</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human CD154 mAb</td>
<td>20-25 mg/kg i.v.</td>
<td>Days -1, 0, 4, 7, 10, 14, then every 7 days (to maintain a trough level of 400-800 ng/ml)</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>20-150 mg/kg/d p.o. x2 daily</td>
<td>From day -2 (to maintain a trough level of 3-6 ug/ml)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Supportive Therapy</th>
<th>Dose</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefazolin</td>
<td>25 mg/kg bid i.v.</td>
<td>For 3 days after surgery</td>
</tr>
<tr>
<td>Famotidine</td>
<td>0.25 mg/kg bid i.v.</td>
<td>From day -3 until day 14</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>5 mg/kg i.v.</td>
<td>From day -4 until day 21</td>
</tr>
<tr>
<td>Ketorolac</td>
<td>0.5 mg/kg i.v.</td>
<td>Before every dose of anti-CD154</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.01 mg/kg bid i.v.</td>
<td>For 3 days after surgery</td>
</tr>
</tbody>
</table>
Diarrhea is not uncommon in humans or baboons receiving MMF (10-12), particularly if the blood levels of the drug are higher than planned. However, the blood levels were within the therapeutic range in all cases. Nevertheless, when diarrhea persisted for >1 week, MMF was discontinued to reduce possible MMF toxicity and to improve the baboons’ immunocompetency. In the two baboons already affected by the condition, persistent diarrhea, dehydration and decreasing weight (Figure 2) necessitated discontinuation of the anti-CD154mAb subsequently. In the remaining two infants, all immunosuppressive therapy was discontinued immediately upon manifestation of similar features.

After onset of diarrhea, all infants showed clinical signs of dehydration (decreased skin turgor, weight loss). Clinical chemistry evaluations were within the normal range except for mild features of dehydration. Multiple fecal cultures proved negative for standard enteric pathogens, although an overgrowth of yeast was seen on one sample. Testing for occult blood in the stool was negative, and blood cultures were also negative in all 4 baboons. Polymerase chain reaction (PCR) against cytomegalovirus (CMV) and norovirus as well as serology for anti-adenovirus and anti-CMV antibodies (all carried out by BioReliance, Rockville, MD) were tested at time of clinical features and were found negative in all four infants.

The diarrhea was unresponsive to treatment with metronidazole (0.5ml p.o. daily, administered for 8-14 days; Flagyl, Pfizer, New York, NY) or subsequently enrofloxacin (0.5ml i.v. daily, Baytril, Bayer, Pittsburgh, PA). Additionally, all infants received daily

Figure 1: Time line of disease onset and progress
Time line of disease onset and progress in 4 immunosuppressed and 2 age-matched infant baboons. All 4 immunosuppressed infants developed the same symptoms within the same period of 4 weeks.
oral fluid support (Pedialyte, Abbott Laboratories, Abbott Park, IL) and loperamide (Imodium, Jansen-Cilag, Beerse, Belgium) for 2-3 weeks.

Despite these measures, in two infants, the condition could not be alleviated. One infant (B7507, age 13 months) died overnight due to severe dehydration, and the other (B5508, age 8 months) was euthanized in view of progressive dehydration that did not improve with medical treatment and fluid replacement. Necropsy was performed on both baboons; histological examination of the colon demonstrated a form of collagenous colitis in both cases (see below).

When the third and fourth infants showed similar clinical progression, colonoscopy and colon biopsies were performed in one (B5008, age 7.5 months). Macroscopically, no lesions or abnormalities were seen; the biopsies did not include layers deeper than the mucosa, and therefore histopathology was inconclusive. However, in the light of the diagnosis in the previous two baboons, treatment with budesonide (at 2.5mg/kg p.o., Entocort, AstraZeneca, Prometheus Laboratories, San Diego, CA) was initiated in both infants. Over the course of 2 weeks, this treatment resulted in cessation of the diarrhea and a return of normal weight gain (Figure 2). Immunosuppressive therapy was not re-started. Budesonide was continued for 7 months and then discontinued. Total follow-up was for 16 months after initiation of budesonide therapy; no further diarrhea occurred in either baboon, and both followed a normal growth curve (Figure 2).

Figure 2: Weight changes of immunosuppressed baboons and controls
Weight changes of the 4 experimental and 2 control baboons over the course of the study. Onset of weight loss associated with diarrhea - 7507 at age 12 months; 5508 at age 7.5 months; 5008 and 5708 at age 8 months.
Gross Pathology Findings
Two baboons underwent necropsy at the time of death or euthanasia, with clinical features of colitis (B7507 and B5508), the other two were euthanized 16 months after all clinical features of colitis had disappeared. In all four animals, the gastrointestinal tract (stomach, jejunum, ileum, and colon) was grossly normal. Additionally, no abnormalities were noted in any thoracic or abdominal organs.

Histopathological Findings
The most consistent histopathologic change was an increased amount of fibrous connective tissue within the colon of all four baboons (Figure 3). Increased collagen deposition was found primarily within the submucosa and, to a lesser degree, within the lamina propria. The most striking changes were found in B7507 (Figure 3A).

Within small intestine, marked villous atrophy and enterocyte necrosis were found in B7507 and B5508 (Figure 4A and 4B). These animals also had extensive collagen deposition within the submucosa and lesser amounts within the lamina propria that mirrors the deposition found in the large intestine. Lesions within the small intestine of B5008 and B5708 (Figure 4C and 4D) were limited to mild villous blunting in B5008 and mild multifocal subacute enteritis. Within all intestinal sections of B7507 and B5508, occasional eosinophilic intranuclear inclusion bodies were present.
Colon, small intestine, stomach and liver of all 4 animals were stained with Gram (bacteria), periodic acid Schiff (fungae), congo red (amyloid; NHP may develop amyloid secondary to infection or inflammation) and trichrome (connective tissue). These stains demonstrated no active infection with microorganisms or amyloid deposition in any of the infant baboons. A generic herpesvirus PCR on formalin fixed and paraffin embedded small intestinal tissues from B5508 was performed since the inclusion bodies found in some intestinal sections suggested a potential viral etiology. Test results were negative.

**DISCUSSION AND REVIEW OF THE LITERATURE**

We report four baboons in which a diagnosis of a form of collagenous colitis was made on the basis of clinical features and, particularly, histopathologic appearances in two out of the four animals. The rapid recovery in response to discontinuation of IS therapy and initiation of budesonide, in combination with unresponsiveness to other treatments and negative bacterial and viral findings, adds further support for the diagnosis in the other two cases.

**Collagenous colitis in humans**

Collagenous colitis is a chronic inflammatory bowel disease that was first described by Lindström in 1976 (13). Clinical features are chronic watery diarrhea that is often of sudden onset, mimicking infectious diarrhea, accompanied by abdominal pain and

**Figure 4: Small intestine H&E and collagen deposition**

H&E staining (top) and collagen staining (Trichrome, bottom) of the small intestine of B7507 (A), B5508 (B), B5008 (C) and B5708 (D) (magnification x10). A marked increase in collagen is found in both the lamina propria (arrow) and submucosa (asterix) of B7507 and B5508 in addition to moderate (B7507) to severe (B5508) blunting and loss of villi with necrosis and loss of surface enterocytes (arrowhead). Mild villous blunting is present in B5008. The small intestine of B5008 and B5708 is normal appearance.

(see color figure on page: 232)
weight loss. Dehydration is rarely seen in humans. The disease is mainly localized to the colon and rectum, and is characterized by a normal appearance of the mucosa on endoscopy and radiology, but with distinct histopathological changes seen on microscopic examination. Biopsy is therefore essential in making the diagnosis. Histopathological features include (i) a diffuse non-continuous thickening of a subepithelial collagen layer throughout the colon, (ii) inflammation of the lamina propria, dominated by lymphocytes and plasma cells, and (iii) flattening and vacuolization of the epithelial cells and detachment of the surface epithelium (14, 15).

The incidence of collagenous colitis would appear to be increasing (or at least being more frequently recognized). Pardi et al (16) reported an increased incidence from 0.3 to 6.2 per million between 1985 and 2001, and others reported an increase from 0.8 to 6.1 per million between 1984 and 1998 (3, 17). The peak incidence is around 65 years of age, and the female: male ratio is reported to be between 6:1 (17) and 9:1 (18).

The etiology and pathogenesis of collagenous colitis remain unknown, although an association with autoimmune diseases is generally accepted (19, 20). Treatment, therefore, remains mainly empirical. Currently, budesonide, a topical intestinal steroid that has efficacy in the terminal ileum and colon, is the drug of choice; it has been studied in several double-blind placebo-controlled trials, and has been demonstrated to significantly improve the patient’s condition within 2-4 weeks, although short-term treatment is associated with an approximate 60-80% relapse rate (21, 22). If treatment is continued for >8 weeks, the response is maintained for >6 months. We elected to maintain budesonide for 7 months to allow sufficient time for the baboons to return to a normal or near-normal rate of growth, similar to age-matched controls. The long-term prognosis of successfully treated collagenous colitis is good. Although the course of the disease is chronic with relapses in the majority of patients, symptoms can be controlled by maintenance treatment to maintain a good quality of life in the majority of patients (23). Serious complications, such as perforation of the colon, are rarely seen. Mortality is not increased compared to healthy subjects (24-26).

Of considerable interest in relation to our presented cases is a recent report indicating an increased risk of microscopic colitis in patients with solid organ transplants (kidney, kidney and pancreas, or liver) (6). The incidence of microscopic colitis was 8.8/1000 patients; three of seven patients were diagnosed with collagenous colitis, which is an approximately 1,000-fold higher incidence than in the general population (16, 18). The seven patients were receiving different immunosuppressive regimens that included drugs such as cyclosporine, tacrolimus, prednisone, sirolimus, MMF, and azathioprine. Six of the seven patients were given budesonide, and all responded
well. The causative factors for the development of collagenous colitis in these patients remain uncertain, but the state of immunosuppression is very likely to be important.

Collagenous colitis in nonhuman primates

There are very few reports of collagenous or microscopic colitis in the literature. In a study at the Southwest Foundation for Biomedical Research (San Antonio, TX, USA) involving 3,200 baboons of <20 years of age, 132 died with chronic diarrhea. All were infants, juveniles, or young adults (i.e., <8 years of age). Diarrhea persisted for several months to >1 year. All were infected with endemic bacterial pathogens, particularly Campylobacter species and/or Balantidium coli, although these microorganisms were also found in healthy animals. Microscopic examination of the bowel was carried out in 86 of the baboons that died, and in only a single case were the appearances consistent with collagenous colitis. The histopathology was similar to that reported in humans, with a thick hyaline band underlying the superficial epithelium and the presence of chronic inflammatory cells (7). The same group also reported a study on 1,600 cynomolgus monkeys (Macaca fascicularis); 90 died with chronic diarrhea, but in no case was a diagnosis of collagenous colitis made (8). We conclude that collagenous colitis is very rare in naïve populations of nonhuman primates.

Diagnosis of collagenous colitis: comparison of findings in baboons and humans

Collagenous colitis was a primary differential diagnosis in all four baboons, although there were some differences from the appearances reported in humans (Table 3). First, the deposition of collagenous tissue in the baboons appeared to be deeper within, and involving a substantially greater portion of, the submucosal layer than described in humans, where it is confined to a region just beneath the surface epithelium. Second, there was no general increase in inflammatory cells in any layer of the large and small intestine except to a minimal degree in the small intestines of B5008 and B5708. However, the combination of clinical features, blood and stool cultures that were

Table 3: Collagenous Colitis in Humans and Baboons: Comparison of Pathology

<table>
<thead>
<tr>
<th>Feature</th>
<th>Humans</th>
<th>Baboons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross intestinal lesions</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Pathogens associated with disease onset</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Collagen deposition</td>
<td>Extensive in colon</td>
<td>Extensive in colon</td>
</tr>
<tr>
<td>Small intestine involvement</td>
<td>Villous blunting and necrosis</td>
<td>Villous blunting and necrosis</td>
</tr>
<tr>
<td>Location of collagen deposition</td>
<td>Primarily subepithelial</td>
<td>Primarily submucosal</td>
</tr>
<tr>
<td>Inflammatory cell infiltrates</td>
<td>Yes; lymphocytes, plasma cells</td>
<td>Minimal</td>
</tr>
</tbody>
</table>
repeatedly negative for enteric pathogens, serum tests that were negative for viral pathogens, the normal macroscopic appearance of the intestinal mucosa, and the distinct deposition of abundant collagen in the colonic mucosa were all comparable to the clinical presentation in humans (Table 3), supporting a diagnosis of a variant form of collagenous colitis. Additionally, there is an association between collagenous colitis and microscopic findings of small intestinal villous necrosis and blunting (27, 28). Interestingly, this change was found in the small intestine of both the baboons B7507 and B5508, which were examined at time of active disease (Table 3, Figure 4).

Comments

In recent years, preclinical models for research in the field of organ and cell Tx have increasingly used nonhuman primates. These species are particularly important in xenotransplantation research as Old World nonhuman primates, e.g., baboons and Old World monkeys, are the only mammals (other than humans) to make antibodies directed to the important Galα1,3Gal antigens expressed in pigs (reviewed by Kobayashi and Cooper (29)). In almost all of these studies, the recipient nonhuman primate is immunosuppressed, which can be associated with complications similar to those in humans, particularly infection (30-32). One fairly common problem is diarrhea, often associated with the drugs the animal is receiving. If drug-induced, it can frequently be reversed by reduction or cessation of drug therapy. However, we have not knowingly seen collagenous colitis previously in >100 immunosuppressed baboons and cynomolgus monkeys, even when the period of immunosuppression extended for several months. Several of these baboons and monkeys -though almost all of these were adults or young adults rather than infants- developed diarrhea. However, the pathogenic microorganism was usually identified and it was generally easily controlled by empirical agents such as loperamide, anti-microbial agents, and/or reduction in immunosuppressive therapy.

Our own experience of collagenous colitis reported here is in some respects in contrast to that in humans, but in other respects similar. Our baboons were very young, rather than elderly, as in humans (3, 16), although there have been rare cases reported in children (4, 5, 33). Three of the four affected animals were male, rather than the predominance of females (as in humans). It is likely to be important that our baboons were immunosuppressed, as collagenous colitis is associated with an autoimmune state in humans. In the two baboons treated with budesonide, this drug rapidly induced remission (as in humans) and, in the absence of continuing immunosuppressive therapy, no relapse occurred either during the 7-month period of therapy or after cessation of therapy.
The four baboons had received immunosuppressive as well as supportive medication for periods ranging between 4-10 months (Tables 1 and 2). A review of the literature suggests an association between microscopic colitis and certain drugs, though not specifically between collagenous colitis and these drugs. Because of the small numbers of patients in these studies, discrimination between collagenous and lymphocytic colitis is often not made. However, drugs that have a high likelihood of inducing microscopic colitis include acarbose, aspirin, lansoprazole, non-steroidal anti-inflammatory agents, ranitidine, sertraline, and ticlopidine (34). The infant baboons in our study received none of these agents, except for the non-steroidal anti-inflammatory agent, ketoprofen, which all four baboons had received only once weekly at low-dose prior to anti-CD154 mAb administration. Olesen et al (3) suggested a weak association between ketoprofen and lymphocytic colitis, but not with collagenous colitis.

Importantly, once the diagnosis was confirmed or suspected, two of the baboons survived as a result of aggressive fluid replacement and treatment with budesonide. We would recommend that collagenous colitis should be suspected and investigated in any baboon, particularly if immunosuppressed, that develops diarrhea and weight loss that does not respond to conventional investigation and therapeutic measures. Awareness of this disease could prevent unnecessary morbidity and mortality in valuable nonhuman primates involved in experimental studies.

A potentially important observation from our own study was the occurrence of all 4 cases within a time span of 4 weeks (Figure 1), despite the fact that the period of time for which the baboons had been receiving immunosuppressive therapy ranged from 4-10 months. The outbreak of collagenous colitis in 4 baboons, housed in the same room, over such a short period of the study suggests to us an infectious etiology. One possible explanation for this could be that the two healthy controls had a subclinical infection to a common viral cause that did not lead to any symptoms, while the state of immunosuppression allowed the 4 experimental animals in the same room to suffer from an opportunistic infection. This contention is also strengthened by the presence of concurrent active inflammatory lesions as well as more chronic change (i.e., villous atrophy) in the small intestine of B7507 and B5508. It should be noted that cases of collagenous colitis associated with small intestinal villous atrophy are reported in the human literature (27, 28).

Whether the infectious agent was introduced to each individual baboon by an animal handler or other vector, or whether one infected baboon passed on the condition to others by direct or close contact remains unclear, but a common infectious agent leading to a pan enterocolitis with consequential severe colonic mucosal fibrosis cannot
be ruled out. We would suggest that efforts to identify infectious agents as a causative factor in patients with proven collagenous colitis should be intensified.

ACKNOWLEDGEMENTS

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Abbreviations

Anti-CD154 mAb - anti-human CD154 monoclonal antibody
IS - immunosuppressive therapy
NHP - Non-human primate
MMF - mycophenolate mofetil
Tx - transplantation
REFERENCES

A syndrome of severe hypoglycemia and acidosis in young immunosuppressed diabetic monkeys and pigs — association with sepsis

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* HZ, DJvdW and EMD contributed equally

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Background
Large animals treated with immunosuppressive drugs for preclinical experiments of transplantation have increased risks of infection, which can be compounded by the induction of diabetes in these animals if islet transplantation is planned.

Methods
We report our experience with severe sepsis in two young cynomolgus monkeys and five pigs that were subjected to diabetes induction, immunosuppressive therapy +/- islet allotransplantation.

Results
In two monkeys and five pigs, infection was associated with a syndrome of profound hypoglycemia accompanied by severe acidosis, which was resistant to treatment. We do not believe this syndrome has been reported previously by others.

Conclusions
Despite treatment, this syndrome complicated the interpretation of blood glucose readings as a measure of islet graft function, and resulted in death or the need for euthanasia in all 7 animals. We tentatively suggest that the syndrome may be related to the presence of microorganisms that metabolize glucose and produce lactate.
INTRODUCTION

The encouraging results of clinical islet allotransplantation (1) have stimulated renewed experimental efforts in nonhuman primate and other large animal models of allotransplantation (2,3) and xenotransplantation (4,5).

The immunosuppressive drug regimens used in preclinical studies of transplantation involving large animals increase the risk of infection, as can a state of diabetes. Here we report our experience with severe sepsis in two young immunosuppressed, diabetic cynomolgus monkeys in a study of islet allotransplantation. The nature of the experiment, which included the establishment of permanent intravascular access via indwelling catheters and induction of a diabetic state, may have further increased the infectious risks. Although housed in clean facilities, fecal contamination of the animal cages is inevitable and creates suboptimal hygienic conditions.

Sepsis is a well-known complication seen in patients with diabetes (6) and in those receiving immunosuppressive therapy after organ transplantation (7). Similarly, sepsis is not uncommon in immunosuppressed nonhuman primates undergoing transplantation procedures. However, the diagnosis of sepsis in nonhuman primates is more difficult than in humans. Lethargy, withdrawal, and/or anorexia may suggest sepsis, as might a significant rise in white blood cell count, though this may not occur when intensive immunosuppressive therapy is being administered. A positive blood or tissue culture is usually necessary to confirm the diagnosis and identify the causative microorganism.

In both of the monkeys reported here, infection was associated with a syndrome of intractable profound hypoglycemia accompanied by severe metabolic acidosis. In the discussion of these cases we include information on five immunosuppressed pigs that also experienced severe hypoglycemic episodes after diabetes induction and islet allotransplantation (8). Together, we suggest these cases represent a syndrome that has not been reported in the literature.

MATERIALS AND METHODS

All animal care procedures were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised
1985), and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

**Case 1**

A 22 month-old (1.9kg) Indonesian cynomolgus monkey (*Macaca fascicularis*) underwent placement of an indwelling central venous catheter through the internal jugular vein and diabetes induction with streptozotocin (*Zanosar*, Sicor Pharmaceuticals, Irvine, CA, 150mg/kg) (Figure 1). (This dose has been utilized by our group with safety in the majority of cases [20] and also by others [34].) Diabetes was confirmed by the absence of C-peptide responses on intravenous glucose tolerance and arginine stimulation tests. Insulin therapy was begun to maintain blood glucose levels at <200mg/dL.

Profound lymphopenia (CD3+ T cells <15cells/mm³) was induced by alemtuzumab (*Campath-1H*, Genzyme, Cambridge, MA) at a dose of 20mg/kg on the day before transplantation (i.e., day -1) (1). Pretreatment was with diphenhydramine 5mg/kg i.v. and metoclopramide 0.5mg/kg i.v. Alemtuzumab administration (10mg/kg) was repeated on day 6 after transplantation to maintain low T cell counts (CD3+ T cells <15cells/mm³ after the second infusion) (1).

On day 0, allogeneic islets were infused into the portal vein through a mini-laparotomy under heparin anticoagulation, and anti-TNF-α anti-inflammatory treatment with etanercept (*Enbrel*, Amgen, Thousand Oaks, CA) and 48h cefazolin antibiotic prophylaxis was initiated. Maintenance immunosuppression consisted of rapamycin 0.5mg/kg/i.m. (aiming for, and achieving, plasma trough levels of 5-15ng/mL). There was definite evidence of islet graft function, with reduction in mean blood glucose level and insulin requirements; C-peptide was detectable on day 1, but later was barely detectable.

---

**Figure 1: Time line of events**

Timing of i.v. catheter insertion, induction of diabetes with streptozotocin, induction of immunosuppressive therapy, islet transplantation, and onset of clinical features of infection.

*alemt = alemtuzumab; cath = catheter; STZ = streptozotocin; Tx = transplantation*
Case 2
This 36 month-old (1.6kg) Indonesian cynomolgus monkey was included in the same research protocol, including placement of an indwelling intravascular catheter and induction of diabetes with streptozotocin. Thirty-five days after induction and confirmation of diabetes, alemtuzumab 20mg/kg was infused after pretreatment as in Case 1.

RESULTS

Case 1
In this monkey, after the transplantation of allo-islets (12,500IEq/kg) into the portal vein (Figure 1), although blood glucose levels during the first 8 post-transplant days were reduced (mean fasting blood glucose 137mg/dL), consistent normoglycemia was not achieved. Insulin requirements were significantly reduced (from 4.4 to 1.5 IU/day). C-peptide was detectable on day 1 (0.8ng/mL), but later was barely detectable (<0.1ng/mL). The monkey remained active, with normal hematological and biochemical parameters. However, it subsequently became profoundly hypoglycemic (blood glucose <20mg/dL) even when no insulin had been administered, and required frequent i.v. boluses or infusions of dextrose. On day 9, hematologic and biochemistry parameters were within normal ranges, but blood analysis revealed metabolic acidosis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10*</td>
<td>12*</td>
<td>0**</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>7.14</td>
<td>7.22</td>
<td>7.16</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td></td>
</tr>
<tr>
<td>Base excess (mmol/L)*</td>
<td>-20</td>
<td>-13</td>
<td>-16</td>
<td></td>
</tr>
<tr>
<td>HCO₃⁻ (mmol/L)</td>
<td>8.9</td>
<td>14.9</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Na⁺ (mmol/L)</td>
<td>150</td>
<td>153</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>K⁺ (mmol/L)</td>
<td>2.8</td>
<td>2.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>iCa²⁺ (mmol/L)</td>
<td>1.38</td>
<td>1.20</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.9</td>
<td>0.7</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>8.5</td>
<td>6</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>4.9</td>
<td>3.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Platelets (×10⁹/L)</td>
<td>183</td>
<td>143</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

*Post islet transplantation
**Islet transplantation was not carried out. Blood test results relate to the day after receiving alemtuzumab.
*Lactate levels and anion gap were not measured.
(pH 7.26, CO₂ 31mm Hg, HCO₃ 13mmol/L, base deficit -13). The cause of the acidosis was uncertain.

On day 10, the monkey became withdrawn, inactive and anorexic, and blood analysis revealed severe metabolic acidosis (Table 1). Although the white blood cell count remained normal, a slight thrombocytopenia and anemia had developed. Physical examination revealed hypothermia (rectal temperature 32°C), but otherwise stable vital signs. Empiric antibiotic treatment with enrofloxacin i.v. and clindamycin i.v. was initiated after blood was drawn for culture. By day 12, despite the antibiotic regimen, the infusion of warm saline, dextrose and sodium bicarbonate, and maximum supportive therapy, the monkey remained hypoglycemic, acidotic, and hypothermic (Table 1). Diarrhea developed and the clinical status of the monkey deteriorated. Lactobacillus species (Gram-positive rods) had grown from the blood culture, and antibiotic treatment was switched to ampicillin i.v. and metronidazole p.o.

Because of a lack of clinical improvement, the monkey was euthanized for humane reasons on day 14 after transplantation. Necropsy showed sporadic bilateral hemorrhagic infarction in the lungs. On histology, portions of the lungs appeared atelectatic and alveoli contained a proteinaceous fluid. In the renal tubules, features of a regenerative epithelium were seen, which suggested recovery from renal injury probably caused by streptozotocin treatment. No growth was observed from lung culture swabs. A blood culture drawn at the time of necropsy grew Klebsiella oxytoca (Gram-negative rods). Normal bacterial flora were cultured from the feces. Culture of the indwelling intravenous catheter remained negative. Histological examination of the liver showed multiple small viable islets with no cellular infiltration.

Case 2
In this monkey, the induction of diabetes was not followed by any untoward effect and the monkey appeared quiet but healthy. Thirty-five days after the successful induction of diabetes, immunosuppressive therapy was initiated (Figure 1). Two days earlier, there had been no abnormalities in hematologic parameters, except for a white blood count of 14.4x10⁹/L, but biochemical parameters were not tested. Two hours after the infusion of alemtuzumab on the day before the planned islet transplantation the monkey became acutely dyspneic, anorexic, hypothermic (rectal temperature 33°C), and demonstrated decreased alertness and lethargy. Laboratory tests showed profound hypoglycemia, severe acidosis, raised serum creatinine, leucopenia (primarily a lymphopenia), and profound thrombocytopenia (Table 1).
The biochemical changes were so severe that we suspect they had been increasing for several days, but had gone unnoticed, although this is conjecture. We suggest that the rapid deterioration might have been associated with cytokine release stimulated by the administration of the alemtuzumab, though we have no data to support this conclusion. There were no differences in selection of the monkey or administration of the monoclonal antibody from our previous study using alemtuzumab in non-diabetic monkeys (9) that might have accounted for this change in clinical condition.

Insulin treatment (up to then at a cumulative dose of 3.83 IU/kg/day) was discontinued and dextrose and bicarbonate infusions established. Blood for culture was drawn and empiric antibiotic treatment with enrofloxacin i.v. was started. In view of the features of sepsis, islet transplantation was postponed (and indeed was never carried out). Despite antibiotic therapy, the monkey’s condition deteriorated rapidly and, after consultation with the veterinary staff, for humane reasons it was euthanized on the following day. Blood cultures grew Gram-negative rods, later classified as *Klebsiella oxytoca*. At necropsy, severe pulmonary edema with pulmonary alveolar hemorrhages were the main findings. Histologic examination of lung tissue confirmed interstitial edema. In the kidneys, acute thromboemboli in most arteries and necrotic glomeruli and tubules were seen, indicating acute diffuse renal cortical infarction, possibly as a consequence of sepsis and/or alemtuzumab administration.

**DISCUSSION**

In this report we describe the occurrence of severe hypoglycemia and metabolic acidosis associated with sepsis in two young cynomolgus monkeys in which diabetes had been induced 4-6 weeks previously, immunosuppressive therapy initiated, and islet transplantation carried out in one case.

As both monkeys had bacterial growth from blood cultures, we conclude that septicemia played an important role in the development of hypoglycemia and acidosis. However, possible side effects of alemtuzumab infusion in Case 2 may have contributed to the clinical condition, although we have not seen this previously in monkeys (n=6) that remained well and non-infected for periods of >1 year after receiving alemtuzumab with or without mycophenolate mofetil (9). Acute alveolar hemorrhage, renal failure and coagulopathy have all been reported after alemtuzumab treatment in human patients (10-12). Nevertheless, we suspect that the monkey in Case 2 had developed an infection before administration of alemtuzumab that had gone unnoticed, but clinical deterioration only became obvious at this time. Early signs of disease or infection
may not always be obvious in nonhuman primates (13). In our previous studies of alemtuzumab, we did not see any infectious complications, but these monkeys were not diabetic (9).

Although the release of stress hormones during sepsis most commonly leads to a hyperglycemic state, hypoglycemic sepsis has been described (14,15), and has been shown to be independently associated with death in critically ill patients (14). Hypoglycemia should be considered a biomarker of disease severity (16), especially in diabetic patients (17). The young age and small size (body weight 1.9kg and 1.6kg, respectively – lower body weights than any of our previous studies) of the two monkeys in this report may have contributed to the severe derangements in glucose metabolism, acid-base balance and body temperature, as we have not observed these signs so severely in our broad experience with nonhuman primate transplant recipients of >2.5kg. Furthermore, their young age and low body weight might have rendered them more vulnerable to the effects of infection.

In previously reported experiments of islet allotransplantation into the gastric submucosal space of young (<2 months, mean 13kg) diabetic pigs immunosuppressed with tacrolimus and mycophenolate mofetil, we observed episodes of profound hypoglycemia in five pigs (8). The clinical picture seen in these pigs was almost identical to that seen in the two monkeys reported here. Diarrhea was observed in four of the five pigs. In the pigs, with functioning or partially-functioning islet grafts, blood glucose levels fell to 25-50mg/dL, resulting in sudden death in two pigs, and the need for euthanasia in the remaining three. Blood cultures grew *Klebsiella oxytoca* in one, and *Streptococcus viridans* and coagulase-negative *Staphylococci* in another. Blood cultures remained negative in the other three; however, at necropsy, signs of infection, such as colitis, cellulitis, and lobar pneumonia, were present. Although at the time we reasoned that infection would have led to hyperglycemia, our more recent experience in the monkeys reported here suggests that sepsis from *Klebsiella, Lactobacillus*, or possibly other pathogens likely played a role in the development of hypoglycemia.

Importantly, we have not seen this syndrome in numerous other nonhuman primates and pigs that have received identical or similar immunosuppressive regimens, but that were not rendered diabetic. We have no suggestions as to how this syndrome can be prevented as the monkeys and pigs were treated similarly to others at our facility that did not develop hypoglycemia and acidosis. We suggest that the nature of the infecting organism may be key in the development of the syndrome.
Our group has considerable experience with induction of diabetes by streptozotocin (in the form of Zanosar) in monkeys (18-20). Although renal injury has been documented on two occasions, the agent has otherwise not been associated with significant complication. In Case 1 of the present report, however, histopathological examination of the kidneys suggested some renal injury may have occurred which may have enhanced the risk of, or reduced the response to, sepsis.

The combination of diabetes and immunosuppressive therapy therefore appears to be important in the development of the syndrome, most likely contributing to an infection with a specific microorganism (or microorganisms). Nonhuman primates would appear to be much less susceptible to this (potential) infecting agent in the absence of either of these two contributory factors. We have not seen this syndrome in nonhuman primates that have been immunosuppressed but not diabetic. Nevertheless, it seems possible that hypoglycemia could develop in the presence of a positive infection and immunosuppressive therapy if the causative microorganisms were lactobacilli (or similar) which metabolize glucose.

Our vigorous efforts to maintain a state of normoglycemia (by i.v. dextrose infusion) and correct the acidosis (by i.v. sodium bicarbonate infusion) unexpectedly proved inadequate, attesting to the aggressive and progressive nature of the biochemical changes taking place. Whether alternative antibiotic therapy, lymphocyte-depleted whole blood transfusion (to correct anemia), and other measures would have proved successful remains speculative, but in our opinion appears unlikely.

*Lactobacillus* species are Gram-positive rods that colonize the gastrointestinal and genitourinary tracts and are considered normal human flora (21). Although rarely seen, *Lactobacillus* infections are opportunistic infections associated particularly with the use of immunosuppressive drugs after transplantation (22), in HIV infection (23), the use of probiotics (24), or the long-term use of indwelling intravascular catheters (25). To our knowledge, *Lactobacillus* infection in nonhuman primates after organ or cell transplantation has not been reported previously. *Lactobacilli* are (facultative) anaerobic bacteria and have a high saccharoclastic activity, i.e., they ferment glucose with at least one-half of the end-product carbon being lactate, which is not further fermented (26). The fermentation of glucose into lactate may have contributed to the treatment-resistant hypoglycemia and metabolic acidosis observed in our Case 1. Furthermore, although not positively cultured, we cannot exclude the possibility that *Lactobacilli* were playing a role in Case 2.
Klebsiella oxytoca, that was cultured from the blood in both of the monkeys reported here and in one of the pigs reported previously (8), is among the Gram-negative rods that can colonize the gastro-intestinal tract (27). The extent of colonization increases markedly during hospitalization, and it is a cause of infection mainly in patients who are immunocompromised or suffering from a debilitating disease, such as diabetes (27). Unlike Lactobacilli, Klebsiella does not ferment glucose into lactate, but may possibly have contributed to hypoglycemia and metabolic acidosis through a different mechanism, such as endotoxemia.

Because of the ability of Lactobacilli to ferment glucose and produce lactate, we suspect that this microorganism may be playing a role in this syndrome. However, the variety of flora cultured in the blood in the monkeys and pigs that developed hypoglycemia and acidosis suggests that perhaps the syndrome can be associated with other infectious agents. Our microbiological data are admittedly limited, and any conclusions drawn must be provisional and cautious.

Other potential factors in the development of the syndrome that might be considered are the absence of the inhibitory effect of insulin on proinflammatory cytokines (28-30). Partial restraint of the monkey in the jacket and tether system, which may possibly be associated with some stress, seems an unlikely factor as we have employed this system for many years without seeing this complication (20, 31-33); furthermore, the pigs that developed the same clinical features were not restrained in any way.

In summary, we have observed a syndrome in which young, diabetic, immunosuppressed monkeys and pigs develop profound hypoglycemia and severe metabolic acidosis, which in several cases was associated with documented sepsis. We suggest that the syndrome might be related to the presence of microorganisms that metabolize glucose and produce lactate. Although the immunosuppressive state almost certainly increases the susceptibility of the animal to an infectious complication, we have not seen this syndrome in non-diabetic immunosuppressed nonhuman primates and pigs. Despite treatment, this intractable syndrome complicated the interpretation of blood glucose readings as a measure of islet graft function, and resulted in death or the need for euthanasia in all animals in which it occurred.

ACKNOWLEDGEMENTS

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Section 2

Alemtuzumab and regulatory T cells for heart transplantation in non-human primates
Non-human primate regulatory T cells: current biology and implications for transplantation

Eefje M. Dons, Giorgio Raimondi, David K.C. Cooper, Angus W. Thomson

Transplantation 2010; 90:811-816.
Regulatory T cells (Treg) offer potential for improving long-term outcomes in cell and organ transplantation. The non-human primate (NHP) model is a valuable resource that can be used to address issues concerning the transfer of Treg therapy to the clinic. Herein we discuss the properties of NHP Treg and prospects for their further evaluation in allo- and xenotransplantation.
REVIEW

The importance of regulatory T cells (Treg) in the control of immunity (1-3) and their involvement in tolerance to auto- and alloantigens (Ags) is well-documented in rodents and humans (4, 5). Treg offer potential for therapy (6-10), and the promise of avoiding many toxicities and morbidities associated with current immunosuppressive drug regimens that, except in rare circumstances, fail to promote clinical transplant tolerance (11). Treg offer the possibility of being highly-specific and effective, with potential to provide long-term tolerance. Naturally-occurring, conventional CD4+CD25+ Treg (nTreg) that constitute approximately 10% of peripheral CD4+ T cells are the most extensively investigated. They are identified by intra-nuclear expression of the transcription factor forkhead/winged-helix box protein 3 (Foxp3) (12), which, at least in mice, is expressed selectively by Treg. Recent reviews (13-18) describe their development and function. nTreg can suppress the activation, proliferation, differentiation and effector function of various immune cells, including CD4+ and CD8+ T cells, B cells, dendritic cells (DC) and natural killer cells (2, 15, 19, 20) via different mechanisms, depending on the target and location of their action.

Multiple ways of improving Treg activity to control alloimmunity are being pursued. Adoptive transfer of purified Treg is the most widely-studied approach. However, many practical concerns need to be addressed, while ethical issues limit the testing that can be done in humans. This singles out the non-human primate (NHP) model as a valuable pre-clinical resource, reinforced by the reported similarity between human and NHP Treg. Adoptive cell therapies are at a very early stage of evaluation in NHP, and there are as yet no reports of conventional Treg therapy in NHP transplantation. However, using an innovative strategy in the rhesus macaque, Bashuda et al (21) administered autologous ‘suppressive T cells’ (approx 10^7/kg) rendered anergic by ex vivo co-culture with donor alloAg in the presence of anti-CD80 and -CD86 mAbs. The anergic T cells were infused 13 days after renal transplantation to splenectomized recipients given brief cyclosporine A and cyclophosphamide (lymphocyte-depleting) therapy during anergic cell preparation from host spleens. Significantly, long-term graft survival and donor-specific tolerance were achieved in 50% of the recipients, without further immunosuppressive therapy, demonstrating the potential of this regulatory suppressive T cell approach in NHP organ transplantation.

Realization of a therapeutic protocol based on adoptive transfer of Treg requires that multiple issues be addressed (Fig 1A): 1) the efficacy of Treg and the number of cells necessary to obtain a therapeutic effect; 2) the Ag specificity necessary for effective control of rejection; 3) the stability of the suppressive phenotype of adoptively-transferred
Treg; 4) the Treg migratory pattern that guarantees the strongest regulatory function; 5) the conditions permissive to regulation of the memory response; 6) the ability of Treg to control the xeno-reactive response; 7) the impact of lymphocyte depletion/concomitant immunosuppressive therapy on Treg function; 8) the influence of Treg on autoimmunity and resistance to infection. In light of the strong similarity reported recently between human and NHP Treg, NHP transplant models are well-positioned to resolve these issues.

Although rare cells, new methods have emerged for the purification and expansion of polyclonal and Ag-specific Treg (22). Phenotypic and functional characterization of NHP nTreg (CD4+CD25+) in blood or lymphoid tissues has been reported for cynomolgus macaques (23, 24), rhesus macaques (25-27) and baboons (28). Their ex vivo expansion following immunomagnetic bead or/and flow sorting has been documented in response to either polyclonal stimuli (25, 29), peripheral blood mononuclear cells (PBMC) (28) or allogeneic DC (23) (Table 1). As in humans, more precise characterization of NHP Treg is needed. Recently, human Treg have been better distinguished from conventional T cells by their lack cell surface CD127 (IL-7Rα); CD127 expression correlates inversely with Foxp3 and the suppressive function of human Treg (30, 31). In cynomolgus macaques, circulating CD4+CD25+CD127- cells that express Foxp3 and exhibit suppressive activity, have been isolated and expanded using ‘semi-mature’ allogeneic DC and IL-2 (23), although the alloAg specificity of the Treg was not ascertained. In rhesus macaques, circulating CD4+CD25+CD127lo/- T cells have

![Diagram](image_url)
Figure 1. (A), Issues that govern the successful application of Treg therapy in transplantation. (B-D), Highly-suppressive Treg can be expanded/induced from cynomolgus monkey peripheral blood. (B), CD4+CD127loCD25+ (nTreg) were flow-sorted from PBMC. They were stimulated in vitro with anti-CD3/CD28-coated beads, in the presence of human IL-2 (500U/ml), with or without TGFβ1 (5ng/ml). In parallel, CD4+CD127hiCD25- T cells were sorted and induced to convert into regulatory cells (iTreg) by stimulation with anti-CD3/CD28-coated beads in the presence of IL-2 and TGFβ1, with or without retinoic acid (RA; 5ng/ml). The fold-expansion obtained under each condition at multiple time points, is indicated. (C), Preservation of Foxp3 expression in expanded nTreg was tested by intracellular staining after 16 days of culture. (D), The suppressive capacity of expanded nTreg was tested in MLR. PBMC were obtained from the same source as nTreg and numbers in vitro with irradiated allogeneic PBMC (1:2 responder to stimulator ratio). Titiated numbers of nTreg or nTreg (TGFβ1) were added to the cultures at the indicated Treg to responder ratios. Responder cell proliferation was quantified by thymidine incorporation on day 4. nTreg expanded with IL-2 alone did not inhibit proliferation significantly (data not shown). (E), as in D, T cells induced to convert into iTreg were tested for their suppressive capacity in MLR. *, p<0.02; **, p<0.05 for all comparisons indicated.

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<table>
<thead>
<tr>
<th>Species</th>
<th>Treg selection (purity)</th>
<th>Cell yield</th>
<th>Expansion method</th>
<th>Expansion rate</th>
<th>Suppressive activity</th>
<th>Reference</th>
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<tr>
<td>Rhesus</td>
<td>T cells from spleen</td>
<td>200 x 10^6 cells per recipient (splenectomized)</td>
<td>Donor splenocytes + anti-CD80/CD86; 13 days (d)</td>
<td>2 - 4 fold or no expansion</td>
<td>In vivo infusion 13d post allo kidney transplant; donor-specific suppression of rejection (21)</td>
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<td>Rhesus</td>
<td>MACS (90%) or FACS (98%) CD4+ CD25^a</td>
<td>Not mentioned</td>
<td>Anti-CD3/CD28 beads, IL-2; 4wk</td>
<td>300-2000-fold</td>
<td>Up to 1:8^a ratio, inhibition of autologous PBMC proliferation (25)</td>
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<td>Anti-CD8 and anti-CD20 Dynabeads, or CD4^+ MACS, followed by anti-CD25 MACS (82%)</td>
<td>10% of CD3^+CD4^+ T cells</td>
<td>Fresh cells used; no expansion</td>
<td>n/a</td>
<td>Proliferation of Teff to anti-CD3 or irradiated PBMC is decreased at 1:1^a ratio, but variation between animals (27)</td>
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<tr>
<td>Rhesus</td>
<td>FACS CD4^+CD25^hi or CD4^+CD25^+CD127</td>
<td>10 x 10^5 cells/ml blood</td>
<td>Anti-CD3/CD28 beads + IL-2; 4 wk</td>
<td>Up to 450-fold</td>
<td>CFSE-MLR, up to 1:100^a ratio; suppression of allo response by responder-specific or 3rd-party Treg (29)</td>
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<td>Rhesus</td>
<td>MACS CD4^+CD127^a</td>
<td>7% of CD4^+</td>
<td>Immature Mo-DC + IL-2 + IL-15; 14d, or 10d followed by 2d without DC</td>
<td>No expansion</td>
<td>Suppression up to 1:40^a ratio; donor-specific (33)</td>
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<td>FACS CD4^+ CD25^+CD127^ (&gt;98%)</td>
<td>0.4% of PBMC</td>
<td>Allo DC (BM / Mφ) + IL-2; 7days</td>
<td>12-25-fold</td>
<td>CFSE-MLR, PBMC ± anti-CD3/CD28+ Treg: 30% inhibition at 1:3^a ratio. Treg suppressive capacity stimulated by BMDC &gt; Mo-DC (23)</td>
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<tr>
<td>Cynomolgus</td>
<td>MACS CD4^+ CD25^+ (&gt;90%)</td>
<td>6.4% of T cells</td>
<td>Fresh cells used; no expansion</td>
<td>n/a</td>
<td>Proliferation to anti-CD3/CD28 or CD3/CD46 stimulation is impaired during active MS (24)</td>
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<td>Baboon</td>
<td>MACS→FACS sorting (&gt;95%) CD4^+CD25^a</td>
<td>1.7% (spleen), 3.1% (LN) 1.9% blood T cells; 10 x 10^4 cells/ml blood</td>
<td>Pig PBMC + IL-2; 3-4 wks + 7-10d without PBMC</td>
<td>200-2000-fold, depending on IL-2 concentration</td>
<td>1:1^a, strong xenogeneic suppression, donor-specific; 4-10x less efficient to third-party (55)</td>
<td></td>
</tr>
</tbody>
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^a Treg:effector cell ratio; BMDC, bone-marrow derived dendritic cells; LN, lymph node; Mo-DC, monocyte-derived dendritic cells; MS, multiple sclerosis; wk, week

FACS, fluorescein-activated cell sorting; MACS, magnetic-activated cell sorting; Mφ, macrophage.
Non-human primate regulatory T cells have been expanded ex vivo using anti-CD3/CD28 monoclonal antibodies (Abs) and IL-2, and shown to potently inhibit effector T cell proliferative responses in a non-specific manner (29). Our recently-published data (33) are the first to show that a highly-suppressive population of alloAg-specific rhesus macaque Treg can be generated in vitro from circulating CD4⁺CD127⁻/lo T cells (6±1% of bulk CD4⁺ T cells) in response to immature allogeneic DC and stimulation with IL-2 and IL-15 (33).

Since the frequency of nTreg is comparatively low, de novo generation of Treg from conventional T cells is an appealing strategy to obtain large numbers of cells. In vitro activation of human CD4⁺CD25⁻ T cells via T cell receptor stimulation in the presence of transforming growth factor (TGF)β1 upregulates Foxp3, conferring suppressive capacity (34-36). As with genetic manipulation of conventional T cells to express Foxp3, the reproducibility of Treg induction (iTreg) using this approach has been questioned, as has the stability of their suppressive capacity (37-39). Despite these concerns, considerable promise has now been shown by the ability of all-trans-retinoic acid (RA) to synergize with TGFβ during T cell stimulation and induce highly suppressive murine or human Foxp3⁺ T cells (40-43). These iTreg suppress immune-mediated disorders in mice (40-42), and potentially represent a valid alternative to expanded nTreg. However, clinical testing is not foreseeable due to safety concerns, and NHP models represent the best tool to establish the feasibility of this approach.

We have characterized and sorted nTreg from cynomolgus monkey peripheral blood and ascertained whether iTreg could be generated from conventional T cells in this species. We tested in vitro conditions for expansion of nTreg and iTreg while, at the same time, preserving their suppressive capacity. Flow-sorted CD4⁺CD25⁺CD127⁺ T cells were stimulated with anti-CD3/CD28-coated beads and IL-2. Additionally, TGF-β1 was tested for its ability to preserve the expression of Foxp3 and nTreg suppressive ability. As shown in **Fig 1B**, both conditions caused significant proliferation of the cells, with up to 700-fold expansion over a 15-day period. In our amplification system, when nTreg were stimulated with IL-2 only, high Foxp3 expression was lost (**Fig 1C**). Addition of TGFβ preserved the appropriate level of this transcription factor necessary for Treg suppressive activity. Expanded nTreg were tested for their ability to suppress proliferation of autologous responder T cells stimulated by allogeneic PBMC. As expected from the absence of Foxp3 expression, nTreg expanded without TGFβ did not exhibit significant suppressive capacity. By contrast, nTreg expanded with TGFβ were highly suppressive (**Fig 1D**).

Cynomolgus monkey CD4⁺CD25⁻CD127hi T cells were also flow-sorted and stimulated in vitro with anti-CD3/CD28-coated beads in the presence of IL-2 and TGFβ1, with or
without RA. Both conditions induced Foxp3 expression and favored significant expansion of the stimulated population (Fig 1B). Interestingly, as reported by Wang et al (43) for human T cells, only cynomolgus monkey T cells stimulated with TGFβ and RA demonstrated profound inhibition of autologous alloreactive PBMC (Fig 1E). These data indicate the feasibility of testing the therapeutic potential of this iTreg population and comparing their suppressive effect to that of expanded nTreg.

Several NHP studies have shown strong in vitro suppressive effects with Treg:responder cell ratios from 1:1 to 1:100, depending on type of expansion. Thus, Anderson et al (29) not only demonstrated impressive expansion of rhesus macaque Treg, but also enhanced suppressive activity of polyclonally-expanded Treg with ratios up to 1:100. Studies in rhesus or cynomolgus macaques using alloAg-specific stimulation indicate however, that cell numbers cannot be expanded significantly (from no increase, up to 25-fold) (23, 33). On the other hand, polyclonal as well as alloAg-specific stimulation, together with high levels of IL-2, followed by 7-10 days polyclonal stimulation, expanded baboon Treg 450-4000-fold after 3-4 weeks of culture (25, 28, 29). Most likely, large numbers of Treg will be required to achieve the desired suppressive effect in vivo. An alternative solution could be incorporation of a T-cell depleting strategy (e.g. alemtuzumab [anti-CD52] or anti-thymocyte globulin [ATG]) in NHP prior to Treg infusion, as fewer Treg would be needed for a favorable Treg:T cell ratio.

Recently, Treg migratory ability has gained increased attention as a correlate of their in vivo suppressive function (16, 44). Thus, recruitment of CD4+ Treg expressing TGFβ1 to the allograft interstitium has been implicated in metastable renal transplant tolerance in rhesus monkeys (45). Multiple studies, including those in transplant models, have indicated that expression of specific chemokine receptors is essential for Treg activity in vivo (46-48). There is evidence that maximal Treg protective activity is achieved when the cells migrate to both the transplant site and draining lymphoid tissue. Based on this paradigm, optimization of a procedure for rendering highly-suppressive NHP Treg should include testing for expression of a pattern of chemokine receptors (CCR2, CCR4, CCR5, CCR7) compatible with their migration to both the graft and lymphoid tissues. Recently, investigations of Treg in lymphoid tissue of simian immunodeficiency virus-infected monkeys have revealed associations between Treg numbers and expression of the ligands for CXCR3, CCR4 and CCR7 (49).

Donor-reactive memory T cells (Tmem) resistant to immunosuppressive agents undermine strategies for tolerance induction. Moreover, recent studies have shown that lymphoablative strategies (e.g. T cell depletion) may lead to preferential expansion and accumulation of Tmem (50), that derive from homeostatic proliferation of
naïve T cells (51). In mice, CD4⁺ and CD8⁺ allospecific Tmem are more resistant to Treg-mediated suppression than naïve T cells (52). However, these Treg still suppress Tmem proliferation when present in equivalent number or at 1:3 ratio (Treg:Tmem) (52). Human nTreg can suppress both naïve and memory T cell proliferation (53). While these reports suggest the potential of Treg to control rejection in sensitized patients, they also indicate the likely need for complementary treatments to control Tmem without affecting Treg activity. Thus it is noteworthy that the proliferation of rapamycin-resistant rhesus monkey Tmem is inhibited by combined use of bortezomib (a proteasomal inhibitor that blocks NFκβ nuclear translocation) and rapamycin, a regimen that preserves pre-existing Treg survival (54).

Although most NHP Treg studies have concerned the allogeneic response, a recent report by Porter et al (55) focused on baboon Treg in a xenogeneic (pig to baboon) setting. Treg were expanded in a donor-specific manner using irradiated pig PBMC. The expanded Treg not only were strongly suppressive at a ratio of 1:1, but donor-specific suppression was achieved. Further development of NHP Treg in the xenogeneic setting, which offers the consistent advantage of donor identification before transplantation, could justify preclinical studies in this field [reviewed in (56)].

Safe and reliable protocols for the selection/induction and expansion of Treg that could be tested clinically are at a very early stage of development. A recent report (57) describes the first two cases of CD4⁺CD25⁺CD127⁻ Treg (donor-derived) therapy in humans with acute or chronic graft-versus-host disease. These reports indicate that the required Treg number may depend on clinical symptoms and underlying disease, as well as CD4⁺ T cell numbers, rather than on host body weight. NHP models have the inherent advantage of closely resembling the human condition and allow for preclinical testing, although such studies are expensive and require specialized resources. An innovative potential approach to assess NHP Treg in vivo could be the use of ‘primatized’ mice, similar to ‘humanized’ mice (58), and engrafted with NHP lymphohematopoietic stem cells leading to a complete repertoire of NHP immune cells. Such a model could be beneficial for initial testing of NHP Treg, without the need for, and limitations of, the large animal model. Taken together, and with due consideration to as questions regarding efficacy and safety, NHP Treg hold considerable potential for preclinical testing in tolerance-promoting strategies.

**Abbreviations**

Ag - antigen  
DC - dendritic cells  
GVHD - graft-versus-host disease
IS - immunosuppressive therapy
NHP - non-human primate
PBMC - peripheral blood mononuclear cells
RA - retinoic acid
Teff - T effector cells
TGFβ - transforming growth factor β
Tmem - memory T cells
Treg - regulatory T cells
Tx - transplantation
REFERENCES


Differential depletion and recovery of lymphocyte subsets in lymph nodes of alemtuzumab-treated cynomolgus monkeys

Eefje M. Dons*, Dirk J. van der Windt*, Michael Nalesnik, Lien T. Lu, Mohamed Ezzelarab, Jan N.M. IJzermans, Fadi Lakkis, David K.C. Cooper, Angus W. Thomson

* EMD and DJvdW contributed equally

Submitted.
Ex vivo-expanded non-human primate regulatory T cells are resistant to alemtuzumab-mediated, complement-dependent cytotoxicity

Eefje M. Dons, Giorgio Raimondi, Hong Zhang, Jay K. Bhama, Lien T. Lu, Mohamed Ezzelarab, Jan N.M. IJzermans, David K.C. Cooper, Angus W. Thomson

Submitted.
Background
Alemtuzumab (Campath-1H) is a mAb directed against CD52 that is expressed on all lymphocytes, including CD4+ FoxP3+ regulatory T cells (Treg), and that depletes lymphocytes and other leukocytes, mainly by complement-dependent mechanisms.

Methods
We investigated the influence of alemtuzumab (i) on *ex vivo*-expanded Treg that could potentially be used in adoptive cell therapy of allograft rejection, and (ii) nTreg following infusion of alemtuzumab in Indonesian cynomolgus monkeys. (i) Treg were flow-sorted from PBMC and lymph nodes and expanded for two rounds using anti-CD2/CD28 beads, IL-2 and TGF-β. CD52 expression, binding of alemtuzumab, and killing of cells were compared between freshly-isolated and expanded Treg and effector T cells. (ii) Monkeys undergoing allogeneic heart transplantation received 3 doses of alemtuzumab and were monitored for Treg and serum alemtuzumab levels.

Results
(i) *Ex vivo*-expanded nTreg were characterized by CD25hi/CD127low and FoxP3+ expression and suppressive capacity by CFSE-MLR. Expanded Treg exhibited progressive downregulation of CD52 expression, absence of alemtuzumab binding, and no complement-dependent killing. (ii) *In vivo* infusion of alemtuzumab resulted in potent depletion of all lymphocytes, but a transient increase in the proportion of circulating Treg. After intravenous infusion of alemtuzumab, monkey serum killed fresh PBMC, but not expanded Treg.

Discussion
Expanded cynomolgues monkey nTreg are resistant to alemtuzumab-mediated, complement-dependent cytotoxicity. Our *in vitro* data suggest that expanded nTreg can be infused into graft recipients given alemtuzumab without complement-mediated killing. Furthermore, our *in vivo* data show that alemtuzumab infusion results in transient relative increase in circulating Treg.
INTRODUCTION

Alemtuzumab (Campath-1H) is a humanized IgG1 mAb directed against the CD52 molecule that is expressed by lymphocytes, natural killer (NK) cells, dendritic cells and monocytes (1). Its administration depletes these cells and it is consequently used to treat lymphoid malignancies (2) or to suppress transplant rejection (3). There is evidence that complement activation, as well as antibody-dependent, cell-mediated cytotoxicity (ADCC) are responsible for alemtuzumab-mediated killing of CD52+ cells (4, 5). In vitro study of human lymphocytes has shown that CD4+ lymphocytes are killed mostly by complement activation (5). By contrast, investigations in a humanized mouse model have revealed that lymphocyte depletion depends mainly on neutrophils and NK cells, and is largely independent of complement (4). The half-life of alemtuzumab in humans is approximately 8 days, but since low concentrations may be sufficient to cause cytotoxicity, depletion of CD52+ cells may persist for up to two months after its infusion (6).

Regulatory T cells (Treg), defined as CD4+CD25highForkhead box P3 (FoxP3)+T cells, can promote allograft tolerance in mouse and humanized normal models following their adoptive transfer (reviewed by Bluestone (7), Wood and Sakaguchi (8), and Wood (9)). This function of Treg has usually been demonstrated in lymphocyte-depleted graft recipients, although there is also evidence of their tolerogenic function in immunocompetent (10). A relative increase in circulating Treg has been demonstrated after alemtuzumab induction in renal transplant patients (11, 12), although this effect appears to be transient (13).

Recently, clinical trials have been initiated to determine the safety and efficacy of Treg infusion in humans (14). Naturally-occurring, circulating Treg display relatively high levels of CD52 compared with other lymphocyte subsets, and are susceptible to killing by alemtuzumab in vitro (5, 15). However, little is known about CD52 expression on Treg after their expansion, that is a pre-requisite for their therapeutic application. We posed the question whether, in the context of organ transplantation and efforts to promote immunological tolerance, expanded Treg are susceptible to alemtuzumab and whether circulating/residual alemtuzumab might destroy administered Treg, thus negating their in vivo function.

We investigated this question in a non-human primate (NHP) model using cynomolgus macaques of Indonesian origin. Unlike humans, most NHP species express CD52 on both white and red blood cells. The Indonesian sub-species of cynomolgus monkey, however, does not express CD52 on its erythrocytes, and can be given alemtuzumab
safely (16). Naturally-occurring macaque Treg can be flow-sorted and expanded using a protocol very similar to that used to expand human Treg with similar suppressive function (17). Moreover, cynomolgus monkey CD52 shares 85% of its primary structure with its human counterpart, including a glycosylation site and the sequence that is the most likely anchor attachment site (18, 19). This homology between human and cynomolgus monkey CD52 renders the latter species an appropriate model in which to explore the influence of alemtuzumab on Treg in a pre-clinical setting.

Our study focused on the influence of alemtuzumab on ex vivo-expanded monkey Treg, as well as on naturally-occurring Treg in vivo. We found that, similarly to humans, alemtuzumab infusion causes profound depletion of all lymphocytes, with a transient increase in the proportion of circulating Treg among the remaining cells. More importantly, we show that expanded monkey Treg downregulate CD52 expression, and are not susceptible to alemtuzumab-mediated complement-dependent cytotoxicity.

**MATERIALS AND METHODS**

**Cynomolgus monkeys**

Healthy cynomolgus monkeys (*Macaca fascicularis*) of Indonesian origin, weighing 3-5kg, were obtained from SPF colonies at Alpha Genesis, Inc, or the NIAID NHP colony (both Yamassee, SC). Some monkeys (n=3) received a heterotopic heart transplant from an ABO-compatible allogeneic donor on day (d)0. On d-2, 5 and 12, the recipient received an intravenous (i.v.) infusion of alemtuzumab (Campath-1H; Genzyme, Cambridge, MA) at doses of 20, 10, and 10mg/kg, respectively. The maintenance immunosuppressive regimen consisted of mycophenolate mofetil (MMF; Genentech USA, Inc, South San Francisco, CA) from d-1 to 18, aiming for serum trough levels of 3-6µg/ml, followed by rapamycin (LC Laboratories, Woburn, MA) monotherapy from d19-54, aiming for serum trough levels of 10-15ng/ml after which it was slowly weaned over 4 weeks and completely discontinued on d84. The results of this in vivo study will be reported separately.

All animal procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985), and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.
Sources of cells and sera
Normal, untreated monkeys were used as blood donors for in vitro experiments. Peripheral blood mononuclear cells (PBMC) were used either immediately upon isolation (fresh PBMC; n=3 experiments) or after storage in liquid N₂ for a maximum period of 1 year (stored PBMC; n=3 experiments). Serum was obtained before alemtuzumab administration (pre-alem) and at multiple time-points thereafter. Blood was drawn weekly to monitor circulating lymphocyte subsets, including Treg (CD4⁺CD25⁺CD127⁻; confirmed by FoxP3⁺) at several time-points) by flow cytometric analysis. Lymph nodes (LN) were harvested from healthy monkeys (used as transplant donors) and cells isolated and stored in liquid N₂ (n=4 experiments).

Treg and Teff cell isolation
Cryopreserved PBMC and LN cells were thawed or PBMC were isolated from fresh blood on day 0 of each experiment (Treg isolation) for 2 rounds of Treg and effector T cell (Teff) expansion. Cells from the same source were also thawed or isolated on day 20, when they served as unexpanded controls, as well as responder cells in carboxyfluorescein diacetate succinimidyl ester (CFSE)-MLR. Naturally-occurring Treg were isolated from PBMC or LN cells by cell sorting (BD Aria, BD Biosciences, San Jose, CA) based on CD4⁺CD25⁺CD127⁻ expression, as described (20). Simultaneously, Teff were sorted based on CD4⁺CD25⁻ expression and served as controls for expanded Treg. To confirm that these cells were Treg or Teff, FoxP3 expression was determined in separate samples.

Treg expansion
The protocol used for Treg and Teff expansion is shown in Figure 1A. Briefly, Treg and Teff were cultured in AIM-V medium, supplemented with 10% heat-inactivated human AB serum and antibiotics/antimycotics (both from Gibco, Carlsbad, CA). Treg were expanded by using NHP-specific anti-CD2/3/28 microbeads (Miltenyi, Biotec, Bergisch Gladbach, Germany) at a cell:bead ratio of 1:2, with high-dose recombinant human (rhu)IL-2 (1000U/ml) and hu transforming growth factor β (TGF-β; 5ng/ml). Teff were expanded using the same microbeads at a cell:bead ratio of 2:1, with IL-2 (500U/ml), but without TGF-β. Medium was refreshed on day 5 and 7. Cells were collected, counted and replated on day 9 (round 1 of expansion), at which time the cells were stained for cell surface CD52 and intracellular FoxP3. Medium was refreshed again on day 14 and 16, and the cells were harvested and counted on day 19 (round 2). At this time, the expanded cells were tested for killing by alemtuzumab. When sufficient cells were obtained, they were tested for suppressive function in CFSE-MLR. A fraction of the expanded cells was maintained in culture for an additional day, analyzed in the binding assay and stained for CD52 and FoxP3 on day 20.
Expression of cell surface markers and intracellular staining

After each round of expansion, a small aliquot of Treg and Teff was collected, washed, and stained for CD3, CD52 and FoxP3 expression. Intracellular staining for FoxP3 was performed using the protocol provided by eBiosciences (San Diego, CA). In the same experiment, a sample of unstimulated cells (freshly-thawed cells or freshly-isolated PBMC) was stained for CD3, CD4, CD25, CD52 and FoxP3, and CD52 and
FoxP3 expression determined for the total unstimulated T cells, as well as for Treg (CD3+CD4+CD25hi) and Teff (CD3+CD4+CD25). The level of CD52 and FoxP3 was expressed as mean fluorescence intensity (MFI) for each cell populations.

**Treg suppressive function: CFSE-MLR**

After round 2 of expansion, unstimulated responder cells were obtained from the same source as do of expansion (i.e., fresh blood, frozen PBMC or frozen LN cells). Pan T cells were isolated using positive labeling for CD2 and magnetic bead separation. CD2+T cells were counted and stained with CFSE. Responder CD2+CFSE+ cells were stimulated with NHP-specific anti-CD2/3/28 beads (Miltenyi Biotec) at a cell:bead ratio of 10:1. Expanded cells were enumerated and stained with Violet Trace (to distinguish them from CD4+CFSE-proliferating responder cells) and added to the responder cells in responder:T cell ratios of 1:2, 1:4, 1:8, 1:16 and 1:32. CFSE-MLR were harvested on d5. Washed cells were stained additionally for CD3, CD4 and CD8 (BD Biosciences). Proliferation was determined as the percentage of CFSE cells within the CD3+CD4+ and CD3+CD8+ cell populations. Proliferation index was calculated by setting the baseline (responder cells + beads only) as 100%. The suppressive function of added Treg was calculated as the percentage of proliferating cells related to this baseline.

**Binding of alemtuzumab to target cells**

Expanded T cells and freshly-isolated or freshly-thawed PBMC or LN cells from the same source were counted, and 0.2×10^6 cells in 90µl PBS added per tube. Alemtuzumab was titrated to concentrations of 1000 to 0.01µg/ml in PBS, and 10µl was added to each tube (making final concentrations of 100-0.001µg/ml). The cells were incubated with alemtuzumab for 30min at 4°C, washed, then blocked with normal goat serum (1:10 final concentration; 20min, 4°C) to prevent non-specific binding. Cells were washed again and stained with fluorescein isothiocyanate (FITC)-goat anti-human IgG-γ (Invitrogen, Carlsbad, CA) and PerCP-Cy5.5 anti-CD3 (BD PharMingen, San Diego, CA). Unstimulated cells were stained additionally for APC-H7 anti-CD4 (BD PharMingen) and PE-Cy7 anti-CD25 (eBiosciences) to enable analysis of binding to Treg and Teff cells within the total cell population. Before running the samples, the cells were stained with DAPI to assess viability, and CountBright beads (Invitrogen) added to determine exact cell numbers remaining in the tube. Analysis of binding was based on the MFI of FITC+ cells within live (DAPI) CD3+ cells, setting the gate based on cells incubated with PBS alone. Relative MFI was calculated as [sample MFI] / [baseline MFI], where baseline MFI is the MFI in the “no alemtuzumab” sample.
Killing of target cells by alemtuzumab

Expanded Treg and Teff, as well as freshly-isolated T cells (NHP CD2+ microbeads, Miltenyi) from the same source were counted, and $0.1 \times 10^6$ cells in 90µl RPMI-1640 added per tube. Alemtuzumab was titrated to concentrations of 1000-0.1µg/ml in autologous serum, and 10µl was added to each tube, making the final concentration 100-0.01µg/ml. Cells were incubated with alemtuzumab for 30 min (at 37ºC), washed, and stained for Annexin-V to detect early apoptosis, following the manufacturer’s protocol (BD Biosciences). Before analyzing the samples, cells were stained additionally with 7-AAD to detect damaged or apoptotic cells. To determine the remaining cell numbers in each tube, CountBright beads (Invitrogen) were added before running the sample. Determination of apoptosis, cell damage, and cell killing for each alemtuzumab concentration was based on the percentage of Annexin-V+, Annexin-V+ 7-AAD+ double-positive cells, and the percentage of CountBright beads within the cell population. In addition, normal cynomolgus monkey serum was compared with heat-inactivated serum (HI serum), RPMI-1640 (no serum), and RPMI-1640 + rabbit complement (10µg/ml), with or without high concentration alemtuzumab (30µg/ml).

Statistical analysis

Differences between tested cell types for expression of FoxP3 and CD52, binding of alemtuzumab (relative MFI) and suppression of proliferation (CFSE-MLR) were evaluated using Student’s paired ‘t’-test. Statistical analyses were conducted using the standard formula in Microsoft Excel software.

RESULTS

Treg isolation and expansion from cynomolgus monkey blood and LN

The incidence of circulating Treg (CD4+CD25hiCD127-) in healthy, untreated cynomolgus monkeys was similar to that in healthy humans (i.e. 5% of total CD4+ T cells), but was higher (~10%) in LN. The size of the monkeys (3-5 kg) allowed for a maximum blood draw of 30-50ml per month. From this volume, 0.57×10^6 Treg could be flow-sorted (n=10 experiments), representing 0.53% of total cells from fresh PBMC (Table 1). A large difference was noted in the number of Treg that could be obtained from the different cell sources. Cryopreserved and fresh PBMC (both n=3 experiments) yielded 0.23% and 0.13% Treg, respectively, from total isolated cells, whereas LN cells (n=4 experiments) yielded 1.05% Treg from total isolated cells (Table 1). Although LN cells yielded higher absolute numbers of Treg, those isolated from fresh blood could be expanded at a much higher rate than Treg isolated from either cryopreserved PBMC
or LN cells, with a mean 80-fold expansion after two rounds versus 24- and 22-fold expansion for stored PBMC and LN cells, respectively (Figure 1B).

FoxP3 expression was determined at the start of culture and after each round of expansion. Teff (CD4+CD25-) from the same source were expanded simultaneously and served as controls. Although not statistically significant, fresh Treg exhibited higher intracellular FoxP3 expression (MFI) than fresh Teff (p=0.060). FoxP3 expression increased in both populations, especially Treg, upon expansion, and was statistically significant for Treg (p=0.048). Whereas Treg comparatively exhibited high FoxP3 levels throughout culture, FoxP3 intensity in Teff diminished after the first round of expansion (Figure 1C).

To verify their suppressive function, when sufficient cells were recovered at the end of the expansion period (d 20), CFSE-MLR were set up to determine their ability to inhibit CD2+ T cell proliferation. Expanded Treg were strongly suppressive at Treg:Tcell ratios up to 1:4, whereas expanded Teff were comparatively ineffective (Figure 1D). These data on FoxP3 and expression suppressive function confirmed that the expanded monkey Treg retained their phenotypic and functional identity.

Expression of CD52
We next determined CD52 expression (MFI) on fresh total T cells (isolated from either PBMC or LN), as well as on fresh and expanded Treg and Teff. CD52 expression on fresh Treg was higher than on fresh Teff (p=0.095) or on the fresh bulk T cell population before expansion. During expansion, CD52 expression decreased progressively on both Treg and Teff. Downmodulation of CD52 was greatest (~60%) on expanded Treg (p=0.041) (Figure 2A).

### Table 1: Recovery of Treg from various sources by cell sorting

<table>
<thead>
<tr>
<th>Treg source</th>
<th>Absolute cell # (x10⁶)</th>
<th>Percentage of total cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n=10)</td>
<td>0.57±0.66</td>
<td>0.53±0.58</td>
</tr>
<tr>
<td>Fresh PBMC (n=3)</td>
<td>0.12±0.06</td>
<td>0.13±0.05</td>
</tr>
<tr>
<td>Cryopreserved PBMC (n=3)</td>
<td>0.19±0.14</td>
<td>0.23±0.11</td>
</tr>
<tr>
<td>Cryopreserved LN cells (n=4)</td>
<td>1.20±0.66</td>
<td>1.05±0.62</td>
</tr>
</tbody>
</table>

Absolute numbers of cynomolgus monkey Treg (CD4+CD25+CD127+), as well as Treg percentages obtained by flow sorting from a single blood draw (cryopreserved or fresh PBMC) were generally low (0.13-0.25% of total cells) compared to those obtained from LN (Treg: 1% of total cells). Values are absolute cell numbers (x10⁶) or percentage of total cells (%) ±1SD (n=10 experiments).
Binding of alemtuzumab to fresh and expanded T cells

To ascertain the relationship between cell surface expression of CD52 and binding of alemtuzumab, cells were incubated with various concentrations of alemtuzumab, and stained with secondary antibody. All freshly-isolated cells (total lymphocytes from PBMC or LN, fresh Treg and fresh Teff) bound alemtuzumab, with a peak at 10µg/ml. Consistent with their higher CD52 expression, fresh Treg bound alemtuzumab slightly more strongly than fresh Teff, although this difference was not statistically significant. Importantly, alemtuzumab did not bind to expanded cells (either Treg or Teff) at any mAb concentration tested (Figure 2B). The difference in binding between expanded

Figure 2: Expanded Treg significantly down-regulate CD52 expression and are not bound by alemtuzumab.

(A) T cells from peripheral blood or LN were stained for CD3, CD4, CD25 and CD52 (n=3). CD52 MFI was analyzed for bulk T cells, fresh (unexpanded) Treg (gated for CD4+ CD25hiCD127-) and fresh (unexpanded) Teff (gated for CD4+CD25). In the same experiment, Treg and Teff cells were analyzed for CD52 after round 1 and 2 of expansion (all conditions n=3). Unstimulated Treg (within the PBMC or LN cell population) expressed a higher level of CD52 than Teff from the same cell source. Expanded cells showed a decrease in CD52 expression after the first round of expansion, which was similar for Treg and Teff, with a further decrease after the second round of expansion (n=3 for all conditions). *p<0.05.

(B) Alemtuzumab does not bind to expanded Treg. Cells were incubated with alemtuzumab at concentrations of 0.001-100 µg/ml to determine binding of alemtuzumab. Binding was expressed as relative MFI. Alemtuzumab exhibited concentration-dependent binding to freshly-isolated PBMC, as well as to freshly-isolated Treg and Teff (n=3). By contrast, alemtuzumab showed no binding to expanded Treg (n=3) and expanded Teff (n=3) at any concentration. *p<0.05; significance calculated between fresh T cells (n=3) and expanded Treg (n=3).

(see color figure on page: 236)
and fresh cells was significant at alemtuzumab concentrations of 100 (p=0.044) and 10µg/ml (p=0.014). This finding correlates with the markedly reduced CD52 expression on expanded cells.

**Cytotoxicity of alemtuzumab**

After observing that expanded monkey Treg had markedly reduced CD52 expression and were not bound by alemtuzumab, we quantified the cytotoxic activity of alemtuzumab against these cells. Fresh T cells (from PBMC) and expanded Treg and Teff were incubated with alemtuzumab at different concentrations, and then analyzed for apoptosis using a combination of Annexin-V and 7-AAD staining, as well as by total cell counts.

![Graphs showing cytotoxicity of alemtuzumab](image)

**Figure 3: Alemtuzumab does not kill expanded Treg.**

(A) Killing of cells was tested using normal monkey serum, heat-inactivated serum (HI serum), RPMI-1640 (no serum), or RPMI-1640 + complement, each with or without 30µg/ml alemtuzumab. Killing is shown as the percentage of cells that became 7-AAD-positive. Normal serum and RPMI-1640+complement were associated with increased killing of cells incubated with alemtuzumab. Heat-inactivated serum and RPMI-1640 were not associated with increased killing. (B, C, D) Cells were incubated with alemtuzumab at the concentrations shown to determine apoptosis and killing of cells. Fresh T cells (n=4) showed increasing 7-AAD (B) and Annexin-V (C) staining as the concentration of alemtuzumab increased. (D) A higher proportion of beads was counted in tubes with higher concentrations of alemtuzumab, indicating that fewer intact cells remained after incubation with high concentration alemtuzumab. In contrast, expanded Treg showed a relatively low baseline level of Annexin-V and 7-AAD staining, which remained unchanged irrespective of the concentration of alemtuzumab. Expanded Teff had a higher baseline level of Annexin-V and 7-AAD staining, but did not show any increase when alemtuzumab was added.
via the CountBright bead percentage (see Materials and Methods). Cytotoxicity was first measured using fresh normal cynomolgus monkey serum (containing complement), heat-inactivated (HI) monkey serum, RPMI-1640 (no serum), and RPMI-1640 + complement (Figure 3A). When cells were incubated with high concentration alemtuzumab (30μg/ml) in either normal serum (containing complement) or RPMI-1640 supplemented with rabbit complement, killing was markedly enhanced compared to the absence of alemtuzumab. There was no significant difference between normal serum and RPMI-1640+complement. However, when cells were incubated with either heat-inactivated serum or RPMI-1640, there was no difference in killing between 0 and 30 μg/ml alemtuzumab. Consequently, we used autologous monkey serum obtained when the cells were isolated (d0, Figure 1A) for all subsequent experiments.

Alemtuzumab was diluted to final concentrations ranging from 100 to 0.01μg/ml to generate a titration curve. Freshly-isolated bulk T cells were killed in a dose-dependent manner (determined by higher Annexin-V, 7-AAD, and CountBright bead percentage) (Figure 3B-D). Killing of fresh T cells was evident at or above mAb concentration of 10μg/ml, correlating with the maximal binding of the mAb to these cells (Figure 2B & C). No killing was detected at <1μg/ml, where values returned to baseline (no alemtuzumab) level. Expanded Treg exhibited a lower baseline level of apoptosis than expanded Teff in each experiment, but both expanded Treg and Teff displayed no alemtuzumab-induced killing. Annexin-V and 7-AAD staining, and the percentage of CountBright beads each remained at baseline levels at each concentration of alemtuzumab tested (Figure 3B-D). These data indicate that, even at high, in vitro concentration, alemtuzumab does not kill expanded cells through a complement-dependent mechanism.

Quantitation of alemtuzumab activity in monkey serum after intravenous infusion

Alemtuzumab has a half-life in humans of ~8d. Serum samples were obtained from 2 heart-transplanted monkeys pre-alemtuzumab and at several times after mAb infusion. The sera were tested for alemtuzumab-induced killing of fresh normal human T cells (as control target cells) as well as of monkey autologous expanded Treg. Similar to the in vitro studies, in which alemtuzumab was added to serum, when fresh T cells were incubated with serum (drawn immediately after infusion of alemtuzumab), killing of the fresh T cells was clearly evident (Figure 4). In contrast, when incubated with serum drawn 7d after infusion, Annexin-V and 7-AAD staining levels had returned to baseline (pre-alemtuzumab) (Figure 4), indicating that any alemtuzumab remaining in the serum 7 days after infusion did not contribute to killing of the cells.
These observations were confirmed by adding alemtuzumab (10ng/ml) to the pre-alemtuzumab serum sample, which resulted in a similar increase in Annexin-V and 7-AAD staining (Figure 4). Similarly to the in vitro experiments, monkey autologous expanded Treg showed no evidence of killing when incubated with either serum taken immediately after alemtuzumab infusion or serum supplemented with 10ng/ml alemtuzumab (Figure 4).

**Figure 4: Alemtuzumab-containing serum, taken immediately after mAb infusion, does not kill expanded Treg.** Monkey serum was drawn pre-alemtuzumab, immediately after a second dose (10mg/kg), immediately before and after a third dose (10mg/kg), and 1 week after the third dose. These sera were incubated with freshly-isolated monkey T cells and expanded autologous Treg. The percentages of Annexin-V and 7-AAD positive cells are shown. Arrows show time-points when alemtuzumab was infused. Fresh T cells showed an increase in apoptosis and cell killing in response to high concentrations of alemtuzumab in the blood (d5 post-alemtuzumab, d12 post-alemtuzumab), as well as when exposed to pre-alemtuzumab serum to which 10ng/ml of alemtuzumab had been added (far right). Values returned to baseline levels of apoptosis and killing when fresh T cells were incubated with serum obtained 1 week after alemtuzumab infusion (d12, d20), indicating that serum contained high concentrations of alemtuzumab early after infusion. Expanded autologous Treg showed no increase in apoptosis or killing when exposed to a high concentration of alemtuzumab in serum, whether the serum was drawn from an alemtuzumab-treated monkey, or whether alemtuzumab had been added to the serum in vitro (far right).

**Alemtuzumab infusion: relative sparing of Treg**

Since alemtuzumab appeared minimally cytotoxic to Treg after their expansion, the influence of alemtuzumab infusion on naturally-occurring Treg remaining in the circulation was investigated. Cynomolgus monkeys (n=3) were given an allogeneic heterotopic heart transplant on day under an immunosuppressive regimen of alemtuzumab weekly (x3) (Figure 5A), plus maintenance MMF for 18d, followed by rapamycin monotherapy until d84.
Pan T cell numbers were depleted >97% after the first dose of alemtuzumab and remained at this low level following subsequent doses. Recovery of total CD4+T cells began by week 7 (increase to >10% of pre-alemtuzumab numbers) (Figure 5A). However, when analyzing Treg within this small population of residual CD4+T cells, all 3 monkeys maintained relatively high numbers of circulating Treg, as measured by the percentage of CD25hiCD127- cells within the CD4+ population (Figure 5B). In view of the overall very low number of circulating lymphocytes, we were able to perform intracellular FoxP3 staining only at bi-weekly intervals. FoxP3+ staining and analysis confirmed that the CD25hiCD127- cells were indeed FoxP3+ Treg (Figure 5C). The increase in the proportion of circulating Treg was transient, as it was evident only from weeks 2-6, after which Treg numbers returned to pre-alemtuzumab baseline levels (Figure 5B).

**Figure 5**: Relative increase in circulating Treg after infusion of alemtuzumab.

(A) Monkeys that had received a heart transplant on day 0 and had received 3 doses of alemtuzumab on days -2, 5, and 12 (n=3 total; n=2 for each time-point) showed a profound depletion of total lymphocytes and, in particular, CD4+ T cells. (B) Within the remaining small circulating CD4+ T cell population, the percentage of CD25hiCD127 Treg increased significantly after alemtuzumab infusion in all 3 monkeys (up to 35% in one case) and returned to baseline values (~5%) by week 7 post-transplant. (C) Intracellular staining for FoxP3 (black line, left vertical axis) pre- and 2, 4, and 6 weeks after alemtuzumab infusion shows a transient increase in FoxP3+ cells within the CD4+ cell population. The MFI for FoxP3 (dark grey bars; right vertical axis) in CD25hiCD127 Treg is markedly higher than that in the CD25 Teff subpopulation (light grey bars) at each time point. Representative values for one monkey are shown.
DISCUSSION

Here we examined whether *ex vivo*-expanded NHP Treg are resistant to alemtuzumab (anti-CD52)-mediated killing and whether it might be feasible to administer these cells to transplant recipients without their being killed by circulating levels of mAb at the time of Treg infusion. We used a NHP model, as Treg in NHP are phenotypically and functionally similar to those in humans (reviewed by Dons (21)) and Indonesian cynomolagus macaques specifically, since CD52 is not expressed on the erythrocytes of this species.

A significant finding was that expanded monkey Treg exhibited considerably lower expression of CD52 than either fresh bulk T cells or freshly isolated Treg. Alemtuzumab showed strong binding to freshly-isolated pan T cells and Treg, with maximum activity at a concentration of 10µg/ml. Therapeutic levels in humans have been reported peak at 13.7µg/ml (range 7.5-16.6µg/ml) after an initial dose of 20mg (6). We could not identify a laboratory to measure alemtuzumab in serum that would allow us determine concentrations of the mAb after infusion. However, the killing assay using monkey serum taken immediately and 7d after mAb infusion suggests that most cytotoxic activity of circulating alemtuzumab had disappeared after 7d. Importantly, alemtuzumab did not bind to expanded Treg at concentrations up to 100µg/ml, and whereas T cells freshly-isolated from blood were susceptible to concentration-dependent killing by alemtuzumab, expanded Treg were not killed at any concentration tested.

These findings suggest that, if Treg can be isolated and expanded from a graft recipient before alemtuzumab administration, then the expanded cells could be administered to the lymphocyte-depleted host at any time after transplantation in the presence of circulating alemtuzumab, without being killed.

We verified complement-dependent killing by alemtuzumab. No killing was observed (compared to baseline) when monkey serum was heat-inactivated, but cytotoxicity was restored when complement was added. Our findings indicate a major complement-dependent component of T cell killing by alemtuzumab. This contrasts with the observed by Hu et al (4), in which it was reported that, using a transgenic mouse expressing human CD52, killing of circulating lymphocytes was largely independent of complement. The principal mechanism identified was antibody-dependent cell-mediated cytotoxicity (ADCC), mediated by neutrophils and NK cells. In our cytotoxicity assay, which was performed on isolated T cells in the absence of neutrophils and NK cells, complement contributed significantly to the death of fresh, but not expanded, T cells. Indeed, Lowenstein et al (5) reported predominantly complement-mediated
killing of human CD4⁺ T cells, which was related to expression of CD52, although only unexpanded cell populations were studied. As we did not assess an ADCC component of alemtuzumab-mediated cytotoxicity the present study, its possible role in killing of expanded Treg cannot be discounted, and should be examined in future investigations.

Analyses of the influence of alemtuzumab on human Treg in vivo have demonstrated a transient increase in circulating CD4⁺CD25⁺FoxP3⁺ Treg after kidney transplantation (11). The mechanism responsible for the initial increase in Treg is not understood, but de novo generation was suggested and could be explained by the induction of Treg in vivo (22). In vitro studies of Treg proliferation have shown that Treg expand preferentially in allogeneic mixed leukocyte reactions (MLR) in the presence of alemtuzumab (23). However, in a similar study, incubation of human PBMC with anti-thymocyte globulin was associated with expansion of Treg whereas, by contrast, alemtuzumab depleted CD25⁺ Treg (15).

A different explanation for the increased proportion of Treg found in the circulation after alemtuzumab administration may be that lymphocyte depletion occurs mainly in the blood and is least in the bone marrow (4). Human bone marrow, where the cells are relatively spared from alemtuzumab-mediated killing (24), has a much higher proportion of Treg than blood, LN, and thymus. This could explain the relatively rapid repopulation of Treg in the blood. Interestingly, when alemtuzumab was added to ex vivo cultures of umbilical blood stem cells, a significant increase in stem cells was observed (25).

Generation or preferential expansion of Treg after alemtuzumab administration appears to be transient in vivo. Bloom et al (11) reported peak Treg numbers (as a percentage of total CD4⁺T cells) 6 months after human renal transplantation, with a subsequent decline, although the percentage remained elevated for 36 months post-transplantation. Long-term follow-up of kidney transplant recipients has indicated that alemtuzumab-based immunosuppression decreases the proportion of CD4⁺Treg when measured several years after transplantation, which correlates with increased anti-donor reactivity in these patients (13). The present results in cynomolgus monkeys indicates a much shorter period of increased percentage of Treg of only 2-7 weeks. This difference may be related to different binding affinity of alemtuzumab in monkeys compared with humans (16).

As alemtuzumab (a humanized mAb) has a comparatively long half-life in humans and cynomolgus monkeys of approximately 8d, and a much longer lymphocyte-depleting effect in the former of about 2 months (6), our finding that expanded Treg are not
affected by complement-dependent killing by alemtuzumab has potentially important clinical relevance.

In summary, we have demonstrated that, following ex vivo expansion, Treg down-regulate CD52 expression and are not susceptible to complement-mediated killing by alemtuzumab. This suggests that expanded Treg can be infused into a transplant recipient at any after alemtuzumab-mediated lymphocyte depletion. In cynomolgus monkeys, a relatively transient increase in the percentage of Treg occurs in vivo, starting about two weeks after the initial alemtuzumab dose, and persists for approximately 4-5 further weeks.

ACKNOWLEDGEMENTS

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Abbreviations
CFSE - carboxyfluorescein diacetate succinimidyl ester;
FoxP3 - Forkhead box P3;
LN - lymph node(s);
MFI - mean fluorescence intensity;
MMF - mycophenolate mofetil;
NHP - non-human primate;
PBMC - peripheral blood mononuclear cells;
Teff - effector T cells;
Treg - regulatory T cells.
REFERENCES

Expanded regulatory T cells resist alemtuzumab killing


Induced regulatory T cells: mechanisms of conversion and suppressive potential

Eefje M. Dons, Giorgio Raimondi, David K.C. Cooper, Angus W. Thomson

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Thymus-derived, naturally-occurring CD4+ CD25+ FoxP3+ regulatory T cells (nTreg) have suppressive activity that is important for the establishment and maintenance of immune homeostasis in the healthy state. Abundant reports have shown that they can suppress pathogenic processes in autoimmune diseases and inhibit transplant rejection and graft-versus-host disease. Far less is known about induced regulatory T cells (iTreg) that are generated from naïve T cells in the periphery or in vitro, by directing naïve T cells to acquire suppressive function under the influence of transforming growth factor-β (TGF-β) and other factors. In this review, we describe mechanisms by which naïve T cells are thought to be converted into iTreg. We also discuss the suppressive potential of iTreg, particularly in comparison to their naturally-occurring counterparts, focusing on those reports in which direct comparisons have been made. Based on current knowledge, we consider the rationale for using iTreg versus nTreg in clinical trials.
INTRODUCTION

Numerous studies have demonstrated the importance of regulatory T cells in maintaining tolerance to auto- and allo-antigens (Ags). Naturally-occurring Treg (nTreg), that are generated in the thymus, were first described by Sakaguchi et al in 1995 [1], and their suppressive function was later associated with expression of the winged helix transcription factor Forkhead Box P3 (FoxP3) [2]. Additionally, nTreg can be identified by expression of the cell-surface markers CD4+ and CD25hi, often in combination with CD127 [3] or CD45RA+ [4]. Adoptive transfer of nTreg has been successful in animal models of autoimmune diseases and transplantation (Tx) [5, 6]. However, as the frequency of nTreg in healthy humans is only ~5% of CD4+ T cells, it is difficult to obtain sufficient numbers of these cells for clinical testing [4, 7]. Many groups have reported the marked expansion of nTreg from a CD4+ CD25hi (in combination with CD127 or CD45RA+) starting population by Tcell receptor (TCR) stimulation in the presence of exogenous IL-2 [8, 9]. However, during prolonged expansion for 3-4 weeks, T effector cells (Teff) outgrow nTreg, resulting in reduction of the suppressive capacity of the cultured cells [3, 4]. A shorter expansion period would avoid this difficulty, but the limited nTreg number that can be generated under these conditions would constitute a problem when Treg are to be used in a clinical setting.

To address this issue, various groups have sought to generate Treg from a naïve T cell starting population. Beginning in 2002, the first reports were published on adaptive or induced Treg (iTreg), that are also CD4+CD25+ and FoxP3+, and have suppressive capacity [10-12]. These iTreg are generated in vivo from naïve T cells in peripheral lymphoid organs during immunological responses to Ag stimulation, a phenomenon that is replicated in vitro through stimulation of mouse or human T cells under specific conditions. Various groups have observed that iTreg can be generated easily by conversion of much more abundant naïve CD4+ T cells in vitro, allowing much higher numbers of Treg to be obtained [10-12]. The percentage of iTreg in the total population of circulating Treg in healthy mice and humans is thought to be in the range <3 to 30% [13, 14]. Here, we review current knowledge on the generation of iTreg and discuss their suppressive function in comparison to nTreg.

THE INDUCTION OF TREG FROM NAÏVE T CELLS

In vivo pathways of iTreg generation

Several pathways through which Treg can be induced in the periphery in vivo have been described. In general, all Ag-presenting cells (APC) have the potential to induce Treg.
Thus, dendritic cells (DC) in the gut-associated lymphoid tissue (GALT) can promote iTreg conversion [15-18]. However, B cells [19]; mesenchymal stem cells [20, 21] and myeloid-derived suppressor cells [22] are also able to promote conversion.

The presentation of self- or foreign Ag is important for conversion to the Treg phenotype. In vivo generation not only occurs as a homeostatic phenomenon, but also during allo immune responses. iTreg reactive to foreign Ag are generated in response to microbes and food Ags in the intestinal mucosa [17], during the induction of tolerance to chicken ovalbumin (OVA) in the mesenteric lymph nodes [23], and by OVA-presenting DC [24]. They are also found in response to self Ag in chronically inflamed tissues [25], in response to self Ag in a mouse autoimmune diabetes model [26], and during homeostatic repopulation in lymphopenic hosts reconstituted with Treg-depleted cells [27, 28]. Moreover, generation of iTreg in response to donor tissue in transplanted organs has been studied extensively. Plasmacytoid DC play an important role in their generation under alloAg stimulation in the transplant setting [29].

Immunosuppressive therapy is also of major influence; different studies show preferential generation of iTreg with use of non-depleting anti-CD4 mAb [30], anti-CD154 mAb+rapamycin [31], or rapamycin alone [32]. By contrast, cyclosporine is detrimental for iTreg generation in vitro as well as in vivo [32].

**Infectious tolerance: nTreg can generate iTreg from conventional CD4+ T cells.**

Although the concept of infectious tolerance has long been recognized as a phenomenon in which the T cells of a tolerant mouse or rat can transfer their suppressive activity to conventional CD4+ T cells in a naïve host [33-35], a possible mechanism underlying this phenomenon has been described much more recently. Two groups have reported the induction of Treg from CD4+CD25- T cells by nTreg [36, 37]. Both studies showed that human nTreg could induce anergic suppressor cells from a CD4+CD25 population. Cell-cell contact between nTreg and naïve CD4+ T cells was necessary for the generation of iTreg, but these iTreg could, in turn, suppress proliferation of Teff in a cell contact-independent fashion. Key cytokines that have been associated with the suppressive activity of iTreg are transforming-growth factor-β (TGF-β) [37] and IL-10 [36].

**KEY COMPONENTS OF IN VITRO GENERATION OF iTREG: IL-2, TGF-β AND COSTIMULATION**

IL-2 (or IL-15) is required for the generation and expansion of nTreg, together with stimulation of the TCR (CD3) and costimulation (via CD28) [6, 9]. By contrast, the
requirements for iTreg generation and expansion are still under investigation. The main factors that have been identified as crucial for induction of FoxP3 expression in CD4+CD25+ cells are IL-2 and TGF-β [10, 12]. Zheng et al [12] first showed that CD4+ suppressor cells could be generated in vitro from human CD4+CD25+ cells with TGF-β and stimulation by irradiated superAg-presenting B cells. The iTreg generated had a CD4+CD25hi cytotoxic T lymphocyte Ag 4 (CTLA4)+ phenotype, exhibited reduced production of interferon (IFN)-γ and IL-10, and suppressed autologous antibody (Ab) production through cell contact as well as TGF-β production. Chen et al [10] reported that TGF-β, together with anti-CD3 and APC stimulation could potently convert mouse CD4+CD25 Teff into Treg (CD4+CD25+CD45RB-) that suppressed allergic responses in a mouse asthma model. Subsequently, several groups have demonstrated that strong costimulation provided by B7 through CD28 during iTreg generation prevents FoxP3 upregulation and renders cells with poor suppressive function [11, 19, 38]. By contrast, upregulation of CTLA4, which is the negative counterpart of CD28 in B7-mediated regulation [39] is required for the suppressor function of iTreg [28, 40-42]. Fantini et al [43] observed that exogenous IL-2 is required for in vivo generation and expansion of iTreg in a murine colitis model, and that TGF-β-induced FoxP3 expression is associated with downregulation of the inhibitory signaling protein and transcription factor mothers against decapentaplegic (Smad)7, which is a TGF-β type 1 receptor antagonist. This makes iTreg susceptible to TGF-β-mediated regulatory effects. These observations were confirmed by in vitro experiments with neutralizing IL-2 and anti-IL-2 Abs in which IL-2 was shown to be crucial for the TGF-β-mediated conversion of naïve CD4+ T cells [44]. Altogether, these observations suggest key roles for IL-2, TGF-β, and negative costimulation through CTLA4 in the generation of iTreg which are summarized in Table 1.

**AUGMENTATION OF iTREG GENERATION**

**The role of retinoic acid in iTreg conversion**

Vitamin A is an important metabolite of the body that influences immune regulation. Its deficiency has been associated with infectious diseases in children [45]. DC in the GALT can produce the vitamin A derivative retinoic acid (RA), which is associated with gut-homing of T cells, as well as enhanced FoxP3 expression of CD4+ T cells in mice [19, 46] and humans [47]. RA functions synergistically with TGF-β in the mouse intestine to induce FoxP3 expression in naïve T cells upon Ag activation [15, 17]. When the active derivative of RA, all-trans retinoic acid (ATRA), is added to cultures of CD4+FoxP3+ T cells, together with IL-2, anti-CD3/28 and TGF-β, a dose-dependent significant increase in FoxP3 expression is found when compared to culture without
ATRA [19]. The cells express high levels of the CC chemokine receptor CCR9 and a4β7, which are homing receptors associated with the intestinal lamina propria [17, 48, 49]. The mechanism by which RA enhances FoxP3 expression and suppressive capacity in iTreg remains under investigation. Different studies demonstrate that it functions either through a cytokine-dependent mechanism, by blocking the secretion of pro-inflammatory cytokines by memory T cells [50], or through cytokine-independent mechanisms, as RA retains its inhibitory effect in the absence of cytokines [51].

The influence of ATRA on iTreg generation has been confirmed in studies showing the generation of human iTreg with stable FoxP3 expression in vitro using ATRA+TGF-β. Combination of ATRA+TGF-β significantly enhances FoxP3 expression of iTreg, as shown by our own studies (Figure 1). Moreover, iTreg generated with ATRA+TGF-β have enhanced suppressive capacity compared to either ATRA or TGF-β alone [52].

By contrast, when human nTreg are cultured with ATRA, this is not associated with increased expansion or suppressor function, although culture of nTreg in ATRA in combination with rapamycin enhances their suppressive capacity [53]. These findings suggest an important role for ATRA in the induction, but not in the maintenance, of regulatory function in nTreg.

Figure 1: Human iTreg generation with TGF-β and ATRA
Human CD4+ T cells were obtained by magnetic bead sorting (CD4+ kit, Miltenyi) of PBMC (purity >90%, 5% FoxP3+) and stimulated with high-dose IL-2 +/- TGF-β (20 ng/ml) +/- ATRA (10 ng/ml). After 6 days, live CD4+ cells were assessed for FoxP3 expression by flow cytometry. Cells cultured in the presence of TGF-β + ATRA showed 50% FoxP3+ expression while cells cultured with only IL-2 were only 25% FoxP3+; the remaining 75% of the cells showed an intermediate level of FoxP3.

(A) Gating for FoxP3+ cells (horizontal bar). Red: isotype; green: no TGF-β, no ATRA; blue: TGF-β only; brown: TGF-β + ATRA.

(B) Quantification of FoxP3+ cells: 50% of cells cultured with TGF-β + ATRA acquired FoxP3+ phenotype.
(see color figure on page: 237)
Rapamycin functions synergistically with TGF-β

The immunosuppressive pro-drug rapamycin is known to selectively inhibit the expansion and function of Teff by blocking the serine/threonine kinase activity of the mammalian target of rapamycin (mTOR), but still allows nTreg to proliferate [54] (for further details see Thomson et al [55]). Several groups have studied the effects of rapamycin in the context of iTreg generation. Rapamycin can enhance the de novo generation of iTreg in bulk CD4+ as well as CD4+CD25- cell populations [56]. Mechanistic studies by Haxhinasto et al [57] have revealed that the active form of the kinase AKT (AKT*) selectively suppresses the de novo expression of FoxP3 induced by TGF-β in murine CD4+CD25- cells, without affecting the already established FoxP3 levels of nTreg. This effect of AKT* is reduced by rapamycin, suggesting a protective effect of rapamycin on the induction of Treg. These findings are supported by the study of Delgoffe et al [58], who showed that mTOR is required for the development of effector T cells from bulk CD4+ T cells. In mTOR-deficient mice, T cells differentiate into iTreg even under strong cytokine-induced skewing conditions for Th1, Th2 or Th17 generation. This is associated with decreased signal transducer and activator of transcription (STAT) activation and hyperactive Smad3 phosphorylation, resulting in higher sensitivity of cells to TGF-β.

In a study of non-human primate (baboon) cells, rapamycin increased the induction of FoxP3+ cells from purified CD4+ cells by ~2-fold (11.2% to 20.6%). CD4+ CD25+ cells isolated from the expanded population potently suppressed a xenogeneic T cell proliferative response [59]. In a recent study of human cells [53], nTreg expanded in rapamycin showed improved capacity to induce infectious tolerance. Thus, CD4+ FoxP3+ cells cocultured with nTreg for 3 days showed 8% FoxP3+ expression and significant IL-2 production; however, if the nTreg were expanded in the presence of rapamycin (or rapamycin+ATRA), there was a 2-fold increase in FoxP3 induction in Teff, and IL-2 production was abrogated.

An important role in the conversion and function of iTreg in relation to mTOR activity has been found for the coregulatory B7 family member programmed death-ligand1 (PD-L1, = B7-H1), which is expressed on hematopoietic and parenchymal cells. Expression of PD-L1 is associated with the inhibition of autoreactive CD4+ T cell responses and peripheral T cell tolerance [60]. PD-L1 synergizes with TGF-β to inhibit the AKT-mTOR axis, thereby promoting iTreg development and function via the same pathway as rapamycin [61]. Cobbold et al [62] have linked the effect of mTOR inhibition to infectious tolerance by showing that enzymes produced by nTreg consume certain amino acids that are essential for T cell proliferation, resulting in inhibition of the mTOR
pathway and induction of FoxP3 expression. These studies suggest that upregulation of PD-L1 may be used as a strategy to further optimize iTreg conversion.

Taken together, these studies show that mTOR inhibition with rapamycin can enhance induction of FoxP3+ Treg from a naïve T cell population, and function synergistically with ATRA (Table 1). This supports the concept that use of rapamycin as maintenance therapy may constitute tolerogenic immunosuppression [63, 64].

**Table 1: Properties of nTreg and iTreg**

<table>
<thead>
<tr>
<th></th>
<th>nTreg (references)</th>
<th>iTreg (references)</th>
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<tbody>
<tr>
<td><strong>Generation</strong></td>
<td>Tissue</td>
<td>Thymus [1, 2]</td>
</tr>
<tr>
<td></td>
<td>Cytokines required</td>
<td>IL-2 or IL-15 (6, 9)</td>
</tr>
<tr>
<td></td>
<td>Costimulation required</td>
<td>CD28 (6, 9)</td>
</tr>
<tr>
<td><strong>Improvement of suppressive function by reagent</strong></td>
<td>ATRA</td>
<td>No (53)</td>
</tr>
<tr>
<td></td>
<td>Rapamycin</td>
<td>Yes (53) / No (57)</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>Self or alloAg (1, 2)</td>
<td>AlloAgs, food allergens, gut microbes, transplanted tissue, self (inflammation) (15, 17, 25, 26, 30)</td>
</tr>
<tr>
<td><strong>Th17 differentiation</strong></td>
<td>IL-6-mediated</td>
<td>Yes (69, 70)</td>
</tr>
<tr>
<td></td>
<td>Antagonized by ATRA</td>
<td>Yes (72)</td>
</tr>
<tr>
<td></td>
<td>Antagonized by rapamycin</td>
<td>Has not yet been addressed</td>
</tr>
<tr>
<td><strong>Specific intracellular marker</strong></td>
<td>FoxP3</td>
<td>Yes (1, 2)</td>
</tr>
<tr>
<td></td>
<td>Helios</td>
<td>Yes (66)</td>
</tr>
</tbody>
</table>

**ALTERNATIVE APPROACH: GENETIC ENGINEERING TO INDUCE FOXP3 EXPRESSION**

An alternative strategy to induce suppressor function in conventional T cells is *ex vivo* FoxP3 gene transfer using a viral vector. This approach was studied by Hori et al [65], who demonstrated the conversion of 30-60% of mouse CD4+CD25+ cells to the Treg phenotype after forced expression of FoxP3 was induced by retroviral transduction. The iTreg generated had reduced cytokine production and a potent inhibitory effect on autologous Teff proliferation. Moreover, the FoxP3+ cells generated were capable of abrogating autoimmune disease in a mouse inflammatory bowel disease model.
Lentivirus-based transduction of the FoxP3 transcription factor was explored by Allen et al [7], after it was found that overexpression of FoxP3 by regular retroviral transduction did not lead consistently to generation of suppressive Treg [66]. In contrast to conventional retroviral vectors, lentiviral vectors have the ability to replicate in non-cycling cells, rendering them very efficient in gene transduction. A lentiviral vector was used to induce FoxP3 in human FoxP3 cells, and a maximum transduction efficiency of 76-97% was achieved. The FoxP3+ cells had an nTreg phenotype and potent suppressor function. Additionally, it was shown that lentivirus-driven FoxP3 expression could be maintained for several weeks, whereas expanded nTreg lost FoxP3 expression upon prolonged culture.

These studies indicate that there are several ways to obtain high numbers of FoxP3+ suppressor cells from a naïve CD4+ T cell population, and promising results provide a strong rationale for the use of viral transduction in further studies.

**IN VIVO PREVALENCE OF iTREG**

The incidence of iTreg in vivo has been determined in different settings. When their frequency was assessed by enumeration of FoxP3+ cells after homeostatic repopulation in a FoxP3- mouse model, it was found to be 8% [28] and 4-7% [27] of total CD4+ cells, respectively. However, in a study without lymphodepletion, little to no conversion was found upon stimulation with self Ag [14]. A solution to the issue of distinguishing nTreg from iTreg would preferably be through a marker that is expressed specifically on one, but not the other cell type. The development of the T lymphocyte lineage is linked to one of the members of the Ikaros transcription factor family, Helios [67]. Expression of Helios is upregulated in nTreg by binding to the FoxP3 promoter; upon Helios elimination, FoxP3 expression and the suppressive function of nTreg is inhibited [68]. A recent study [13] used Helios expression to assess nTreg/iTreg proportions in healthy mice and humans. Helios was expressed by 100% of thymic CD4+FoxP3+ nTreg, but was not expressed when mouse or human FoxP3+ iTreg were generated in vitro. Both mouse and human CD4+FoxP3+ cells from healthy subjects are 70% Helios+[13], suggesting that 30% of circulating FoxP3+ Treg are peripherally-induced iTreg. Based on these studies, it can be concluded that there is a significant percentage of circulating iTreg in healthy individuals, although the exact proportion of iTreg within the total FoxP3+ population needs to be better defined.
TREG PLASTICITY: TH17 DIFFERENTIATION

During the activation of murine naïve CD4⁺ T cells in the presence of TGF-β, there is a crucial role for IL-6, a common pro-inflammatory cytokine produced by Teff and macrophages. When combined with TGF-β, this acute-phase protein is associated directly with differentiation of CD4⁺ T cells into pro-inflammatory Th17 cells, rather than into the FoxP3⁺ iTreg phenotype in the mouse model, suggesting that an inflammatory milieu can drive naïve cells to convert into effector rather than suppressor cells [69, 70]. Moreover, IL-6 can convert already established FoxP3⁺ nTreg into Th17 cells [71, 72]. The capacity of IL-6 (or an inflammatory milieu in general) to direct T cells to an immune effector rather than regulatory phenotype is a problematic issue for expansion of a pure Treg population in vitro, and could also be of crucial importance when Treg are used in a clinical setting of autoimmune disease or transplantation, where, despite immunosuppressive therapy, some degree of inflammatory response can be expected.

In addition to the influence of IL-6, high levels of TCR/CD28 costimulation are associated with induction of nuclear factor-κB (NF-κB) activation, which potently inhibits iTreg differentiation in naïve CD4⁺ T cells. Indeed, when naïve CD4⁺ T cells are stimulated with anti-CD28 and high doses of anti-CD3, expression of the proinflammatory cytokines IL-17 and IL-9 is enhanced significantly, and the frequency of FoxP3⁺ cells diminished [38]. As the main goal of iTreg induction is to generate potent suppressor cells, contamination by outgrowth of effector cells is likely to have a detrimental effect on their potential application. These recent observations raise concerns with regard to the preparation of Treg for therapeutic application, and indicate that a reliable method to assess suppressive capacity might become a necessary goal.

A promising and straightforward solution to overcome the detrimental outgrowth of Th17 cells in the Treg population is the addition of ATRA or rapamycin during the expansion of naïve T cells. ATRA potently suppresses Th17 differentiation of naïve T cells and drives them to iTreg conversion with stable FoxP3⁺ expression, despite an IL-6-induced inflammatory environment [23, 73]. Recently, it has been shown that ATRA has similar effects on nTreg stability as on iTreg, since it prevents their conversion to Th17 cells [74]. Similar to the inhibitory effect of ATRA on TGF-β-mediated iTreg/Th17 differentiation, rapamycin inhibits the development of Th17 cells from bulk CD4⁺ cells, as well as CD4⁺CD25⁻ populations under Th17-skewing conditions [56]. Therefore, it is likely that iTreg generated in vitro under the influence of ATRA or rapamycin will have a stable phenotype and function. It is not clear if this same effect can be obtained by in vivo administration of ATRA or rapamycin.
Importantly, in experiments involving human cells, it has been found that naïve T cells do not differentiate into Th17 cells when stimulated with TGF-β and IL-6 alone, but that this requires additional stimulation by IL-1β ([75], IL-23 [76], or DC [77]. Unfortunately, reports on human iTreg have not determined the Treg-skewing effects of ATRA or rapamycin on iTreg/Th17 differentiation. Although this knowledge is perhaps not essential for *in vitro* generation of these cells (since human naïve T cells need additional stimulation to differentiate into Th17 cells, and cells and cytokine levels can be strictly controlled during laboratory experiments), it will certainly be an important issue for future clinical application of iTreg.

**ITREG VS NTREG: DIRECT COMPARISON**

Induced Treg have significant suppressive function when compared to baseline levels of T cell proliferation (baseline = responder cells + stimulation only; no Treg or control T cells added), but a wide range of suppressive efficacy has been reported depending on methods of induction [10, 12, 52, 56]. To adequately compare the function of iTreg with that of nTreg, it is necessary to test both cell types in the same system and under identical conditions. Interestingly, only a few groups that have generated iTreg have undertaken this direct comparison of the function of iTreg versus nTreg. The few studies on iTreg in the mouse model that have done so all show the level of suppression of *fresh* nTreg compared to *expanded* iTreg.

Conversion of CD4+CD25 T cells by 5 days stimulation with anti-CD3, irradiated APC and IL-2 +/- TGF-β showed, when compared to fresh CD4+CD25 T cells, a significant increase in normalized FoxP3 mRNA levels which was about 50% of the nTreg level. These 5-day expanded iTreg potently suppressed baseline CD4+CD25 Teff proliferation to levels comparable to those when nTreg were assessed [11]. In another study using a similar method of induction, murine CD4+CD25 T cells were cultured for 5 days with plate-bound anti-CD3 and soluble anti-CD28 +/- IL-2 +/- TGF-β. The frequency of CD25+FoxP3+ iTreg increased significantly from 3% (no TGF-β) to 53% when IL-2+TGF-β was added to the cultures. In a suppression assay, the addition of TGF-β-expanded iTreg or fresh nTreg (1:1 to responders) resulted in similar suppression of Teff proliferation to 5-10% of baseline under both conditions. These findings were then translated to an *in vivo* colitis model, where iTreg infusion resulted in a marked decrease in colonic inflammation, although these results were not compared to nTreg [78]

Very promising results have also been reported by Benson et al [19], who showed almost 100% conversion to CD4+FoxP3+ iTreg by 4 days expansion of CD4+FoxP3+ Tcells
with anti-CD3, anti-CD28, IL-2, TGF-β and ATRA. Expanded iTreg exhibited similar suppressive activity as fresh nTreg when added to responder CD4+ cells in a 1:1 ratio. At a ratio of 1:8 (Treg:responder), iTreg retained some suppressive effect, whereas fresh nTreg had lost their suppressive capacity. These studies in the mouse model provide evidence that iTreg can exhibit suppressive function at a similar strength as nTreg.

In a study in which baboon bulk CD4+ T cells were expanded in the presence of anti-CD3/28 beads, IL-2 and rapamycin for 4 weeks, increased expression of FoxP3+ of 20% (11% when cultured without rapamycin) was found. The CD25hi cells were purified after expansion, and added to an MLR of autologous baboon CD4+CD25- responders stimulated with xenogeneic (porcine) irradiated PBMC. Compared to freshly-isolated nTreg, iTreg cultured with rapamycin showed similar, potent inhibition of the xenogeneic T cell response [59].

Interestingly, a recent study describes the induction of human Treg by bacterial superAgs, which provide potent polyclonal activation [79]. Stimulation of human CD4+CD25- T cells with Streptococcal pyrogenic exotoxin and autologous APC alone (without the addition of IL-2 or TGF-β) resulted in the induction of 20% CD25+FoxP3+ T cells after 3 days stimulation (5% without bacterial Ag stimulation). Subsequent addition of Treg in an autologous CD4+CD25- proliferation assay showed that, at 1:1 ratio, fresh nTreg could reduce proliferation to 20% of baseline, compared to 40% for iTreg; however, at 1:10, nTreg were still suppressive (55%), whereas iTreg had lost their suppressive effect (80%).

Overall, these studies provide promising evidence that, similar to the findings in the mouse model, in humans and primates iTreg generated from naïve T cells can acquire suppressive function of similar strength as their nTreg counterparts.

FUTURE PROSPECTS

Many questions remain unresolved regarding pathways and optimal conditions for the induction of Treg from a naïve T cell population and how the resulting population compares to nTreg. Although recent reports are promising, there are very few studies that provide a direct comparison of nTreg and iTreg. This will be of great importance when such studies are translated to clinical trials in the near future, especially in regard to the quantity of cells needed. One concern that may arise when translating these studies to clinical trials, is that not all cells from the starting population would be converted, thus leaving a remainder of contaminating Teff. However, this concern
could be overcome by sorting the iTreg after expansion (e.g., based on expression of CD25^{hi}, CD45RA^{+}, or CCR9, depending on the method of iTreg generation).

Another critical point is the potential to drive a subset of naïve T cells to the Th17 or other effector phenotype, rather than to iTreg during stimulation - a risk that cannot be underestimated. Although it is well-demonstrated that this can be abrogated by the use of ATRA or rapamycin in \textit{in vitro} culture, the potential of Th17 differentiation \textit{in vivo} may remain. Interestingly, RA has also been associated recently with promotion of Teff responses, suggesting a dual function for proinflammatory as well as suppressive T cell responses [80]. A possible solution to overcome the release of pro-inflammatory under RA stimulation would be by blocking IL-15 during iTreg stimulation [81]. Moreover, most studies on iTreg have been carried out in the mouse model, but Treg in humans have already proven different from those in the mouse. These differences can be found, for example, in the conversion of naïve T cells to Th17, which in humans does not occur when stimulation is with TGF-β and IL-6 alone; or in the expression of FoxP3, which in the mouse is strictly related to suppressor function, while human activated T cells can transiently express intermediate levels of FoxP3 while lacking suppressive function [82].

Remaining concerns that need to be addressed before clinical trials are initiated, include the stability of iTreg (and nTreg) phenotype, particularly considering their interaction with inflammatory cells and immunosuppressive therapy; the importance of appropriate migration of the cells (reviewed by Zhang et al [83]), and a detailed comparison of the efficacy of nTreg versus iTreg \textit{in vivo}. In this regard, non-human primate models might provide valuable information on the \textit{in vivo} function of iTreg before use of human cells is translated to clinical trials.

\textbf{CONCLUDING REMARKS}

Regulatory T cells have been the subject of intense investigation for the last decade, and as the first clinical trials to ascertain the safety of ex-vivo expanded nTreg infusion in humans are being initiated [63], determination of the necessary numbers of Treg has become increasingly pressing. The induction of a regulatory phenotype from naïve CD4^{+}CD25^{-} Teff has become a well-established approach to convert large numbers of T cells into functional Treg. Although various methods for the generation of iTreg have been published, a consensus exists in regard to the basic procedure of induction, using IL-2, TGF-β and low costimulation to generate potently suppressive FoxP3^{+} iTreg. Rapamycin and ATRA are proven to be valuable, as these reagents not only enhance
FoxP3 induction, but also actively prevent the differentiation of naïve T cells into Th17 cells, thus providing a more stable phenotype. iTreg have proven to be stable and potent suppressor cells that seem of similar suppressive strength to nTreg. Perhaps optimal results will be achieved when both cell types are being used in concert, a concept that still needs to be explored.

Altogether, the induction of a regulatory phenotype provides a promising means to generate potently suppressive Treg with stable FoxP3 expression in significantly higher numbers than can be obtained by expansion of nTreg, thereby having the potential for use in large-scale clinical trials.

ACKNOWLEDGEMENTS

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Abbreviations

Ab - antibody
Ag - antigen
APC - antigen-presenting cells
ATRA - all-trans retinoic acid
CTLA4 - cytotoxic lymphocyte antigen 4
DC - dendritic cells
FoxP3 - Forkhead box P3
iTreg - induced regulatory T cells
mTOR - mammalian target of rapamycin
nTreg - naturally-occurring regulatory T cell
RA - retinoic acid
Teff - T effector cells
TGF-β - transforming growth factor β
Treg - regulatory T cells
Tx - transplantation
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Induced regulatory T cells

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Controlled release formulations of IL-2, TGF-ß1 and rapamycin for the induction of regulatory T cells

Siddharth Jhunjhunwala, Stephen C. Balmert, Giorgio Raimondi, Eefje M. Dons, Erin E. Nichols, Angus W. Thomson, Steven R. Little

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Background
The absence of regulatory T cells (Treg) is a hallmark for a wide variety of disorders such as autoimmunity, dermatitis, periodontitis and even transplant rejection. A potential treatment option for these disorders is to increase local Treg numbers. Enhancing local numbers of Treg through in situ Treg expansion or induction could be a potential treatment option for these disorders.

Methods
Current methods for in vivo Treg expansion rely on biologic therapies, which are not Treg-specific and are associated with many adverse side-effects. Synthetic formulations capable of inducing Treg could be an alternative strategy to achieve in situ increase in Treg numbers. Here we report the development and in vitro testing of a Treg-inducing synthetic formulation that consists of controlled release vehicles for IL-2, TGF-β and rapamycin (a combination of cytokines and drugs that have previously been reported to induce Treg).

Results
We demonstrate that IL-2, TGF-β and rapamycin (rapa) are released over 3-4 weeks from these formulations. Additionally, Treg induced in the presence of these formulations expressed the canonical markers for Treg (phenotype) and suppressed naïve T cell proliferation (function) at levels similar to soluble factor induced Treg as well as naturally occurring Treg. Most importantly, we show that these release formulations are capable of inducing FoxP3+ Treg in human cells in vitro.

Discussion
Our data suggest that controlled release formulations of IL-2, TGF-β and rapa can induce functional Treg in vitro with the potential to be developed into an in vivo Treg induction and expansion therapy.
INTRODUCTION

Over the past two decades, regulatory T cells (Treg) have been identified as one of the central components of the mammalian immune system [1-4]. The most commonly described, widely studied, and possibly most abundant regulatory T cells in the body are those that express CD4, CD25 [1, 5] and FoxP3 [6-8]. These CD4+ CD25+ FoxP3+ cells (Treg) play important roles in suppressing the activity of self-reactive immune cells and in re-establishing homeostasis following infection. Moreover, increased numbers of Treg suppress diverse inflammatory diseases such as autoimmunity [2, 9], transplant rejection [10, 11], dermatitis [12], psoriasis [13, 14] and even periodontitis [15, 16]. Given this evidence, it is not hard to perceive that strategies to boost local Treg numbers could be developed into potential therapeutics to treat these diseases.

Enhancing numbers of Treg at local tissue sites can be achieved by (i) ex vivo expansion of Treg followed by their local administration or systemic re-infusion, or (ii) in vivo manipulation of immune cells in order to tip the balance between Treg and effector T cells towards Treg. The latter approach is preferable given the stringency associated with ex vivo culture of human cells under Good Manufacturing Practice (GMP) conditions [17-19]. One possible means to achieve increased number of Treg in vivo is the use of biologic therapies that selectively enhance Treg numbers and function. Various antibodies (Abs), such as anti- IL-2 monoclonal (m) Ab [20], superagonistic anti-CD28 mAb [21], and agonistic anti-CD4 mAb [22], have been used in the past to increase in vivo Treg numbers. However, their exact mechanism of action has still not been characterized, and their safety in humans remains questionable. In fact, phase I clinical trials of the superagonistic anti-CD28 Ab (TGN1412) resulted in severe negative reactions (cytokine ‘storm’) in all 6 human subjects who received the Ab [23].

An alternative approach to increase Treg numbers in vivo is through the establishment of a local immunosuppressive environment that selectively favors Treg expansion. An environment rich in IL-2, transforming growth factor- β1 (TGF-β) and rapamycin (rapa), an inhibitor of the serine-threonine kinase mammalian target of rapamycin, has been shown to favor Treg development, even under inflammatory conditions [24-26]. However, providing a continuous presence of these factors in vivo, has proven difficult. Controlled release vehicles for such factors offer a potential solution to these problems.

In this study we describe the development and testing of controlled release formulations for IL-2, TGF-β and rapa. We show that a combination of these formulations (called FactorMP henceforth) is capable of Treg induction in vitro using either mouse or human cells. Further, we demonstrate that the FactorMP-induced Treg maintain
their proliferative capacity and functional ability \textit{in vitro} and express phenotypic surface markers that are consistent with soluble factor-induced Treg.

\textbf{MATERIALS AND METHODS}

\textbf{Mice}

Six-eight week old C57Bl/6 (B6) and B6.SJL-Ptprca/BoyAiTac (CD45.1) were purchased from Taconic and used within two months. All animals were maintained under specific pathogen free conditions. Experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use for Laboratory Animals and under Institutional Animal Care and Use Committee-approved protocols.

\textbf{Microparticle Preparation}

IL-2 and TGF-β microparticles (IL-2MP and TGFβMP, respectively) were prepared using the double emulsion-evaporation technique, as described [27, 28]. For the IL-2MP the following conditions were used. Five µg of recombinant (r) mouse IL-2 (from R&D Systems Minneapolis, MN, prepared in 50 mM ammonium acetate and 1 mM DTT) was mixed with 2 mg of BSA and 5 mM NaCl in 200 µl of de-ionized water. This solution was added to 4 ml of dichloromethane containing 200 mg of poly lactic-co-glycolic acid (PLGA; RG502H, 50% glycolate 50% lactate blend, viscosity 0.16-0.24 dl/g, Boehringer Ingelheim Chemicals Inc., Petersburg, VA), and the mixture was agitated using a sonicator (Vibra-Cell, Newton, CT) at 25% amplitude for 10 sec, creating the primary emulsion. This emulsion was then mixed with 60 ml of 2% polyvinyl-alcohol (PVA, MW ~25,000, 98% hydrolyzed; Polysciences) under homogenization (L4RT-A, Silverson, procured through Fisher Scientific) at 3000 rpm for 1 min, creating the second emulsion. The resulting double-emulsion was then added to 80 ml of 1% PVA, and left for 3 hr spinning at 600 rpm. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized (Virtis Benchtop K freeze dryer, Gardiner, NY; operating at 80 mTorr).

For the TGFβMP the following conditions were used. One µg of r-human TGF-β (CHO cell-derived, PeproTech, Rocky Hill, NJ; prepared in 10 mM sodium citrate) was mixed with 10 mg D-mannitol, 1 mg of BSA, and 15 mM NaCl in 200 µl of de-ionized water. This solution was added to 4 ml of dichloromethane containing 200 mg of PLGA (RG502H), and the mixture agitated using a sonicator at 25% amplitude for 10 sec, creating the primary emulsion. This emulsion was then mixed with 60 ml of 2% PVA (containing 125 mM NaCl) under homogenization at 3000 rpm for 1 min, creating the second emulsion. The resulting double emulsion was then added to 80 ml of 1%
PVA (containing 125 mM NaCl), and left for 3 hr spinning at 600 rpm. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized.

The rapaMP were prepared using the single emulsion-evaporation technique as described [29, 30]. Briefly, 1 mg of rapa (LC labs, Woburn, MA) dissolved in DMSO was mixed with 4 ml of dichloromethane containing 200 mg of PLGA (RG502H). This solution was mixed with 60 ml of 2% PVA under homogenization at 3000 rpm for 1 min creating the microparticle emulsion. The resulting emulsion was then added to 80 ml of 1% PVA and left for 3 hr spinning at 600 rpm. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized.

**Microparticle Characterization and Release Assays**

Scanning electron micrographs of the microparticles were obtained using a scanning electron microscope (JSM-6330F, JEOL, Peabody, MA). The size distribution of microparticles was determined using volume impedance measurements on a Multisizer 3 (Beckman Coulter, Brea, CA).

Release assays were conducted by incubating a suspension of particles on a rotoshaker at 37 °C; (i) 10 mg in 1 ml of cell culture media for IL-2MP and TGFβMP, and (ii) 10 mg in 1 ml of PBS containing 0.2% Tween-80 for rapaMP (due to the low solubility of rapa in aqueous solutions, release assays were conducted in PBS containing Tween-80 to avoid a obtaining a release profile that was dissolution dependant). At regular time intervals, particle suspensions were centrifuged (250g, 5 min), the supernatant removed, and the particles re-suspended in 1 ml of appropriate solution. The amount of each cytokine in the supernatant was measured using a cytokine-specific ELISA (R&D systems, Minneapolis, MN), and the amount of rapa was measured using spectrophotometry (absorbance at 278 nm).

**Mouse T cell isolation**

Spleen and lymph nodes were dissected from B6 or CD45.1 mice. Following mechanical digestion, the tissue suspension was passed through a 70 µm nylon filter to obtain a single cell suspension of leukocytes. Predominantly naïve CD4+ T cells (> 90% pure) were isolated from this suspension with a CD4+ T cell negative isolation kit (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. These purified CD4+ T cells were used in cell culture and suppression assays. Natural regulatory T cells (nTreg) were isolated from this purified population of CD4+ T cells through positive selection of CD25 expressing cells using the CD25 microbead kit (Miltenyi Biotec, Auburn, CA) as per manufacturer’s instructions.
Induction of mouse regulatory T cells (Treg)

For Treg induction experiments, naïve T cells were cultured either in direct contact with FactorMP (in 96-well round bottom cell culture plates), or separated from FactorMP by permeable transwell inserts (HTS Transwell®-96, 0.4μm pore size; Corning, Lowell, MA); (n=3 experiments with FactorMP in direct contact with T cells, and n=2 experiments in transwell® plates with particles separated from the cells). Dynabeads® mouse T-activator CD3/CD28 beads (Dynabeads®; Invitrogen, Carlsbad, CA) were used at a 2:1 (beads:T cells) ratio to activate T cells, and cultures maintained for 4 days. For cultures in the presence of soluble factors, the following concentrations of factors were used: 10 ng/ml IL-2, 5 ng/ml TGF-β and 10 ng/ml rapa (corresponds to a total amount of 2 ng IL-2, 1 ng TGF-β and 2 ng rapa). The following quantities of microparticles were used for the induction experiments in 200 μl of cell culture media: 2 mg TGFβMP, 0.5 mg IL-2MP, and ~ 0.01 - 0.05 mg rapaMP. TGFβMP were pre-incubated in media for 18-22 days prior to use in the Treg induction experiments to account for the initial lag in release of TGF-β from the microparticles. To determine the phenotype of cells after culture, cells were stained with anti-CD4 (L3T4), anti-FoxP3 (FJK-16s), anti-CD25 (PC61.5), anti-glucocorticoid-induced TNFR-induced protein (GITR; DTA-1), anti-folate receptor-4 (FR4; eBio12A5) (antibodies from eBiosciences, San Diego, CA) and anti-cytotoxic T-lymphocyte antigen 4 (CTLA4; UC10-4B9, from Biolegend, San Diego, CA). To determine iTreg proliferation, naïve T cells were stained with carboxyfluorescein diacetate succinimidyl ester cell tracer (CFSE; Invitrogen, Carlsbad, CA) prior to activation with Dynabeads®. Stained cells were then analyzed on a BD-LSRII flow cytometer.

Suppression assay

Freshly-isolated naïve CD4+CD45.1+ T cells were stained with CFSE (Invitrogen, as per the manufacturer’s instructions) and co-cultured with induced Treg (generated as described above) at different ratios in 96-well plates. For suppression assays, iTreg were generated in Transwell® cell culture plates, such that the iTreg could be easily separated from the FactorMP prior to co-culture with naïve CD4+CD45.1+ T cells. The number of naïve CD4+CD45.1+ T cells was kept constant at 50,000 cells / well. For stimulation, 50,000 Dynabeads® were used per well. Co-cultures were carried out for 4 days, after which cells were stained for flow cytometry.

Human T cell culture

Anti-coagulated peripheral venous blood was obtained from healthy adult volunteers under a University of Pittsburgh Institutional Review Board approved protocol. CD4+ T cells were isolated from mono nuclear cells using the CD4 negative isolation kit (Miltenyi Biotec, Auburn, CA). Cells (500,000) were cultured in 0.5 ml of media (with
serum) in the presence of human T cell activation beads (anti-CD2, anti-CD3 and anti-CD28 coated beads, Miltenyi Biotec, Auburn, CA). Cell culture media was supplemented with additional factors or FactorMP at the following concentrations: 500 U/ml recombinant-human IL-2, 10 ng/ml TGF-β, 2 ng/ml rapa, 8 mg/ml of TGFβMP, and/or 0.02 mg/ml of rapaMP. Following 4 days of culture, cells were collected, stained and Treg induction analyzed by flow cytometry. Soluble IL-2 was used in all of these cultures instead of IL-2MP, as IL-2MP encapsulated mouse rIL-2 and not the human protein.

RESULTS

Microparticle Characterization

IL-2MP, TGFβMP, and rapaMP were all prepared under similar conditions, using the same polymer (RG502H, viscosity 0.16-0.24 dl/g). Scanning electron micrographs (Figure 1A) show that IL-2MP have a porous exterior surface (as these particles were

Figure 1: Microparticle Characteristics. Scanning electron micrographs of intact microparticles (A) and the cross-section of microparticles (B) showing the internal architecture. C – Microparticle size distribution determined using volume impedance measurements.
formulated with a 20-30 mOsm higher soluble concentration in the primary emulsion when compared to the bulk aqueous phase, while the TGFβMP are slightly porous (a 0-5 mOsm difference in solute concentration) and the rapaMP were smooth without pores on the surface. Additionally, particle sections (Figure 1B) show emulsion pockets in the IL-2MP and TGFβMP, while there are no interior pockets in the rapaMP as these particles were formulated using the single emulsion-evaporation fabrication procedure. Further, the particles were prepared such that they were large enough (IL-2MP = 25.5 ± 7.5 µm; TGFβMP = 16.7 ± 6.3 µm; rapaMP = 16.7 ± 6.4 µm; errors indicate standard deviation from the mean for each particle set) to remain at a site of injection and not be taken up by phagocytic cells as shown in figure 1C. The size distribution observed in these microparticle formulations is characteristic of the double emulsion-evaporation procedure and has been previously described [27, 28, 31, 32]. Finally, as shown in figure 2 we observed a high initial burst followed by continuous release from IL-2MP (loading efficiency = 37.1 ± 6.1 %), a linear release of TGF-β (loading efficiency = 24.9 ± 9.9 %) following a ~2 week lag phase, and a continuous release from rapaMP (loading efficiency = 63.9 ± 3.0 %).

![Graphs of IL-2, TGFβ, and Rapamycin Release Profiles](image)

**Figure 2: Release Profile.**

In vitro release profiles of IL-2, TGFβ (in cell culture media) and rapa (in PBS containing 0.2% Tween-80). Error bars on release profiles are based on n = 6 measurements for IL-2MP and TGFβMP, and n= 3 measurements for rapaMP.
Treg Induction
Soluble IL-2, TGF-β and rapa have been shown previously to induce Treg (iTreg) [24, 25]. We wanted to determine if degradable polymer-based formulations designed to sustain the release of these factors could induce Treg reliably. Indeed, we observed that the microparticle formulations were similar to soluble factors in their in vitro Treg induction efficacy, as measured by FoxP3 expression (Figure 3A and 3B). Furthermore, we observed similar Treg induction efficiencies in experiments with microparticles that were in direct contact with T cells, and those with microparticles separated from T cells by transwell membranes. In addition, the FactorMP were capable of inducing Treg by releasing equivalent (2-3 ng IL-2 and 2-10 ng rapa), or reduced (0.2-0.4 ng of TGF-β) total amounts of the factors over 4 days of culture. Further, we observed that iTreg were capable of robust proliferation (as observed through CFSE dilution, Figure 3A).

Figure 3: FactorMP induce mouse Treg.
A – representative flow cytometry dot plots (gated on CD4-expressing cells) of naïve T cells stimulated in the presence of soluble factors or FactorMP. The X axis on these plots represents CFSE, which is a cell proliferation marker and the Y axis represents intracellular FoxP3, which is a definitive marker for mouse Treg. B – quantitative analysis of the percentage of CD4+ T cells that express FoxP3 after culture for 4 days under different conditions; * indicates p<0.05 based on n ≥ 3 independent experiments.
Phenotype and function of microparticle-induced Treg

In addition to FoxP3, Treg are known to express many other characteristic surface proteins. We tested for 3 canonical surface markers: CD25, FR-4 and GITR. CD25 is the high-affinity IL-2 receptor, which increases sensitivity to IL-2 and is important for Treg proliferation. FR-4, a folate receptor, is required for folic acid sensing and uptake, which in turn prolongs Treg survival. Finally, GITR is a surface receptor that has been suggested to play an important role in Treg survival and suppression. We observed that FactorMP-iTreg expressed these surface markers at levels equivalent to those on soluble factor-iTreg (Figure 4A). Importantly, although the expression of these surface proteins, along with FoxP3, suggests that these cells are Treg, it does not guarantee suppressive function. In order to test the ability of FactorMP-iTreg to suppress naïve T cell proliferation, we adopted an in vitro co-culture system described previously [33]. In these co-culture suppression assays, we observed that FactorMP-iTreg indeed possessed suppressive capabilities similar to Treg induced by soluble factors and natural Treg (Figure 4B).

Microparticle formulations induce Treg from human T cells

Human T cells isolated from peripheral blood mononuclear cells can also be induced to a Treg phenotype using soluble IL-2, TGF-β and rapa [34, 35]. For potential clinical application of our technology, we needed to determine if the microparticle formulations were capable of inducing Treg from human T cells. To this end, human T cells were cultured in the presence of soluble factors or FactorMP. We observed that the microparticles were equally capable of inducing Treg when compared to the soluble factors (Figure 5), while releasing equivalent (1-10 ng/ml rapa) or reduced (2-4 ng/ml TGF-β) amounts of factors.

DISCUSSION

Therapies that enhance Treg numbers and function have the potential to suppress transplant rejection and autoimmunity [2, 3]. Clinical trials are currently testing cellular therapies involving Treg as potential therapeutics for treating graft versus host disease [34, 36]. However, Treg-based cellular therapies face many challenges, which include, but are not limited to: (i) difficulties in isolating pure and homogenous populations and large quantities of Treg from the blood, (ii) inconsistent maintenance of the Treg phenotype and suppressive function post-proliferation, and (iii) the need for GMP facilities [17-19]. Hence, acellular therapies that can increase numbers and/or the suppressive potency of Treg without the need for ex vivo culture could be transformative.
One potential method to increase the ratio of Treg to effector T cells is to establish an environment rich in IL-2, TGF-β and rapa as described [24-26, 37]. Such an immunosuppressive, Treg-inducing environment can be attained through the sustained release of these factors at a local site. To this end, release formulations were fabricated using an FDA-approved, widely-used polymer, PLGA. We prepared porous IL-2MP (Figure 1) with a high initial burst, followed by a slow continuous release of the factor over a 5 week time frame (Figure 2). Such a release formulation for IL-2 was suitable for the induction of Treg, as it has been suggested that high initial doses of IL-2 might help Treg grow better and resist apoptosis [38, 39]. Additionally, we sought a way to continuously release TGF-β, as it has been shown that continuous presence of TGF-β is required for FoxP3 expression in naïve T cells [24, 40]. Other groups have reported release of TGF-β from similar PLGA microparticles [28, 32] with the expected burst and lag that typically accompanies protein release [41, 42]. In our formulations, we wished to avoid this initial burst of TGF-β, and indeed our formulations were successfully able to abstain from that initial burst (Figure 2). However, despite multiple attempts, we were unable to circumvent the 2 week lag phase. Unpublished data from our lab suggest that the initial lag phase could be due to ionic interactions between TGF-β

Figure 4: FactorMP-iTreg express canonical Treg surface markers and suppress effector T cells.
A – representative flow cytometry dot plots (gated on CD4-expressing cells) showing the expression of surface markers and intracellular FoxP3 on naïve T cells stimulated in the presence of soluble factors or FactorMP. B – representative plots of CFSE dilution showing that the FactorMP-iTreg can suppress naïve T cell proliferation. Gates on individual plots indicate the percentage of proliferating cells. Ratios (1:1, 1:4 and 1:8) indicate the number of Treg in culture to the number of naïve T cells. Data are representative of at least 2 independent experiments.
(with a high isoelectric point of ~8.6) and degrading PLGA polymer. Regardless, to overcome the problem of the initial lag phase, we were able to simply pre-incubate the TGFβMP (18-22 days) prior to their use in cell culture, which ultimately results in the initially targeted, linear release over a 3 week period of time (Figure 2). Finally, rapaMP were also formulated to be similar in size to the IL-2MP and TGFβMP and to release continuously over a 2-3 week time frame as previously demonstrated (Figure 2) [29, 30].

Importantly, the combination of these microparticle formulations (FactorMP) is as effective as soluble factors at inducing Treg from naïve T cells in in vitro cultures (Figure 3B). Additionally, we determined that the FactorMP iTreg were capable of robust proliferation (Figure 3A), expressed canonical surface markers representative of Treg (Figure 4A), and were able to suppress naïve T cell proliferation in an in vitro suppression assay (Figure 4B). Further, it was observed that Treg induction and proliferation occurred even when the cells were in contact with microparticles, suggesting that the microparticles do not have adverse on these cells. Finally, we observed that these

Figure 5: Microparticle formulations generate human-iTreg.
A – representative plots displaying FoxP3 expression profile on human T cells cultured under different conditions. Numbers in plots represent the median fluorescence intensities (MFI). Grey plots indicate the FoxP3 expression in naïve unstimulated T cells. B – quantitative analysis of normalized FoxP3 MFI as determined from 2 independent experiments (n≥3). MFI was normalized by determining the ratio of experimental MFI and control (soluble IL-2 treated cells) MFI.
Formulations for the induction of regulatory T cells

microparticles are equally effective at inducing human Treg. The human-iTreg showed high expression of FoxP3 (Figure 5) and were also capable of proliferation (data not shown). Overall, our data suggest that these FactorMP have the potential to be used \textit{in vivo} for local Treg induction at sites of transplant rejection or autoimmunity.

We envisage these particulate formulations could be explored as an ‘off-the-shelf’ therapeutic for creating a local immunosuppressive environment and increasing the presence of Treg at sites of inflammation. We are currently testing these particles in \textit{in vivo} mouse models of destructive inflammation and autoimmunity where induction of immunological homeostasis may alleviate disease symptoms. Another possible application for such formulations would be to create an immunosuppressive lymph node like environment \textit{in vivo}, when used in combination with formulations that can recruit \cite{43} and activate \cite{44, 45} naïve T cells.

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REFERENCES


Formulations for the induction of regulatory T cells


Discussion, Future Perspectives and Summary
MAIN RESULTS AND INTERPRETATION

In this dissertation several studies and reviews are presented on the development of methods and therapies to improve the survival after heart transplantation.

Studies in Section 1 focus on infants requiring a heart transplant, whose immature immune system can serve as a window of opportunity to carry out a transplant with a much milder anti-donor response than that observed in adults. We investigated the effects of T cell-directed immunosuppressive therapy given to infant baboons, with or without a transplant, to study the immature immune response and improve this window of opportunity. Additionally, we investigated one common and one rare complication of immunosuppressive therapy that are seen both in humans and non-human primate (NHP) models, and discuss ways to reduce the incidence of these complications and the importance of early diagnosis.

Studies in Section 2 focus on novel therapies to reduce rejection, by testing regulatory T cells (Treg) for suppression of the anti-donor response in NHP. We studied the effect of alemtuzumab, a potent lymphocyte-depleting drug, on those Treg by in vitro and in vivo experiments, so that in future experiments these therapies could be used together. Furthermore, we investigated the possibility of generating Treg from a naïve T cell population, so that much larger numbers of suppressive cells could be obtained. These induced Treg (iTreg) could be used separately or in concert with naturally-occurring Treg therapy.

The immature immune system: opportunities for transplantation

A major hurdle in transplantation is the long waiting time to obtain a donor organ. This problem is particularly striking in infants with congenital heart defects, who often require heart Tx at a very young age (1).

During the neonatal period, the T cell compartment of laboratory mice is relatively lymphopenic and undergoes lymphopenia-induced proliferation (LIP) of T cells (2). It exhibits remarkable plasticity and is prone to tolerance induction after transplantation (reviewed in 3). Unlike rodent species, human neonates are born with an almost entirely functional immune system. However, there are several indications that the human neonatal immune system still has immature features. For example, the immune system of neonates is still antigen-inexperienced and consists of naïve T lymphocytes (4).
Natural anti-blood group A/B antibodies are usually absent during the first 3 months of life in humans and baboons, but subsequently develop during the first year (5, 6). The relative absence of antibodies during the first few months provides a ‘window of opportunity’ during which ABO-blood group-incompatible (ABO-I) heart Tx can be carried out successfully (7).

We investigated the effect of T cell-directed immunosuppressive therapy on both T cell homeostasis and antibody development in infant baboons. Some of these infant baboons received an artery patch transplant, as a model for heart transplantation. The transplants were either from an A or B blood group-incompatible donor baboon (AB-I allograft) or from a donor pig (xenograft).

Chapter 2 describes that T cell-based immunosuppressive therapy inhibits the development of natural anti-AB blood type and anti-pig antibodies in infant baboons. The main results are that in experimental group 1 (controls; healthy infant baboons that did not receive immunosuppressive therapy or a transplant and were followed from 1 – 18 months of age), anti-A/B and cytotoxic anti-pig IgM increased steadily during the first year. Experimental group 2 (infants who received a transplant at 3 months of age, but no immunosuppressive therapy) became sensitized to donor-specific AB-I or pig antigens within 2 weeks and showed strong donor-specific antibody production. This demonstrates that, despite the young age of the recipients, a small artery patch graft from either an AB-I or pig donor is sufficient to elicit donor-specific sensitization in infant baboons. The experimental groups 3 (transplanted at 3 months) and 4 (no transplant) infants that received anti-CD154 mAb made no or minimal anti-A/B and anti-pig antibodies while receiving immunosuppressive therapy. A striking observation was the finding that all infants receiving anti-CD154 mAb (n=5) showed a marked inhibition and delay in development of antibodies, even in the absence of an allograft or xenograft.

Chapter 3 demonstrates that the immune system of infant baboons is relatively lymphopenic and naïve, and T lymphocytes significantly increase in number with a transient appearance of memory T cells. Thus, we confirmed that neonatal NHP undergo lymphopenia-induced proliferation to establish T lymphocyte homeostasis. Unlike the results described in chapter 2, where delayed antibody production created a window of opportunity for transplantation, the T cell immune system was able to readily and actively mount an immune response against transplanted antigens. In group 3 infants, lymphocyte depletion by an anti-CD154 mAb was associated with the expansion of memory T cells, that may form an obstacle if immunologic tolerance is the goal. On the basis of the assays performed, we did not identify a T cell window of
opportunity for tolerance induction, possibly due to the lymphopenia-induced proliferation of memory T cells. However, anti-donor T cell responses could be suppressed by immunosuppressive therapy (artery patch transplants were not rejected) and T cell responses did not increase after immunosuppressive therapy was discontinued.

Altogether, these results lead to the conclusions that the development and homeostasis of the non-human primat (NHP) immune system is similar to that of humans and is therefore an adequate study model. Both humans and NHP show an absence of anti-blood group- and anti-pig antibodies until 3 months of age, after which they gradually develop. The immune systems of infants baboons and humans are both relatively lymphopenic and naïve at birth, and T lymphocytes significantly increase in the first months of life. Although we did not show B- or T-cell tolerance in our infant baboon study model, we do show that if an infant receives T-cell directed immunosuppressive therapy, even in the absence of a graft, the natural antibody production is delayed. This results in a longer window of opportunity for an ABO-incompatible transplant. This finding significantly increases the chance to find a suitable donor.

Complications of immunosuppressive therapy

One of the major problems of transplantation is the need for life-long immunosuppressive therapy to prevent rejection of the donor organ. Because this is a systemic therapy that affects the entire immune system, it is associated with many side effects; its main consequences are infections and malignancies (8).

Two different complications are presented in this thesis. The first, collagenous colitis, is an example of a rare side-effect that is difficult to diagnose and requires very specific treatment; the second is sepsis, a very common side-effect that is one of the major complications of IS therapy leading to a lot of co-morbidity in patients. Together, these studies do not only make us aware of the frequency and variety of the problems that can occur, but also make us aware of the delicate balance that transplant patients need in their medication; the daily line between illness due to too much immunosuppression, and rejection of the organ due to too little immunosuppression is very thin.

Chapter 4 describes a collagenous colitis-like condition in immunosuppressed infant baboons. It has been reported only once previously in baboons (9). However, we report four baboons suffering from similar clinical features. In two, the diagnosis of collagenous colitis was confirmed after euthanasia by histology; the other two survived because adequate treatment could be initiated once the diagnosis was made in the first two cases, strongly suggesting that collagenous colitis was the cause of disease in these infants as well. All four baboons received the same immunosup-
pressive therapy at time of onset of the disease. Interestingly, it has been reported in infants very rarely (10). Collagenous colitis has occasionally been reported in patients with organ transplants, but it is not a common side effect (11). Although most patients fully recover within weeks after administration of budesonide, it requires histology to make the diagnosis. In this study we provide a NHP model for the disease. Awareness of this disease, but more importantly, awareness of rare complications, could prevent unnecessary morbidity and mortality in transplant recipients.

Chapter 5 describes a syndrome of severe hypoglycemia and acidosis in young immunosuppressed diabetic monkeys and pigs, and its association with sepsis. Infection and sepsis are common side effects in patients with diabetes (12) and in those receiving immunosuppressive therapy after organ transplantation (13). The diagnosis of sepsis is more difficult in immunosuppressed laboratory animals and is mainly made by clinical features (lethargy, withdrawal, and/or anorexia). A rise in white blood cell count could lead to the diagnosis, but this may be absent in an immunosuppressed animal. A positive blood or tissue culture is usually necessary to confirm the diagnosis. We describe seven cases in which diabetes had been induced in laboratory animals (monkeys and pigs) that received immunosuppressive therapy. In all cases, sepsis developed and was further complicated by the occurrence of severe hypoglycemia and metabolic acidosis, resulting in death or the need for euthanasia in all animals in which it occurred.

Altogether, these findings illustrate the diversity of complications after immunosuppressive treatment. The high incidence of sepsis, that can lead to more serious complications, indicates the need for very cautious dosing of immunosuppressive drugs. The occurrence of rare complications such as collagenous colitis or hypoglycemia illustrates the importance of close observation and aggressive use of diagnostics when transplant patients present any symptom of concern. The short life expectancy of transplant recipients - 37 +/- 15 years for patients who received a heart transplant in childhood (14), and a 70% 5-year survival for adult heart transplant recipients who suffered from heart failure (15) - strongly shows the risk of complications that result from the drugs that are given to prevent rejection and keep these patients alive.

Tip the balance: T cell depletion and regulatory T cells

The main problem of immunosuppressive therapy is that it suppresses the patients' entire immune system, resulting in complications as discussed above. Regulatory T cells (Treg) are important in the control of immunity (16) and tolerance to auto- and allo- antigens in humans and mice (17, 18). These cells offer the possibility of being highly-specific and effective in suppressing the anti-donor response in the transplant
setting, with potential to provide long-term tolerance, without affecting the patients' immune response to common pathogens, such as viruses and bacteria.

Naturally-occurring Treg (nTreg), defined as CD4+ T cells that express the intracellular marker FoxP3+ (19) are about 5-10% of total CD4+ T cells in humans and NHP. nTreg can suppress the activation, proliferation, differentiation and effector function of various immune cells, including CD4+ and CD8+ T cells and B cells (16, 20, 21). nTreg can be isolated from the blood and expanded \textit{in vitro} to increase Treg cell numbers (22). However, the starting number of nTreg from a single blood draw is very low, and a high Treg number is needed to “tip the balance” between Treg and effector T cells (Teff), and suppress the remaining CD4+ T cells that will otherwise attack the donor organ.

Alemtuzumab is a humanized anti-CD52 monoclonal antibody that profoundly depletes lymphocytes, including CD4+ T effector cells, from the blood (23) and can be used to avoid transplant rejection (24). If alemtuzumab would be given to a transplant patient to suppress the immune response, the Treg-to-Teff ratio would instantly be much higher because most T cells are Teff, which would be depleted. However, many CD4+ T cells are not in the circulation, but remain in the lymph nodes.

We investigated several aspects of alemtuzumab depletion in relation to future Treg therapy. First, we carried out a literature search on the application of NHP Treg. We then studied the effect of alemtuzumab on depletion of lymphocytes and specifically Treg in the lymph node of NHP \textit{in vivo}. Furthermore, we investigated how alemtuzumab affects nTreg in the circulation in NHPs, and whether remaining alemtuzumab could deplete Treg that are expanded \textit{ex vivo}. We used a NHP model, as Treg in NHP are phenotypically and functionally similar to those in humans (reviewed in chapter 6), and Indonesian cynomolgus macaques specifically, since CD52 is not expressed on the erythrocytes of this species (25).

\textbf{Chapter 6} reviews the application of NHP Treg. NHP models have the inherent advantage of closely resembling the human condition and allow for preclinical testing. In this report too we find that in respect to Treg, the NHP model is very similar to the human. NHP Treg can be identified, isolated and expanded following practically identical protocols as used for human Treg, and suppressive capacity after expansion is comparable. Of clinical importance, several reports indicate that the Treg number required to achieve suppression of rejection may depend on several individual factors such as clinical symptoms, underlying disease, or CD4+ T cell numbers, rather than only on host body weight. The NHP model is the most suitable model to test these conditions prior to testing a novel cell therapy in a clinical trial.
Chapter 7 describes how alemtuzumab significantly depletes lymphocytes from the lymph nodes in NHP. Lymph nodes were obtained from healthy controls (Group 1, n=10), monkeys that only received alemtuzumab (Group 2, n=6), or monkeys that received alemtuzumab and a heart transplant (Group 3, n=5).

The main results showed a profound depletion of ~80% of absolute cell numbers of lymphocytes from the lymph nodes, though not as thoroughly as in the blood (>98% depletion). Lymphocytes remained significantly depleted until 3 months after alemtuzumab administration, after which they started to recover; at 12 months, absolute cell numbers reached near-normal levels. Furthermore, CD3⁺T cells and CD20⁺B cells were depleted equally at day 2, suggesting that alemtuzumab had an equally strong affinity for both cell types. However, there was a striking difference in recovery of these cells at later time-points; the proportion of CD3⁺T cells remained significantly reduced at 12 months while B cells had recovered back to normal. A similar pattern was observed in circulating lymphocytes in the blood (25). The ratio of CD4⁺ to CD8⁺ within the CD3⁺ T cell population remained unchanged at all time-points. Naïve and memory T cells were depleted equally, but as reported for repopulating cells in the blood, a faster recovery of memory T cells was observed that persisted for 12 months post-alemtuzumab. These results correlate with observations made in human and cynomolgus monkey blood, where effector memory T cell proportion of total lymphocytes was 2-3 fold increased (25).

Interestingly, when alemtuzumab was administered, a gradual decrease of FoxP3⁺ Treg (as percentage of total CD4⁺T cells) was observed in the lymph node; indeed, this reached statistical significance at 2-3 months post-alemtuzumab. In contrast, a transient increase in FoxP3⁺ Treg has been reported by others (26) and by our own institute (27) in the blood of human patients, which we confirmed in our Group 3 monkeys (Dons et al, submitted).

Chapter 8 describes the finding that ex vivo-expanded NHP Treg are resistant to alemtuzumab-mediated, complement-dependent cytotoxicity. We investigated the influence of (i) in vitro added alemtuzumab on ex vivo-expanded nTreg that could potentially be used in adoptive cell therapy and (ii) serum containing in vivo infused alemtuzumab on ex vivo-expanded nTreg in Indonesian cynomolgus monkeys.

The main results are that expanded monkey nTreg exhibited considerably lower expression of CD52 than either fresh bulk T cells or freshly isolated Treg. Correlating with the decreased expression, the expanded Treg exhibited no binding and no complement-mediated killing by high dose alemtuzumab in vitro. From these results
we concluded that expanded monkey Treg are resistant to alemtuzumab-mediated, complement-dependent cytotoxicity. Furthermore, after intravenous infusion of alemtuzumab *in vivo*, monkey serum killed fresh lymphocytes, but not expanded Treg *in vitro* experiments. Alemtuzumab has a comparatively long half-life in humans and cynomolgus monkeys of approximately 8d, and a much longer lymphocyte-depleting effect in the former of about 2 months (28). Our data suggest that if Treg can be isolated and expanded from a graft recipient before alemtuzumab administration, then the expanded cells could be administered to the lymphocyte-depleted host at any time after transplantation in the presence of circulating alemtuzumab, without risk of complement-mediated killing.

In addition to the *in vitro* experiments, the depletion and recovery of FoxP3⁺ nTreg in the circulation was analysed after alemtuzumab was administered to the monkeys. All lymphocytes in the circulation were depleted significantly (>98%), including nTreg. However, the recovery of Treg was much faster than of other CD4⁺ T cells, resulting in a transient increase in the percentage of Treg starting about two weeks after the initial alemtuzumab dose, and persisting for approximately 4-5 further weeks. This transient increase in circulating Treg has also been demonstrated, although for a longer period, in human kidney transplant recipients receiving alemtuzumab (26). In the study by Bloom et al, *de novo* generation of Treg was suggested and could be explained by the induction of Treg *in vivo* (described in chapter 9). In contrast, as we described in chapter 7, FoxP3⁺ Treg in the lymph nodes were reduced at 2-3 months after alemtuzumab administration. It is known that lymphocyte depletion occurs mainly in the blood and is lowest in the bone marrow (29). Human bone marrow, where the cells are relatively spared from alemtuzumab-mediated killing (30), has a much higher proportion of Treg than blood. This could explain the relatively rapid repopulation of Treg in the blood. Additionally, Treg remaining in the lymph nodes, which also has a higher proportion of Treg than the circulation, may receive a similar signal that promotes their entry into the circulation rather than remaining in the lymph nodes. This would explain the decrease in FoxP3⁺ Treg in the LN after alemtuzumab infusion.

Altogether, these findings suggest that the NHP is an adequate model to study *in vitro* and *in vivo* Treg properties prior to clinical application in human transplant patients. We have demonstrated that *in vivo* infused alemtuzumab profoundly depletes lymphocytes from the blood as well as the lymph node in NHPs. We show that NHP Treg can be isolated and expanded following a similar protocol as is used for human Treg, and expanded Treg exhibit a similar suppressive function. Following *ex vivo* expansion, Treg downregulate CD52 expression and are not susceptible to complement-mediated killing by alemtuzumab. This suggests that expanded Treg can be infused
into a transplant recipient at any time after alemtuzumab infusion, without the risk of alemtuzumab-mediated depletion.

**A novel approach: the induction of regulatory T cells**
Adoptive transfer of naturally occurring FoxP3+ Treg has been studied abundantly and is successful in animal models of autoimmune diseases and transplantation (31, 32). However, as the frequency of nTreg in healthy humans is only ~5% of CD4+ T cells, it is difficult to obtain sufficient numbers of these cells for clinical testing (33, 34).

To address this issue, various groups have sought to generate Treg from the much more abundant naïve CD4+ T cell starting population. Beginning in 2002, the first reports were published on induced Treg (iTreg), that are CD4+CD25+ and FoxP3+, and have suppressive capacity (35, 36, 37), similar to nTreg. Unlike nTreg, that are generated in the thymus, these iTreg are generated *in vivo* from naïve CD4+ T cells in peripheral lymphoid organs during immunological responses to antigen stimulation, a phenomenon that is replicated *in vitro* through stimulation of mouse or human CD4+ T cells under specific conditions.

**Chapter 9** presents a review of the current literature on iTreg, and describes the mechanisms of conversion and suppressive potential that have been reported. The induction of a regulatory phenotype from naïve CD4+CD25− Teff has become a well-established approach to convert large numbers of T cells into functional Treg. Although various methods for the generation of iTreg have been published, a consensus exists in regard to the basic procedure of induction. The specific combination of the cytokines IL-2 and TGF-β together with low costimulation can generate potently suppressive FoxP3+ iTreg. Rapamycin and all-trans retinoic acid are proven to be valuable, as the addition of these reagents not only enhances FoxP3 induction, but also actively prevents the differentiation of naïve T cells into Th17 cells, thus providing a more stable phenotype. iTreg have proven to be stable and potent suppressor cells that seem to be of similar suppressive strength to nTreg. As significantly higher numbers of Treg can be obtained through iTreg than by expansion of nTreg, this novel approach has the potential for use in large-scale clinical trials.

**Chapter 10** describes controlled release formulations of IL-2, TGF-β1 and rapamycin for the induction of Treg. We report the development and *in vitro* testing of a Treg-inducing synthetic formulation (FactorMP) that consists of controlled release vehicles for a combination of cytokines and drugs that have previously been reported to induce Treg (IL-2, TGF-β and rapamycin). We demonstrate that IL-2, TGF-β and rapamycin are released over 3-4 weeks from these formulations. Additionally, Treg induced in
the presence of these formulations expressed the markers representative for Treg phenotype, and suppressed naïve T cell proliferation (function) at levels similar to expanded nTreg. Most importantly, we show that these release formulations are capable of inducing FoxP3⁺ Treg in human cells in vitro. Overall, our data suggest that these FactorMP have the potential to be used not only for in vitro expansion, but also in vivo for local Treg induction at sites of transplant rejection or autoimmunity. We envisage these particulate formulations could be explored as an ‘off-the-shelf’ therapeutic for creating a local immunosuppressive environment and increasing the presence of Treg at sites of inflammation.

Altogether, these findings illustrate how novel methods can provide alternative means to achieve suppression of the immune response against a transplant, and potentially lead to tolerance to the graft by tipping the balance between regulatory T cells and T effector cells. The induction of Treg could be of major importance to help tip this balance toward tolerance instead of rejection. The application that allows controlled release of the appropriate cytokines may enable an in vivo approach to achieve immunological homeostasis in transplant patients in the future.

METHODOLOGICAL CONSIDERATIONS

Studies that use in vitro experiments such as presented in this dissertation, have the limitation that each of the assays can not adequately reproduce the in vivo situation. Results based on only laboratory experiments are therefore never fully representative of the clinical setting. On the other hand, the number of experiments that can be carried out in vivo in a preclinical setting, such as presented here, are limited in a different way. These large animal models are costly and time-consuming, and are mostly used as proof of principle. The number needed to obtain statistical significance, which is the foundation of evidence-based medicine, is not always reached. Nevertheless, large animal models are considered indispensable to translate results found by in vitro assays and studies in the rodent model, before application in the clinical setting.

FUTURE PERSPECTIVES

This dissertation has lead to various new insights in the field of heart transplantation. However, improvement of the clinical outcome is the ultimate goal, while the experiments carried out for this thesis were either in vitro assays or in vivo large animal
modesl, which are different from the clinical situation. Here we try to envision potential clinical applications for the results of these studies.

The Window of Opportunity in the immature immune system
Our finding that anti-CD154mAb-based immunosuppressive therapy can delay natural antibody production in infants has implications for clinical organ transplantation. An infant needing a heart transplant at birth could possibly be maintained on anti-CD-154mAb-based immunosuppressive therapy to prevent natural antibody production while waiting for a donor organ. Although in our study in baboons this effect was only seen with anti-CD154mAb, further studies involving combinations of immunosuppressive agents might possibly achieve the same result. This window of opportunity was only seen for antibody production, and not for T cell development. Therefore, T cell-directed immunosuppressive therapy would still need to be given. Furthermore, if a xenograft were to be transplanted into a human, the immature immune system of infants would be advantageous, as it offers the possibility of modulating its immune response.

Theoretically, if a pig organ was transplanted early in infancy, the child might never develop antibodies to the graft, and conventional T cell-directed immunosuppressive therapy might be sufficient to maintain the graft. The pig heart could provide at least a ‘bridge’ for the patient until a suitable human donor heart becomes available (38). This might be a particularly successful approach if the heart is taken from a genetically-modified pig (39, 40, 41), thus reducing the humoral and cellular immunological responses further (42, 43, 44). Pig hearts of suitable size for infants could readily be obtained.

However, our experience is that the response to a pig organ in NHP is complex. In addition to natural antibodies and the adaptive cellular response, it involves an innate cellular response, coagulation dysregulation, and a marked inflammatory response (43). Nevertheless, particularly with regard to genetically-modified pig heart transplantation, it might provide a bridge to allotransplantation.

Regulatory T cells to overcome complications of immunosuppressive drugs
Systemic immunosuppressive therapy is known to cause frequent and serious complications and side effects. This is a particularly striking problem in transplant recipients who are required to take these drugs for the remainder of their lives. Chapters 4 and 5 illustrate examples of these complications that were observed in our NHP model, but that are also relevant in the clinical setting. One therapy that has great potential to bypass these systemic drugs, is the development of Treg, particularly if these cells can
be made suppressive in a donor-specific manner. If Treg therapy can be optimized for clinical use, lower doses of systemic immunosuppressive therapy would be needed, which may result in fewer or less severe complications. This would greatly enhance the quality of life as well as survival of these patients.

Regulatory T cells in combination with T cell-directed immunosuppression
We have shown that it may be feasible to administer ex vivo expanded Treg to transplant recipients without the Treg being killed by circulating levels of alemtuzumab remaining at the time of Treg infusion. We have also shown that alemtuzumab profoundly depletes lymphocytes not only from the blood, but also from the lymph nodes in non-human primates. These findings further support the theory that alemtuzumab is an effective T cell-directed induction therapy. It could be used to deplete the blood as well as the lymph nodes from effector T cells, which, in combination with adoptive transfer of ex vivo-expanded regulatory T cells, would give a very favourable Treg-to-Teffector ratio. This combination could be tested in a preclinical setting, such as the NHP transplant model. Further development of NHP Treg could be in the xenogeneic setting (pig-to-primate, with pig-to-human as the ultimate goal), which offers the consistent advantage of donor identification before transplantation. If Treg can be developed to specifically suppress the NHP (or human) anti-pig response by in vitro assays, this could justify preclinical studies in this field.

Induced regulatory T cells
Regulatory T cells have been the subject of intense investigation for the last decade, and as the first clinical trials to ascertain the safety of ex vivo expanded nTreg infusion in humans treated for graft-versus-host disease are being initiated (45, 46), determination of the necessary numbers of Treg has become increasingly pressing. However, Treg-based cellular therapies face many challenges, which include, but are not limited to: (i) difficulties in isolating pure and homogenous populations and large quantities of Treg from the blood, (ii) inconsistent maintenance of the Treg phenotype and suppressive function post-proliferation, and (iii) the need for good manufacturing practice (GMP) facilities (47, 48, 49). Hence, acellular therapies that can increase numbers and/or the suppressive potency of Treg without the need for ex vivo culture could be transformative. The generation of iTreg from a much more abundant naïve T cell starting population may be the solution. Perhaps optimal results will be achieved when both cell types are being used in concert, a concept that still needs to be explored. Importantly, a detailed comparison of the efficacy of nTreg versus iTreg in vivo would be required before this can be translated to clinical trials in human transplant patients. We envisage these particle formulations could be explored as an off-the-shelf therapeutic for creating a local immunosuppressive environment and increasing
the presence of Treg at sites of inflammation. Another possible application for such formulations would be to create an immunosuppressive lymph node like environment in vivo, when used in combination with formulations that can recruit (50) and activate (51, 52) naïve T cells.

SUMMARY

In summary, studies in section 1 show that T cell-directed immunosuppressive therapy decreases production of natural antibodies when administered to immature non-human primate recipients. This results in a larger window for transplantation of a blood-type incompatible, or possibly a xenogeneic heart transplant, thereby increasing the number of potential donors. However, it does not result in T cell tolerance; infants would still need to receive immunosuppressive therapy to suppress the cell-mediated rejection. Other studies in this section show that immunosuppressive therapy, which is necessary to keep the recipient's immune system from rejecting the transplanted organ, can lead to significant side effects such as sepsis and gastro-intestinal disease in immature recipients.

Studies in section 2 show the potent lymphocyte-depleting effect of alemtuzumab not only in the blood but also in the lymph nodes of adult non-human primates receiving a heart transplant. By depleting the reservoir of lymphocytes in the lymph nodes, a prolonged time of depletion can be achieved. Regulatory T cells are known to enhance tolerance to a donor organ. We show that monkey regulatory T cells are functionally suppressive after their number was expanded by ex-vivo culture, and that after expansion these cells were no longer subject to alemtuzumab-mediated cell killing. This observation will enable studies where high numbers of these cells can be administered to a patient who has received alemtuzumab. Moreover, we studied an alternative way to generate a high number of regulatory T cells, by converting naïve T cells during ex vivo culture. These cells were indeed suppressive, and can potentially be used in concert with their naturally-occurring counterparts.
5. Fong SW, Qaqundah BY, Taylor WF. Developmental patterns of ABO isoagglutinins in normal children correlated with the effects of age, sex, and maternal isoagglutinins. Transfusion 1974;14(6):551-559.
Samenvatting
SAMENVATTING

Harttransplantatie kan de levensverwachting van hartpatienten significant verlengen. Voor zowel volwassen patienten die lijden aan hartfalen, als neonaten die ter wereld komen met een aangeboren hartafwijking, kan een harttransplantatie levensreddend zijn. Het ultieme doel van transplantatie is het vinden van een behandeling die leidt tot immunologische tolerantie ten opzichte van het donor orgaan, zonder dat daarbij systemische bijwerkingen optreden, en waarbij geen levenslange medicatie noodzakelijk is. Dit proefschrift, onderverdeeld in 2 secties, beschrijft studies gericht op het verbeteren van deze verschillende aspecten.

Sectie 1: T cel-gerichtte immuunsuppressieve therapie bij transplantatie in neonatale primaten

Studies in Sectie 1 zijn gericht op harttransplantatie voor aangeboren hartafwijkingen bij neonaten. Het jonge immuunsysteem biedt perspectieven voor transplantatie gedurende de eerste 3 levensmaanden (window of opportunity). Gedurende deze periode hebben zij nog geen natuurlijke antistoffen ontwikkeld tegen de niet-eigen bloedgroep en tegen andere diersoorten die afstoting kunnen veroorzaken. Hoofdstuk 2 laat zien dat T cell gerichtte immuunsuppressieve therapie de productie van deze natuurlijke antistoffen vermindert wanneer deze therapie aan jonge primaten wordt gegeven. Dit resulteert in een verlenging van de “window of opportunity”. Door deze therapie aan zuigelingen te geven die geboren worden met een aangeboren hartafwijking waarvoor harttransplantatie noodzakelijk is, zullen zij deze natuurlijke antistoffen niet ontwikkelen en kan er een hart van een andere bloedgroep worden getransplanteerd.

In hoofdstuk 3 wordt de ontwikkeling van de T cel component van het immuunsysteem in jonge primaten beschreven. Wij observeerden dat zij bij de geboorte een lymfopeen en naïef immuunsysteem hebben, wat overigens wel in staat is tot afstoting van een transplantaat indien er geen immuunsuppressieve therapie wordt toegediend. Deze zuigelingen zullen dus na transplantatie wel T cel gerichte immuunsuppressieve therapie moeten krijgen om afstoting te voorkomen. Wanneer deze therapie werd toegediend, werd een homeostatische proliferatie van geheugen T cellen waargenomen.

Andere studies in sectie 1 laten verschillende voorbeelden zien van significante bijwerkingen en complicaties van systemische immuunsuppressie, zoals sepsis en gastro-intestinale aandoeningen, die wij geconstateerd hebben in jonge primaten. In hoofdstuk 4 en 5 wordt beschreven hoe deze complicaties kunnen worden gediagnostiseerd en behandeld.
**Sectie 2: Alemtuzumab en regulatoire T cellen voor harttransplantatie in primaten**

Studies in Sectie 2 beschrijven nieuwe therapieën om afstoting te voorkomen door gebruik te maken van regulatoire T cellen (Treg). Treg zijn een specifieke subgroep van T cellen die tolerantie van een donor orgaan kunnen bewerkstelligen. Echter, in de natuurlijke situatie bestaan zij uit slechts ~5% van het totale aantal T cellen; de overige 95% zijn effector T cellen die juist voor afstoting zorgen. Om een effectieve onderdrukking van de afstoting te kunnen bewerkstelligen, zal ofwel het aantal Treg sterk vermeerderd moeten worden, ofwel het aantal effector T cellen verminderd.

**Hoofdstuk 6** geeft een overzicht van de literatuur omtrent Treg in primaten, welke erg vergelijkbaar zijn met humane Treg. Er wordt beschreven welke technieken er beschikbaar zijn om de Treg van de overige lymfocyten uit het bloed te isoleren, en in aantal te vermeerderen voor gebruik in pre-klinische studies.

Hoofdstuk 7 en 8 beschrijven toepassingen van de immuunsuppressieve medicatie alemtuzumab in relatie tot Treg en transplantatie. Alemtuzumab bindt zich aan lymfocyten en laat deze afsterven; in mensen en primaten verwijdert het vrijwel alle lymfocyten uit de bloedbaan. Deze cellen verzamelen zich echter vooral in de lymfeklieren. In **hoofdstuk 7** wordt aangetoond dat alemtuzumab 75-80% van de lymfocyten verwijdert uit de lymfeklieren in primaten. Dit kan verklaren waarom alemtuzumab een langdurig lymfocyten-verlagende werking heeft. Echter, Treg verzamelen zich van nature in relatief hoge proporties in de lymfeklieren, en ook de Treg werden door alemtuzumab vernietigd. Vergelijkbaar met de homeostatische proliferatie in de bloedbaan (hoofdstuk 3), wordt ook in de lymfeklieren een proliferatie van geheugen T cellen waargenomen na de toediening van immuunsuppressieve therapie.

In **hoofdstuk 8** laten wij zien dat Treg van primaten kunnen worden geïsoleerd en in het laboratorium kunnen worden vermeerderd (door middel van kweek). Na deze kweek, waarbij de cellen 100-maal in aantal vermeerderd werden, hebben de Treg nog steeds een immuun-onderdrukkende functie. Bovendien werd aangetoond dat de Treg na de kweek resistent zijn tegen de toxiciteit van alemtuzumab. Een mogelijke klinische toepassing kan zijn dat Treg van een patiënt geïsoleerd worden, waarna de patiënt een (hart) transplantatie en alemtuzumab immuunsuppressie krijgt. Hierna zouden de Treg gekweekt kunnen worden en enkele weken later in groot aantal aan de patiënt worden teruggegeven. Dit heeft de voordelen dat (1) de patiënt op dat moment zeer lage aantallen T effector cellen heeft, en de Treg-tot-T effector ratio dus relatief zeer gunstig is, en (2) de gekweekte Treg zullen niet door de alemtuzumab afsterven.
Omdat natuurlijk-voorkomende Treg slechts in kleine aantallen voorkomen, wordt er ook gezocht naar alternatieve manieren om grote aantallen Treg te genereren. Een veelbelovende methode is het omzetten van naive T cellen naar Treg, door gebruik te maken van specifieke stimulatie en cytokines. In hoofdstuk 9 worden de meest recente inzichten op dit gebied besproken, waarbij ook aandacht wordt geschonken aan het bevorderen van de stabiliteit van deze cellen.

In hoofdstuk 10 laten wij zien dat humane T cellen door middel van specifieke kweek kunnen worden omgezet naar Treg, waarbij deze cellen na de kweek een immuunonderdrukkende functie hebben. Een vergelijkbaar experiment in muizen liet zien dat deze omzetting niet alleen in kweek, maar ook in een levend organisme wordt gerealiseerd. Bovendien werd in deze studie de omzetting van T cellen naar Treg gestimuleerd door middel van micropartikels. Wij hebben micropartikels gegenereerd die gedurende enkele weken de gewenste cytokines gereguleerd afgeven. Een mogelijke toepassing kan zijn dat de micropartikels lokaal kunnen worden aangebracht, bijvoorbeeld bij het transplantaat, zodat lokale aanmaak van Treg gestimuleerd wordt. De omgezette Treg kunnen samen met de natuurlijk voorkomende Treg als therapie gebruikt worden.
Appendices

- Color Figures
- Acknowledgements / Dankwoord
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- About the author
COLOR FIGURES

Chapter 2 - Supplementary Figure 1: Photograph of segment of donor carotid artery placed as an onlay patch graft into the wall of the recipient infra-renal aorta. The graft is measured approximately 1.0 x 0.5 cm (arrow).
Chapter 2 - Supplementary Figure 2: Anti-A, -B and -Gal staining of artery patch grafts in Group 3 infant baboons.

(A) Positive B5008 (blood group A) AB-I graft stained for the B antigen, showing remaining B antigens on the graft endothelium (X40)

(B) Positive B7507 (blood group B) AB-I graft stained for the A antigen, showing remaining A antigens in the graft vasa vasorum (X40)

(C) Positive B5508 (blood group B) WT pig graft stained for Gal, showing no remaining Gal antigens in the graft endothelium or adventitia (X20)

(D) Positive B5708 (blood group B) WT pig graft stained for Gal, showing remaining Gal antigens in the graft endothelium and vasa vasorum (X10)

(E) Positive control for A staining; native aorta of blood group A infant baboon (X40) (similar to B staining in native blood group B aorta)

(F) Negative control for A staining; native aorta of blood group B infant baboon (X40) (similar to B staining in native blood group A aorta)

(G) Positive control for Gal staining; carotid artery of WT pig (X40)

(H) Negative control for Gal staining; native aorta of blood group B infant baboon (X40)

Arrows indicate positive staining; Solid arrows: anti-A or -B staining; Dotted arrows: anti-Gal staining; Red: Anti-A, -B or -Gal staining [FITC]; blue: cell nucleus [DAPI]; green: collagenous fibers [autofluorescent].
Chapter 3 - Figure 4: Stimulation index after mixed lymphocyte reaction (MLR) in control and transplanted (Group 2) baboons. Responses in healthy infant baboons (Control) are compared with responses in baboons after AB-I allo-Tx (A, B) and WT pig xeno-Tx (C, D) in the presence (IS) or absence (No IS) of immunosuppressive therapy. Data for B7607 after allo-stimulation are missing due to technical error (E). (E) Graft histology 6 weeks after Tx without IS (Group 2A) showed fibrosis and heavy lymphocytic infiltration. Normal vascular histology can no longer be recognized.
Chapter 4 - Figure 3: Colon H&E and collagen deposition
H&E staining (top) and collagen staining (Trichrome, bottom) of the colon. B7507 (A), B5508 (B), B5008 (C) and B5708 (D) (magnification x4). A moderate to extensive increase in collagen can be seen within the submucosa (asterix, top figures) and is illustrated by blue staining by Trichrome (bottom figures). Mild to extensive collagen deposition is also present between the glands within mucosa (arrow). The most severe changes are illustrated in B7507 with a thick layer of dense submucosal fibrous connective tissue and regions of complete mucosal epithelial cell loss secondary to collagen deposition.

Chapter 4 - Figure 4: Small intestine H&E and collagen deposition
H&E staining (top) and collagen staining (Trichrome, bottom) of the small intestine of B7507 (A), B5508 (B), B5008 (C) and B5708 (D) (magnification x10). A marked increase in collagen is found in both the lamina propria (arrow) and submucosa (asterix) of B7507 and B5508 in addition to moderate (B7507) to severe (B5508) blunting and loss of villi with necrosis and loss of surface enterocytes (arrowhead). Mild villous blunting is present in B5508. The small intestine of B5008 and B5708s has a normal appearance.
Chapter 6 - Figure 1. (A), Issues that govern the successful application of Treg therapy in transplantation.
Chapter 7 - Figure 7: Immunofluorescence: loss of lymphocytes in LN visualized

LN tissue was fixed in 10% formalin and stained for CD3⁺ (green) and CD20⁺ (red). Pre-alemtuzumab samples (left side) from M173-08 (A) and M170-08 (C) showed a dense population of both CD3⁺ and CD20⁺ cells with normal architecture. LN biopsies at 2 weeks post-alemtuzumab (right side) showed evidence of depletion of both CD3⁺ and CD20⁺ lymphocytes when either 1 dose (M173-08, B) or two doses (M170-08, D) of alemtuzumab had been administered. The remaining cells in the LN appeared to have a higher proportion of CD20⁺ cells (red) in both post-alemtuzumab samples.
Chapter 7 - Figure 8: Continuing loss of cells in LN with cumulative doses of alemtuzumab

LN biopsies of M171-08 (3 weekly doses of 20-20-20mg/kg) and M175-08 (5 weekly doses of 20-10-10-10-10 mg/kg) showed a dense population of CD3+ and CD20+ lymphocytes in the pre-treatment biopsy (A and D), similar to M173-08 and M170-08 (Figure 7). When weekly doses of alemtuzumab were administered for a longer period of time, a continuing depleting effect was observed. Both M171-08 and M175-08 showed some depletion of T and B cells in the biopsy taken at 2 weeks (B and E, respectively), which was more profound at 1 month (C and F, respectively).
Chapter 8 - Figure 2: Expanded Treg significantly down-regulate CD52 expression and are not bound by alemtuzumab.

(A) T cells from peripheral blood or LN were stained for CD3, CD4, CD25 and CD52 (n=3). CD52 MFI was analyzed for bulk T cells, fresh (unexpanded) Treg (gated for CD4+ CD25hiCD127-) and fresh (unexpanded) Teff (gated for CD4+CD25+). In the same experiment, Treg and Teff cells were analyzed for CD52 after round 1 and 2 of expansion (all conditions n=3). Unstimulated Treg (within the PBMC or LN cell population) expressed a higher level of CD52 than Teff from the same cell source. Expanded cells showed a decrease in CD52 expression after the first round of expansion, which was similar for Treg and Teff, with a further decrease after the second round of expansion (n=3 for all conditions). *p<0.05.

(B) Alemtuzumab does not bind to expanded Treg. Cells were incubated with alemtuzumab at concentrations of 0.001-100 µg/ml to determine binding of alemtuzumab. Binding was expressed as relative MFI. Alemtuzumab exhibited concentration-dependent binding to freshly-isolated PBMC, as well as to freshly-isolated Treg and Teff (n=3). By contrast, alemtuzumab showed no binding to expanded Treg (n=3) and expanded Teff (n=3) at any concentration. *p<0.05; significance calculated between fresh T cells (n=3) and expanded Treg (n=3).
Appendices

Chapter 9 - Figure 1: Human iTreg generation with TGF-b and ATRA

Human CD4+ T cells were obtained by magnetic bead sorting (CD4+ kit, Miltenyi) of PBMC (purity >90%, 5% FoxP3+) and stimulated with high-dose IL-2 +/- TGF-b (20 ng/ml) +/- ATRA (10 ng/ml). After 6 days, live CD4+ cells were assessed for FoxP3 expression by flow cytometry. Cells cultured in the presence of TGF-b + ATRA showed 50% FoxP3+ expression while cells cultured with only IL-2 were only 25% FoxP3+; the remaining 75% of the cells showed an intermediate level of FoxP3.

(A) Gating for FoxP3+ cells (horizontal bar). Red: isotype; green: no TGF-b, no ATRA; blue: TGF-b only; brown: TGF-b + ATRA.

(B) Quantification of FoxP3+ cells: 50% of cells cultured with TGF-b + ATRA acquired FoxP3+ phenotype.
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A special thanks goes out to my Japanese colleagues Hidetaka, Hayato, Osamu and Koshi: thank you for teaching me your language...or at least some of the important words! Osamu and Shoko, thank you for the Farewell run. It nearly made me miss my flight back but it was so worth it!
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I’ve shared my house with several roomies – and I’ve been very lucky that all of them were fun and friendly. JAY! Thank you for bringing Kait and Gunner into the house. And robots. Living with you really made it feel like a home. I miss our house!

De “Dutch in Pittsburgh”; Paul & Silvia, Judith, Mark, Martin: ontzettend leuk om Koninginnedag en Sinterklaas samen te vieren, en het WK natuurlijk niet te vergeten! Tijd voor een “Yinzers in Holland” club voor Pittsburgh veteranen...?

Living in Pittsburgh surely has made me become very sporty, and that is reflected in the friends that I’ve made...Camila, it is because of you that I first picked up running again, and only because you tricked me into it. “Do you want to make a weekend trip to Chicago? Oh, there is also a half marathon.” Thank you for that...and for introducing me to the girls’ nights with Sangeetha and Amira. My rowing team Rif Raf: I feel very lucky to have ended up in this team! Every week it felt like a summer vacation, rowing along that beautiful Pittsburgh skyline and grilling out after the training. Are we still doing that race in Venice? Amy and I are counting on you. Captain Mike, thank you for also getting me started in the wonderful world of road biking! Aaron, thank you for introducing me to Cortney and setting up the marathon training...I could not have made it through the Pittsburgh marathon without our huge-breakfast-after-training schedule. Cortney! I am so happy I got to know you and Dale...thank you for taking me to that triathlon weekend, cheering me all the way through the 10k, and for organizing your wedding before I had to leave. “Pittsburgh is my Playground” Amber: you are awesome. The first time we met, you made me run up all the 36 flights of stairs in the Cathedral of Learning. Five times. So I just knew we had to become friends. I am very proud of you for finishing your first Ironman, and hope to one day be able to accomplish the same thing. Keep smiling!

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Lieve Maurice. We zijn samen aan dit avontuur begonnen en hebben ontzettend veel meegemaakt. Het is niet altijd alleen maar gemakkelijk geweest, maar ik hoop dat je er met net zoveel plezier op terugkijkt als ik!

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Lieve papa, mama, en Charles, bedankt voor alles! Jullie zijn er altijd voor mij.
CURRICULUM VITAE

Opleiding:
- Sept 2000 – Mei 2001: Geneeskunde, Universiteit Antwerpen, Belgie

Klinische training:
- Dec 2007 – Feb 2008: Oudste co-schap Heelkunde, Sint Franciscus Gasthuis, Rotterdam (Dr. Kerver, MD)
- Aug 2007 – Sept 2007: Co-schap Public Health bij Child In Need Institute (CINI), Kolkata, India
- Juni 2004 – Juli 2004: Klinische uitwisselingsstage, kindergeneeskunde, Ospedale Sant'Anna, academisch ziekenhuis van Ferrara, Italie
- Feb 2004: Keuzevak: kinderchirurgie, Erasmus MC-Sophia

Klinische werkervaring:
- Mrt 2008 – Sept 2008: ANIOS spoedeisende hulp, Lievensberg ziekenhuis, Bergen op Zoom. Tevens kleine verrichtingen op polikliniek heelkunde (Dr. T. Bikkers)
- Apr 2005 – Juli 2005: PIAMA onderzoek (Prevention and Intervention of Asthma and Mite Allergy) in Erasmus MC-Sophia; anamnese, longfunctie tests, bloed afname en allergie-huidtests bij kinderen van 7-8 jaar oud.
Chapter 13

Studententeam Thoraxchirurgie, Erasmus MC. Pre- en post-operatieve zorg voor cardiale en pulmonale patienten.


Research:

Oct 2008 – Aug 2012 Promotieonderzoek aan Thomas E. Starzl Transplantation Institute, UPMC, University of Pittsburgh, Pittsburgh, PA, USA. (mentoren: Prof. D.K.C. Cooper, MD, PhD, FRCS (UPMC), Prof. A.W. Thomson, PhD, (UPMC) en Prof. J.N.M. IJzermans, MD, PhD (Erasmus MC Rotterdam)


Sept 2003 – Dec 2003 Research project: stretch of fibroblasts, Department of Plastic and Reconstructive Surgery, Erasmus MC.

Beurzen:

Juli 2010 – Aug 2012 5-jarige beurs van National Institute of Health (NIH, USA), voor onderzoeksvoorstel “Alemtuzumab and Regulatory T cells for Heart Transplant Tolerance in Monkeys”, deels gebaseerd op mijn preliminaire data (postdoctoral research associate)

Jan 2010 – Juli 2010 Stichting Professor Michael van Vloten Fonds beurs


Bestuursfuncties:

Jan 2009 – Jan 2011 “Dutch in Pittsburgh” activiteitencommissie


Sept 2004 – Feb 2005 Gala commissie van MFVR.


Cursussen:
Jan 2009 – Feb 2009  Flow cytometrie, Starzl Transplantation Institute, University of Pittsburgh, USA.
Juni 2008, April 2012  ATLS: Advanced Trauma Life Support course via UPMC, University of Pittsburgh (recent hernieuwd)
Mei 2008  LAS: Laboratory Animal Science via Erasmus Universiteit Rotterdam.
Nov 2002 – Mei 2003  Medische filosofie via de Capita Selecta commissie van MFVR (Medische Faculteits Vereniging Rotterdam)

Overige interesses:
Juni 2006  Sculptuur expositie op Talent Festival, Rotterdam (eigen werk)
Sept 2000 – Mrt 2002  Sculpturen voor carnavals vereniging Variant, Bergen op Zoom gedurende 2 seizoenen, beide keren 1e prijs.
PHD PORTFOLIO

Name: Eefje M. Dons
Erasmus MC Department: Surgery
PhD period: 2008 - 2012
Supervisors: Prof. Jan N. M. IJzermans, MD, PhD
Prof. David K.C. Cooper, MD, PhD, FRCS

Research period: 2008 – 2012 Starzl Transplantation Institute, Pittsburgh, USA

COURSES

Jan 2009 – Feb 2009  Flow cytometry. By Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, PA.
June 2008, April 2012  ATLS: Advanced Trauma Life Support course. UPMC, University of Pittsburgh, Pittsburgh, PA (+ 4-year renewal)
May 2008   LAS: Laboratory Animal Science. Erasmus University Rotterdam.
Nov 2002 – May 2003  Medical philosophy. By Capita Selecta committee, MFVR (Medische Faculteits Vereniging Rotterdam), Rotterdam, the Netherlands

PUBLICATIONS


the development of natural anti-AB blood type and anti-pig antibodies in infant baboons. Transplantation. 2012 Apr 27;93(8):769-76. (original article)


Fujita M, McGrath KM, Dons EM, Kumar G, Bottino R, Echeverri GJ, Hata J, Haruma K, Cooper DKC, Hara H. Technique of Endoscopic Biopsy of Islet Allografts Transplanted into the Gastric Submucosal Space in Pigs. Status: Accepted for publication, Cell Transplantation (original article)
Dons EM, Zhang H, Raimondi G, IJzermans JNM, Cooper DKC, Thomson AW. *Ex Vivo Expanded Regulatory T cells are Resistant to Alemtuzumab-Mediated, Complement-Dependent Cytotoxicity*
Status: Submitted (original article)

Dons EM, Van der Windt DJ, IJzermans JNM, Cooper DKC. *Alemtuzumab Significantly Depletes Lymphocytes from the Lymph Nodes.*
Status: Submitted (original article)

Status: Final preparations (original article)

**SCIENTIFIC PRESENTATIONS**
American Transplant Congress May 2013, Seattle, WA, USA, Poster presentation: *Ex vivo-expanded non-human primate regulatory T cells are resistant to Alemtuzumab-mediated, complement-dependent cytotoxicity*

American Transplant Congress May 2013, Seattle, WA, USA, Poster presentation: *Differential depletion and recovery of lymphocyte subsets in lymph nodes of Alemtuzumab-treated cynomolgus monkeys*
Young Investigator Award

Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Presentation for Award winners of the Annual Thomas E. Starzl Prize in Surgery and Immunology, Drs. A.B. Cosimi, PhD, and Dr. D.H.Sachs, MD, PhD, Professors of Surgery at Harvard Medical School.
May 2012; Oral presentation: *Lymphodepletion with Alemtuzumab for Heart Transplantation in cynomolgus monkeys*

Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Research in Progress seminar January 2012; Oral presentation: *Lymphodepletion with Alemtuzumab for Heart Transplantation in cynomolgus monkeys*

Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Research in Progress seminar February 2012; Oral presentation: *non-human primate natural and induced regulatory T cells with allo-antigen specific suppressive function*
Cell Transplant Society – International Xenotransplantation Association 2011 Joint Congress, October 2011, Miami, FL, USA; Oral presentation: *Non-human primate natural and induced regulatory T cells suppress proliferative response to WT pig cells*

Cell Transplant Society – International Xenotransplantation Association 2011 Joint Congress, October 2011, Miami, FL, USA; Oral presentation: *T cell-directed immunosuppressive therapy prevents development of natural anti-AB and anti-pig antibodies in infant baboons*

American Transplantation Congress May 2011, Philadelphia, PA, USA; Poster presentation: *Expansion and function of non-human primate induced regulatory T cells*  
Distinguished Poster Award

Genzyme Annual Fellows Conference, April 2011, Cambridge, MA, USA; Oral presentation: *Expansion and function of non-human primate induced regulatory T cells*

Keystone meeting: Immunoregulatory Networks, April 2011, Breckenridge, CO, USA; Poster presentation: *Expansion and function of non-human primate induced regulatory T cells*

The Transplantation Society Meeting August 2010, Vancouver, Canada, Poster presentation: *Collagenous Colitis in Immunosuppressed Baboons – an Infectious Origin?*

American Transplantation Congress May 2010, San Diego; Oral presentation (late-breaking abstract): *T cell-Directed Immunosuppressive Therapy Prevents Development of Natural Anti-AB and Anti-pig Antibodies in Infant Baboons*
ABOUT THE AUTHOR

Eefje Marloes Dons was born January 8th 1981 in Hilversum, The Netherlands. She attended the Juvenaat gymnasium college in Bergen op Zoom from 1993-1999. After a 2 year delay caused by numerus fixus, she was admitted to medical school in 2001 through decentral selection at the Erasmus University Rotterdam, where she was one of the 30 students selected out of more than 500 candidates.

During her medical studies she organized several international clinical traineeships, to Italy (pediatrics), Norway (surgery, pediatrics and gynaecology) and India (public health). She passed all her Medical School exams in one stroke resulting in her MD degree in February 2008. In 2008 she obtained a prestigious research “Ter Meulen Fund” grant from the Royal Dutch Academy of Science for a research project at the Thomas E. Starzl Transplantation Institute under supervision of Dr. David K.C. Cooper. She could start several months later, and worked as emergency room doctor at Lievensberg Hospital, Bergen op Zoom, during the 6 months in between. There she gained experience in clinical work at the emergency room, and had the opportunity to work with the surgeons carrying out daycare procedures in her spare time.

She started her research career in October 2008 at the Thomas E. Starzl Transplantation Institute, Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA, with mentors Prof. dr. David K.C. Cooper (University of Pittsburgh) and Prof. Jan N.M. IJzermans (Erasmus Medical Center Rotterdam). A year later her work was further supported by the Dutch Surgery Fund Prof. Michael Van Vloten. During the first two years of her research she worked on the studies presented in section 1 of this dissertation. In parallel, she carried out the laboratory experiments that resulted in the data leading to a 5-year National Institutes of Health (NIH, USA) U01 grant on the project “Alemtuzumab and Regulatory T cells for Heart Transplant Tolerance in Monkeys” (mentors: Prof. David K.C. Cooper and Prof. Angus W. Thomson, both at University of Pittsburgh) for which she held main management responsibility during the last two years. This included organization of the monkey heart transplants and follow-up animal care, carrying out most of the laboratory assays, as well as management, financial overview, logistics, and overall planning. Studies that are part of this project are presented in section 2 of this dissertation.
Today she is working at the surgical department of the Albert Schweitzer Hospital Dordrecht, the Netherlands where she is dedicated to start her training in general surgery.

Eefje enjoys running, triathlons, rowing and hiking. In Pittsburgh she lived right next to Frick Park, a large city forest with steep hills, narrow trails and creeks, where she practiced offroad running (preferably with Gunner). She started cycling on her road-bike in the surrounding suburbs, powering up the flowing hills with her cycling friends. She was part of Rif Raf, a Pittsburgh-based mixed-8 rowing team that won amateur competitions every year. She completed several on- and off-road half-marathons, marathons and triathlons in the Netherlands and the USA, and has won several prizes in her age category over the past few years. She has every intention to continue the outdoorsyness now that she is back in the Netherlands, and has already completed a half-marathon on the beach.

Contact the author at eefjedons@gmail.com
T CELL-DIRECTED IMMUNOSUPPRESSIVE THERAPY AND REGULATORY T CELLS FOR HEART TRANSPLANT SURVIVAL IN NON-HUMAN PRIMATES

EEFJE M. DONS