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Hua Pan

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Hua PAN

**Induction de tolérance au cours des greffes de tissus composites
chez le porcelet nouveau-né.**

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Résumé

L'objectif de mon projet de thèse était de mettre au point un modèle préclinique d'allogreffe de tissus vascularisés composites (ATC) avec induction de tolérance par injection de cellules souches hématopoïétiques chez le porcelet nouveau-né. L'objectif à plus long terme est de pouvoir proposer cette option thérapeutique à des enfants nouveau-nés souffrant d'anomalies congénitales sévères de la main ou du visage.

Dans l'introduction bibliographique, nous avons abordé en détail les mécanismes de tolérance néonatale chez la souris, ainsi que la transplantation *in utero* de cellules souches hématopoïétiques dans des modèles animaux et humains. Les caractéristiques du système immunitaire du nouveau-né humain ont ensuite été décrites. Une étude des différents protocoles de conditionnement non-myéloablatifs utilisés pour l'induction de tolérance dans les greffes d'organes solides a également été menée, et la greffe de thymus et de moelle osseuse vascularisée en association avec l'ATC ont été abordés. Enfin, une revue exhaustive des différentes études d'induction de tolérance dans le cadre d'ATC chez les humains et les grands animaux a été faite.

Dans la partie Résultats, un modèle préclinique expérimental d'ATC a été élaboré chez le porcelet nouveau-né avec: 1- une validation de la faisabilité chirurgicale de l'ATC chez le porcelet nouveau-né; 2- l'élaboration d'un traitement immunosuppresseur compatible avec l'ATC chez le porcelet nouveau-né. Le choix des molécules immunosuppressives est le suivant: Cyclosporine A, globulines anti-thymocytes de porc et Mycophénolate Mofétil. Des études indépendantes et successives des mécanismes d'action de chaque immunosuppresseur ont alors été menées chez le porcelet nouveau-né.

Les résultats de mon projet de thèse ont permis d'élaborer: 1- un modèle chirurgical d'ATC chez le porcelet nouveau-né; 2- un protocole complet d'immunosuppression chez le porcelet nouveau-né. La perspective à court terme de ce travail est de réaliser l'expérience "finale" d'induction de tolérance dans le cadre

d'une ATC chez le porcelet nouveau-né, expérience qui sera menée au printemps 2014. Si la tolérance à l'ATC est induite dans notre modèle préclinique porcin, l'objectif à plus long terme de ce travail sera de transférer ce protocole chez l'humain en élaborant un protocole d'induction de tolérance et d'allogreffe de main pour les nouveau-nés.

Tolerance Induction for Vascularized Composite Allografts through Mixed Hematopoietic Chimerism in Neonatal Swines

Abstract

This present research is devoted to the exploration of performing vascularized composite allografts as a treatment for severe congenital hand or face anomalies in neonates or very young infants.

The bibliographic studies at first revised the discovery and mechanisms of neonatal tolerance in mice, as well as *in utero* hematopoietic stem cells transplantation in large-animal models and human fetuses. Then the properties of human neonatal immune system were described; and the non-myeloablative or non-toxic conditioning regimens for solid organ transplant tolerance induction were also studied, in order to give the clue to a applicable conditioning regimen for tolerance induction in neonates. The potent thymus and vascularized bone marrow transplantation in neonatal VCA were considered as advantages. Finally, the researches concerning tolerance induction for VCA in large animal models and in human patients were reviewed.

In experimental studies, the preclinical VCA was firstly established in neonatal swines. Subsequent experiments thus studied the immunosuppressive agents, as well as conditioning regimen, including the administration of cyclosporine A, rabbit anti-pig thymocyte globulin and mycophenolate mofetil for VCA in pig neonates.

The findings in these experiments were then concluded. Based on these finding, a general tolerance induction protocol for VCA in neonatal swines was designed and experiment will be performed in year 2014-2015. If donor-specific tolerance for VCA could be induced with present protocol, we will subsequently elaborate an applicable tolerance induction protocol and hand allotransplantation program in human newborn infants.

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***Pediatric Vascularized Composite Allografts
It's time?***

General Introduction

Vascularized transplantation of body structures composed of multiple tissues derived from ectoderm and mesoderm, such as upper or lower extremities, face, knee, larynx and abdomen wall, is termed as vascularized composite allografts (VCA) or composite tissue allografts (CTA) [1]. VCA has achieved great development in limb or face reconstructions since the first hand transplantation was successfully performed in Lyon, France in 1998 [2] and the first face transplantation in France in 2005 [3]. Until 2010, more than 60 cases of unilateral or bilateral upper extremities transplant, 20 cases of partial or full face transplant, as well as several cases of transplantations of other body structures such as larynx, knee, abdomen wall, et al. were reported by the international registry on hand and composite tissue transplantation [4]. Thus, nowadays in clinical VCA setting, researchers have accumulated a lot of experiences in (a) recipient screening, (b) donor choosing by human leukocyte antigen (HLA) matching and also appearance similarity), (c) surgical team organization and transplantation operating, (d) immunosuppressive therapy management and monitoring, (e) treatment of complications and side-effects related to immunosuppressive therapy, as well as (f) functional rehabilitation and care of psychological disorders. Today, VCA appears to be a more and more promising therapy because of optimized immunosuppressive regimen, proper rehabilitation and careful follow-up [5, 6].

VCA could be considered as an acceptable therapy in adult patients for limb loss or severe craniofacial defects subsequent to injuries, malformations or tumors. VCA may also become a potential therapy for severe congenital limb or face defects in neonates and infants. The requirement of VCA has been presented since several years, for congenital limb aplasia (especially upper limb transverse aplasia) [7] or craniofacial malformations such as Treacher-Collins syndrome, hemifacial microsomia and some severe hemangioma [8].

The surgical feasibility of performing VCA in very young infants has been proved. In year 2000, first successful hand transplantation in newborn identical twins was

performed in Malaysia [9]. Then in 2006 in Canada, first successful lower-extremity transplantation was performed between newborn identical twins [10], of which the 6-year follow-up showed an excellent functional recovery [11]. On the other hand, allotransplantations of solid organs such as heart, lung or liver in young infants have accumulated adequate experiences for the exploration of VCA in infants [12]. However, as a non-life-saving therapy, hand or face allotransplantation in the vulnerable newborn infants remains not acceptable because of the obligatory subsequent life-long immunosuppression. This long-term immunosuppression is associated to an important morbidity and mortality related opportunistic infections, risk of secondary malignancies and nephrotoxicity of calcineurin inhibitors, such as cyclosporine A (CsA) and tacrolimus (FK506). In addition, immunosuppressive therapy use of corticosteroids is still common and may be associated to severe side-effects such as hypertension, hyperglycemia and childhood growth and development [6]. Induction of donor-specific tolerance might be a very attractive solution to avoid these difficulties and to allow this high-risk intervention to be performed in neonatal patients [1].

Nevertheless, performing VCA in newborn might have some advantages.

(1) Neonatal stage is considered to be an immune “window phase” which is susceptible for induction of tolerance [13]. Thus, donor-specific tolerance induction through hematopoietic stem cells transplantation may be benefit for this special stage and could be successful without intensive conditioning therapy.

(2) Donor-derived bone marrow graft used in composite allograft transplantation, and especially the bone marrow component in limb allograft from a newborn donor will contain more hematopoietic stem cells compared to those from an aged donor, therefore will have greater chance to achieve an adequate donor chimerism inducing tolerance without development of graft-versus-host-disease (GVHD).

(3) VCA in neonatal stage should have less difficulty in functional rehabilitation because of a more potent plasticity of neonatal brain cortex than adult one.

(4) Neonatal face transplant patient should have no psychological disorder in the recovery of self-image as in that age, sense of self-image may not yet be developed.

The final principal objective of our present study will be devoted to induce donor-specific tolerance to VCA in neonatal large-animals through bone marrow stem cells transplantation after non-toxic conditioning therapy. In order to be applicable in further clinical study, we develop a pre-clinical large-animal model (neonatal swine) following different steps (a) surgical feasibility of VCA in neonatal swines; (b) feasibility, toxicity and efficacy of a conditioning protocol in present study is based on lymphocyte-depleted induction therapy using a novel rabbit anti-pig thymocyte globulin (pATG) followed by a maintenance therapy including CsA and mycophenolate mofetil (MMF); (c) harvest of bone marrow cells (BMC) and detection of BMC composition; (d) the final objective will be tolerance induction to VCA through donor-derived BMC infusion with this non-toxic conditioning therapy.

Part I: Bibliographic Studies

I. Discovery of Tolerance in Fetuses and Neonates

The principle of immune tolerance was used in embryonic developmental study before it was essentially discovered and described [14]. Embryonic tissues such as limb buds have been successfully transplanted onto other embryos and would sometimes survive beyond the embryonic stage. However, the underlying property of this phenomenon had not been noticed and the interest of embryologists was confined to the developmental processes of these transplants. The question why the grafts could survive in embryonic hosts but would be rejected in adult allogeneic hosts had never been posed.

The phenomenon of fetal immunologic tolerance was noticed in 1945 by Ray D. Owen who published an article in the journal of Science [15]. Owen studied blood groups in twin cattle at the University of Wisconsin and found that dizygotic twin cattle had two distinct sets of red blood cells (RBCs): each twin had its own set of RBCs as well as another set of RBCs which could only have been derived from the other twin sibling during fetal life. This discovery was linked to another earlier finding from Frank R. Lillie, who illustrated that the fetuses of dizygotic twin cattle almost invariably develop a placental anastomosis with each other. Owen therefore hypothesized that RBCs and their precursor cells should have been exchanged between the cattle fetuses. These precursor cells could engraft and survive in their hosts, subsequently continue to differentiate into RBCs as host grew into adulthood. But unfortunately, this brilliant finding had not appealed the attention of other immunologists until it was highlighted by the Australian virologist and immunologist Frank M. Burnet and his co-author Frank Fenner in their famous book "The Production of Antibodies" which was published in 1949 [16].

Considered as a pillar of immunologic concepts, the publication of "The Production of Antibodies" marked that immunology had moved from a chemical study to a biological discipline. In his book, Burnet introduced the concept of "self" and "non self" in immune response, that is a founder dogma of the 20th century immunology. Burnet regarded that the "self" of the host body is actively orchestrated during

embryogenesis due to complex interactions between immune cells and other cells/molecules present within the embryo. Link to the concept of “self”, Burnet proposed the concept of immune tolerance, based on the hypothesis that the body failed to make antibodies to its own components in order to avoid autoimmunity. He hypothesized that if cells from a genetically distinct individual are implanted and established in the embryo, no antibody should develop against the foreign cell antigen when the embryo takes on independent existence. At that time, the concept of cellular immune was not yet developed and people believed that allograft rejection was caused by humoral response.

Burnet had well predicted the existence of tolerance; however, he was unable to prove it by experimental approaches. This task was accomplished 4 years later by Peter B. Medawar and his colleagues Rupert E. Billingham and Leslie Brent. They performed the engraftment of splenocytes isolated from A-line adult mice into CBA mouse fetuses by *in utero* intravenous infusion. After these *in utero* transplanted CBA recipients were born and had grown to adult age, they demonstrated that engrafted adult CBA recipients could accept skin graft from the A-line adult mouse donor but not from another AU mouse strain. Their article, “‘Actively Acquired Tolerance’ of Foreign Cells”, was published in *Nature* in 1953 [17], dating the discovery of immune tolerance. Four important notions can be highlighted from this pioneer article.

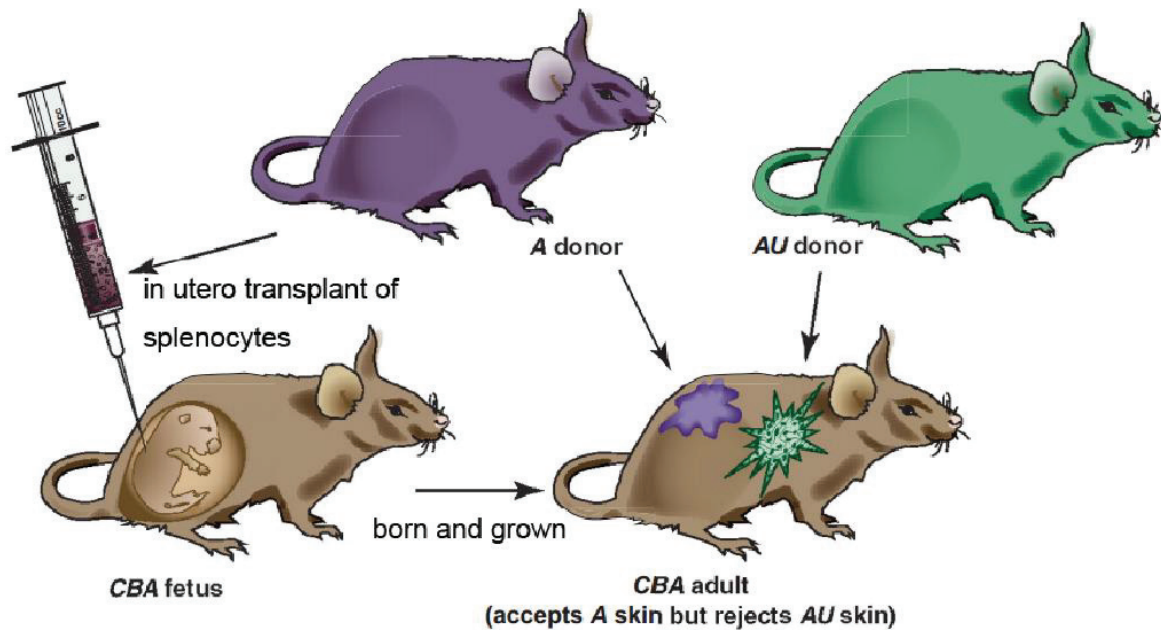


Figure 1: Discovery of fetal/neonatal tolerance.

In the seminal experiments of Billingham, Brent and Medawar, mouse fetuses from the CBA-strain were in utero injected with live adult tissue cells from a genetically disparate mouse strain (strain-A). Eight weeks after birth, the CBA-mice were challenged with skin grafts from an adult A-strain donor. The majority of the recipients did not mount a rejection response, indicating that they had become tolerant. The same mice rejected skin grafts from a third-party donor (AU-strain), indicating that tolerance is specific [18].

1. Mice never develop (or only develop to a limited extent), the capacity to react immunologically against foreign homologous cells with which they have been inoculated in fetal life. Such animals are tolerant not only to the foreign cells of the original inoculum, but also to skin grafts freshly transplanted in adult life from the original donor or from a donor of the same immunogenetic background.

2. Acquired tolerance is immunologically specific: mice tolerant to skin graft from splenocyte donor keep the capacity to reject skin grafts from other donors of different immunogenetic background.

3. Acquired tolerance is due to a specific failure of the host's immunological response.

4. The fertility of tolerant mice if unimpaired.

Subsequent studies demonstrated that tolerance could be induced by injecting neonates shortly after birth, rather than during fetal period [19, 20].

Seven years later in 1960, Medawar and Burnet won the Nobel Prize for Physiology/Medicine for the discovery of immune tolerance.

II. Mechanisms of Neonatal Tolerance

Neonatal tolerance was discovered 60 years ago, however underlying mechanism still lack of a thorough illustration. As demonstrated by Medawar and colleagues in the article “‘Actively Acquired Tolerance’ of Foreign Cells”, neonatal tolerance includes two essential components:

(1) Tolerance to foreign cells of the original inoculum. The engraftment of allogeneic splenocytes or bone marrow cells in neonatal recipients doesn't require any conditioning regimen prior to transplantation of splenocytes.

(2) Tolerance to flesh transplanted skin grafts from the same donors in adult recipients.

The latter tolerance to skin graft in adult life should be regarded as the maintenance of neonatal tolerance. It's widely accepted that it relies on central tolerance, a mechanism that leads to the clonal deletion of donor-reactive thymocytes from the T-cell repertoire, which results from the contact and interaction among recipient thymocytes and thymic antigen-presenting cells that derive from both recipient and donor precursor cells. Tolerance induction through donor-derived stem cells infusion in the setting of solid organ transplantation is at least partially based on this central tolerance mechanism.

However, the explanation of the former tolerance to the original inoculum is more obscure and is still a matter of debate nowadays.

Generally, under the “self/non-self” notion from Burnet, in the domain of cellular immune, two main categories of interpretation, active model and passive model, were involved in the studies. Moreover, the Th1 to Th2 immune deviation in neonatal stage was also proposed as one important factor. Besides, according to the “danger” notion, a novel concept of immunology which was proposed by Polly Matzinger in 1990s, some other explanations were also presented.

This chapter will focus on these different models and propose how they may be involved in the mechanism of neonatal tolerance.

II.1. Passive Model

The passive model is based on clonal selection theory named by F. Burnet. It was a thymus-involved central tolerance model suggesting that neonatal mice present an immature immune system and are therefore incompetent to mount a cytotoxic T lymphocyte (CTL) response to reject the donor cells, which would therefore engraft in the neonatal mouse recipients and circulate to the thymus to induce tolerance by clonal deletion as natural self-tolerance. Natural self-tolerance is shaped by self-antigens expressed and presented by various types of thymic antigen-presenting cells (APCs), such as medullary thymic epithelial cells (mTECs) and thymic dendritic cells (DCs) present in the medulla [21]. Depending on the overall signal quality and/or quantity delivered during interactions between thymocytes and APCs, self-reactive thymocytes get to either apoptosis or anergy by regulatory T cells (negative selection).

According to the passive models, when semi-allogeneic cells are infused intravenously to induce neonatal tolerance, donor antigen-specific thymocytes would be quickly depleted in thymus during the process of negative selection. Remarkably, the donor antigen-specific thymocytes are purged from the neonatal thymus through a very rapid mechanism. Within 72 hours after injection, thymocytes that are able to respond to alloantigens from the tolerance-conferring inoculum are no longer observed in mixed lymphocyte reaction (MLR). Moreover, recipient T cells with receptor for donor-derived molecules could no longer be detected. During the same interval, cells bearing donor alloantigens enter into the host thymus, spleen, and bone marrow, creating a chimerism status in the recipients [22, 23].

The importance of chimerism within thymus for the induction and maintenance of central tolerance was also investigated in the passive model. In the study of M. Hosono et al., BALB/c (H-2^d, MIS-I^b) mice were neonatally tolerized by the intravenous administration of either bone marrow (BM) cells or peritoneal cavity (PerC) cells from (BALB/c X AKR) F1 (H-2^{d/k}, MIS-I^{b/a}) mice. BM cell inoculation resulted in 1 year-long deletion of MIs-1^a-reactive T cells, which was significantly different to that of PerC cell inoculation which caused only a transient loss of MIs-1^a-reactive T cells (1

week after birth). This functional profile of the tolerant state correlated well with the degree and persistence of the intra-thymic presence of F1 type Ia⁺ cells [24]. M. Hosono et al. next revealed that inoculated cells accumulating in the thymic medulla, but not in the cortex, induced tolerance to the MHC-II alloantigens [25].

M. Eto et al. studied the role of chimerism in the establishment of allograft tolerance in adult mice that were conditioned with cyclophosphamide and then received infusions of allogeneic donor BM and spleen cells. Even if 1.2×10^8 cells were infused in these experiments (10 folds more than that were infused to neonatal mice), in the thymus of the tolerant mice, neither mixed chimerism nor the clonal deletion of antigen-specific thymocytes was observed at 2 weeks, whereas both of them were observed at 5 weeks. The mixed chimerism and clonal deletion in the thymus were then lasting for more than 10 weeks after treatment. These results may argue that micro-anatomical barriers limiting entry of foreign cells into the neonatal thymus are less rigorous than that in the adult thymus. This difference may therefore explain why transplantation tolerance is relatively easy to achieve in neonates [26].

In sum, the passive models well explained the long-lasting maintenance of tolerance by clonal deletion in thymus. However, mechanisms leading to the immediate acceptance of donor cells remained very obscure and other explanations such as Th1 (type 1 helper T cells) to Th2 bias theory may be relevant.

II.2. Active Model

The active model is based on the immune network theory that has been developed since 1974 mainly by Niels K. Jerne [27] and Geoffrey W. Hoffmann [28].

The active model suggests that during neonatal stage, the introduction of (CBA X A) F1 antigen into the developing immune system of recipient (CBA mice) may favor a “network interaction”. Under such interaction, the nascent membrane receptors on the F1 antigen-specific peripheral T cells are especially immunogenic for their “anti-idiotypic” counterparts (suppressor T cells) and selectively expanded this population to predominantly suppress the alloreactive response [29].

The existence of suppressor T cells proposed by active model was proved to be

inaccurate nowadays, but this notion gave rise to the discovery of regulatory T cell (T_{reg}) populations. The term of “suppressor T cells” was used operationally for many years in immunology before the discovery of T_{reg} , merely to indicate the “negative side” of T-cell response. Early investigation on the existence of “suppressor T cells” took place in 1969 [30] when it was demonstrated that female mice that underwent thymectomy on day 3 following birth developed autoimmune diseases that could be prevented by reconstituting these mice either with thymocytes or splenocytes from normal adult syngeneic mice. Subsequent investigations demonstrated the existence of a population of thymic-derived lymphocytes that prevented the development of autoimmune diseases [31]. The “Suppressor T-cell population” was first described by R.K. Gershon and K. Kondo who demonstrated their ability to achieve and transfer antigen-specific tolerance in naïve animals [32].

However, the existence of suppressor T cells gradually fell into ill-repute for two main reasons. First, there were no specific markers to identify and isolate suppressor T-cells. Second, it was postulated that these suppressor T cells had functional gene rearrangements and produced antigen-specific soluble factors encoded by an “I-J” locus mapped within the major histocompatibility (MHC) region in mice [33, 34]. When molecular biology techniques became powerful enough to investigate T-cell receptor (TCR) gene rearrangements, it was evident that suppressor T-cells did not have any functional gene rearrangements. Moreover, it was demonstrated that the putative “I-J” locus simply did not exist [34].

In 1990, a major breakthrough in characterization of suppressor T cells came from the report of Hall and colleagues. They demonstrated that a subset of $CD4^+$ T-cells characterized by surface expression of CD25 could specifically transfer tolerance from rats with long surviving cardiac allografts [35]. Following this study, Sakaguchi et al. demonstrated that suppressive properties were enriched in the $CD4^+CD25^+$ T-cell population that could prevent organ-specific autoimmunity induced by syngeneic $CD4^+CD25^-$ T-cells in nude mice [36]. The term “regulatory T-cells” has subsequently gained widespread popularity instead of “suppressor T cells”, erasing the long stigma associated with this first nomenclature.

Although the predictions of the active models were falsified by the development of TCR research and the discovery of T_{reg} , and some of the suppressive effects of “suppressor T cells” were re-explained by Th2-biased response, the exact role of T_{reg} in the mechanism of neonatal tolerance remains unknown. Gao et al. reported that neonatal BALB/c mice injected with semiallogenic CAF1 (BALB/c \times A/J) splenocytes can induce antigen-specific tolerance to A/J that lasts into adulthood. Authors demonstrated that A/J-specific anergic $CD8^+$ cells were present in neonatal primed mice that develop tolerance but not in neonatal primed mice that reject A/J skin grafts. Anergic $CD8^+$ cells were regulated by $CD4^+CD25^+$ T_{reg} and showed decreased proliferation and no CTL activity against A/J targets [37]. In conclusion, donor-derived T_{reg} may be dispensable in the induction of neonatal tolerance [38], but host T_{reg} should have played an important role in the maintenance of tolerance [39].

II.3. Th1 to Th2 Immune Deviation

Th1 to Th2 deviation is a non-thymus involved and non-clonal deletion mechanism. It suggests that in neonatal tolerance mice the newborn T cells generate Th2 biased immune response that protects the donor cells from rejection.

Th1/Th2 paradigm is a central tenet of modern immunology for understanding adaptive immune responses. By the Th1/Th2 paradigm, mammalian adaptive immune system is able to polarize functions into the type-1 and type-2 immune pathways, which respectively resolve infections with intracellular or extracellular pathogens [40].

The polarization of immune responses is largely regulated by antigen specific helper T (Th) cells. Antigen-activated T_h cells can differentiate into either Th1 or Th2 cells. Th1 cells mainly secrete IL-2, IFN- γ , IL-10 and IL-18. They promote cell-mediated immune responses. Th1-type immune responses are oftentimes associated with autoimmune diseases, allograft rejection, and delayed-type hypersensitivity reactions. In contrast, Th2 cells mainly secrete IL-4, IL-5, IL-9, IL-13 and IL-19, driven by IL-4 and IL-13. They promote humoral immune responses [41]. Th1-type immune responses are principally directed against helminth infections and can cause allergy [39].

Th1 and Th2 effector helper T cells tend to develop in a mutually exclusive manner. Th1-associated cytokines such as IFN- γ and IL-12 promote helper T cells differentiation into Th1 cells while inhibiting the development of Th2 cells, whereas Th2-associated cytokines such as IL-4 promote the differentiation of Th2 cells while inhibiting the development of Th1 cells [39, 42-45].

In organ transplantation, graft rejection is generally accompanied by a Th1-type immune response, whereas tolerance in the allograft model is generally accompanied by a Th2-type immune response [39].

Neonatal mice are considered as having a Th2 cytokine bias due to: (a) in primary immune response, the limited CD4⁺ Th1 and CD8⁺ CTL responses observed when they are initially inoculated with foreign antigen; (b) in secondary immune response, mice immunized in neonatal stage mount Th2 dominated memory responses when they follow a re-exposure to the same antigen in adult stage. Th1 to Th2 immune deviation in neonatal stage is also considered to play an important role in the immediate acceptance of donor cell inoculum [46-49].

Several factors have been identified in order to explain the Th1 to Th2 bias immune response in neonatal mice, whereas a conclusive explanation for the existence and evolution of Th1 to Th2 bias has not yet been reached.

Increased levels of Th2 cytokines (IL-4 and IL-13) and anti-inflammatory cytokines (IL-10 and TGF- β) [50-53] could be related to the increased ratio of CD4:CD8 cells in neonates. Neonatal CD8⁺ cytotoxic T cells have been found to produce increased levels of IL-13 and IL-4 and increased expression of their receptors, IL-13R and IL-4R, respectively. The increased expression of these cytokines and their receptors is thought to induce a Th2 bias in primary responses while increasing CD8⁺ cells to apoptosis upon secondary exposure [54, 55]. Th1 cytokines induction may be actively inhibited by neonatal regulatory cells [56, 57]. Defective production of IL-12 by professional neonatal APCs (macrophages and DCs) may also dampen the initiation of a Th1 response [55, 58-60]. Finally, a lack of CD40 ligand (CD40L) up-regulation of following T-cell activation [61-63] and the influence of the environment (e.g. lymph node or spleen) have also been suggested to influence

Th2 bias [64].

Although Th2 bias of cytokine secretion may be crucial during the initial phase of neonatal tolerance induction, it was also concluded that Th2 bias doesn't account for maintenance of the long-term tolerant state. Because Th2 bias in mice was apparent only at 2-3 weeks of age and completely disappeared at 6 weeks, thereafter, tolerance should be maintained by another mechanism, e.g. donor-reactive T cells undergo clonal depletion in the thymus or undergo anergy by T_{reg} in the peripheral lymphoid organs.

II.4. Neonatal Tolerance and Danger Model

The self/non-self notion which is the predominant doctrine in immunology since 1950s started to be challenged in late 1980s. Immunologists began to recognize that in order to be activated, T cells require APCs to pick up and then present antigens to corresponding T cell receptors. Moreover, T cell activation also requires APCs to send them the co-stimulation signals.

In 1989, Charles Janeway argued that the innate immune system is the real gatekeeper of whether the immune system would respond or not to foreign antigens. He also argued that the innate immune system used the conserved pattern-recognition receptors (PRRs) to recognize the pathogen-associated molecular patterns (PAMPs) [65]. In 1994, Polly Matzinger published an article entitled "Tolerance, Danger and the Extended Family" which marked a watershed in the field of basic immunology. In this article, Matzinger proposed the idea that APCs respond to what she called "danger signals", that correspond to molecules released by cells undergoing injury, stress or necrosis (in contrast to apoptosis, programmed cell death). The "danger signals" released by these cells stimulate the immune system to overcome the "dangerous events" by launching an immune response. Matzinger argued that the immune system orchestrates immune responses neither because of a neonatal definition of "self/non-self" (proposed by Burnet), nor because of old definitions of pathogens (proposed by Janeway), but due to a dynamic and constantly updated response to "danger signal" defined by cellular damage [66].

The “danger” model also highlighted the role of APCs in mechanism of neonatal tolerance. In 1996, the “common sense” considering that neonates are inherently susceptible to tolerance induction was challenged by a series of findings published in Science [67-69]. Surprisingly, these findings showed that neonates are competent to mount *in vivo* T-cell responses under certain conditions.

Based on the "danger model", John Paul Ridge and Polly Matzinger analyzed the possibility that critical components in experimental neonatal tolerance are donor cells, not responding T cells. Ridge and Matzinger showed that the proportion of APCs in Medawar's classical donor inoculum [17] is the factor controlling tolerance or immune activation. Medawar's transfer of splenocytes or BM cell suspensions contain very few professional APCs [70] and a large percentage of T and B cells which cannot co-stimulate virgin T cells [71-73]. The tiny number of virgin T cells in newborn mice might easily be overwhelmed by interactions with tolerogenic cells present in the inoculum before having the opportunity to meet activating APCs such as DCs. In sum, the authors emphasized that neonatal tolerance was depending on the donor-derived tolerogenic cells. The authors also expected if isolated the critical components of the inoculum, the neonates should become primed by an injection of professional APCs such as DCs and tolerized by injection of non-professional APCs such as B cells. In this respect neonatal T cells do not differ from virgin T cells in adults [70, 71, 74]. Thus in the experiment, by administering a pure population of DCs, the authors demonstrated that neonatal T cells are neither innately tolerizable nor able to launch only Th2 responses. Neonatal T cells could be activated to produce CTL responses. Finally, they also showed that the number of cells administered is important, and that it is possible to induce tolerance in adults, provided the number of non-purified splenic cells administered was large enough.

The importance of quantity of infused cells was re-emphasized by Sarzotti et al. [69]. They showed that neonatal mice infected with Cas-Br-M murine leukemia virus (MLV) did not produce protective CTL and IFN- γ responses that observed in adult mice, instead producing non-protective Th2 responses. Consequently this led to the development of virus-induced neurological disease. However, if the dose of Cas-Br-M

MLV was decreased proportionately according to the small T-cell number in neonatal mice, a switch from Th2 to Th1 occurred and protection was induced. Adkins B, et al. demonstrated that neonates are competent to develop vigorous major histocompatibility complex-class I (MHC-I) restricted CTL activity in vivo upon exposure to either fully or semi-allogeneic spleen cells [75]. Specific CTL activity was generated using doses of cells approximately ten-fold lower than levels used for the induction of GVHD or tolerance. Thus, mouse neonates are fully competent in their capacity to develop alloreactive CTL activity, as long as the dose of donor cells is low enough.

To the neonatal Th2 bias theory, Forsthuber et al. analyzed the induction of Th1 and Th2 immunity in neonatal mice and proposed that neonates are immunocompetent [67]. CD4+ T cell response to prototypic antigens of neonatal mice does not essentially differ from that of adult mice. Therefore, the neonatal period may not be an immunologically privileged window during ontogeny that is beneficial to the establishment of self-tolerance, but an immunologically competent period.

Theories of danger model are far from perfect. Subsequent studies made the situation to be more complicate. The explanation from “danger model” which hypothesized that neonatal tolerance is induced by the predominance of “non-professional” APCs such as B lymphocytes in the tolerizing inoculum was challenged by other experiments. Y. Modigliani et al. [38] directly addressed this hypothesis and proved that neonatal tolerance can be induced by infusion of splenocytes and BM cells from Rag-1 deficient mice which have small lymphoid organs that do not contain mature B and T lymphocytes [76]. The results showed that donor lymphocytes are not required to achieve neonatal tolerance.

II.5. Conclusion

After more than five decades of research we have come to realize that tolerance is not a single phenomenon, nor is it achieved by a unitary mechanism. In fact, hypotheses to explain tolerance are numerous, diverse, sometimes contradictory and are developing with the evolution of basic immunological notions. Moreover, neonatal

tolerance in mice can only be achieved under appropriate experimental conditions.

Although neonatal immune responses had been described before 1996, collectively these findings engendered resurgence in the investigation of neonatal immunity and vaccination, with the emphasis on immune responsiveness rather than tolerance or the lack of response. At present it is clear that neonates are able to mount various immune responses, ranging from deficient to deviant to fully mature, depending on the conditions of antigen exposure.

Under the concept of “danger model”, future research may pay more attention to the study of the role of innate immune system, such as APCs, PRRs, complement components, thrombocytes, etc, in transplantation immunology. However, as mentioned at the beginning of this part, maybe no single theory can explain the function of immune system as a whole. Tolerance should be defined as a phenomenon that evolves with time, but not as an immobile status.

III. Tolerance Induction in Neonates: Implication from *in utero* Hematopoietic Stem Cells Transplantation

It has been known for decades that in neonatal mice the injection of proper dose of semi-allogeneic splenocytes can result in neonatal tolerance and chimerism without any conditioning regimen [17, 75, 77]. However, neonatal tolerance induction protocols which have been proved to be effective in rodent species are rarely as effective as in large-animals and human who are born with almost entirely functional T cell immune system [13, 78, 79]. Even for tolerance induction in fetus in which the immune system is more immature than neonates, *in utero* hematopoietic stem cells transplantation (IUHSCT) has not yet been realized in human fetus except in cases of immune deficiencies [80].

Comparison of immune maturation between mouse/human		
Function	Mouse	Human
Length of gestation	21 days	40 weeks
Lymphohematopoietic cells colonize primordial thymus	Day 11–12	Week 9
Morphologic division of thymus into cortex and medulla	Day 13–14	Weeks 11–14
Expression of $\gamma\delta$ TCR then $\alpha\beta$ TCR	Day 14–16	Weeks 11–13
Proliferative response demonstrable in MLR	Day 16–18	Week 12 (thymus)
Mitogen responsiveness	Week 19 (spleen) (weak until week 23)	Week 13–14 (thymus)
	Day 18 (thymus) (to some mitogens only after birth)	Week 16–18 (spleen, peripheral blood)
Cytotoxic response demonstrable (CML)	Weak until postnatal day 7	Beginning from about Week 20–23 (thymus)

Table 1: Comparison of immune maturation between mouse/human.

Table from [79].

Because there are rare reported studies on tolerance induction in large-animal and human neonates, we focused on the studies of IUHSCT which can be regarded as tolerance induction in fetus of mice (MHC-mismatched donors and recipients), large-animals and human, in order to give some implications to tolerance induction in human neonates or very young infants.

III.1. Induction of Tolerance in Non-defective Mice after

MHC-mismatched IUHSCT

Evidence of immune barrier after IUHSCT has been illustrated by a rodent model using MHC-mismatched donors and recipients. In research of Carrier et al., 11 to 13 days gestation of Balb-c (H2^d) and C57BL/6 (H2^b) fetal mice received intraperitoneal or transplacental transplants of either allogeneic or syngenic fetal liver cells [81]. Engraftment was tested by quantitative polymerase chain reaction (qPCR) for the Y chromosome in female recipients. Results were listed as below.

(1) Only 8% (3 of 36) of allogeneic mismatched recipients and 25% (3 of 12) of syngenic recipients demonstrated a durable engraftment (male donor cells detected beyond 20 weeks of age) based on analysis of peripheral blood leukocytes.

(2) However, when spleen and liver were analyzed, 51% (17 of 33) of allogeneic recipients and 50% (6 of 12) of syngenic recipients showed durable engraftment.

(3) The percentage of donor cells that durably engrafted was very low, varied from 0.0001% in spleen and liver to 0.6% in peripheral blood of recipients.

(4) Postnatal boosting with single dose of allogeneic MHC-mismatched donor cells in tolerant, engrafted mice resulted in a significant increase of chimerism in the peripheral blood from 0.2% pre-boost to 5% at 6 months after the boost.

(5) Allogeneic recipients were also evaluated for donor skin graft acceptance at 6 to 12 months of age. Authors found that 3 mice with engraftment in blood and/or tissue permanently accepted donor skin grafts, 1 of them with donor cells detectable only in the liver; 6 additional mice that showed prolonged skin graft acceptance had no evidence of durable engraftment in the blood but were engrafted in the liver and/or spleen.

Authors concluded that a very small number of circulating donor cells in the blood or the tissues ($\leq 0.1\%$ donor cells) is sufficient for the induction and maintenance of tolerance. Moreover, the presence of donor cells in the circulating blood is not

necessary for prolonged skin graft acceptance or maintenance of permanent skin graft acceptance.

In the study of Peranteau et al., 14-day gestation Balb/c and C57BL/6 fetal mice received transplants of either allogeneic or syngenic bone marrow using a new intravascular technique that allowed definitive administration of much higher doses of donor cells and resulted in high percentage of chimerism detectable by flow cytometer [82]. The results showed that all of the survival recipients demonstrated engraftment at 1 week after birth. However, 70% of allogeneic recipients lost engraftment by 1 month of age, and 80% ultimately failed to sustain long-term chimerism. In contrast, all syngenic recipients maintained stable, long-term, multilineage chimerism. From 1 to 6 months of age, in allogeneic recipients, the level of chimerism in peripheral blood CD45⁺ cells decreased from about 7% of donor cells to less than 2%; but level of chimerism in syngenic recipients maintained at about 8%. These results strongly supported an immune barrier to allogeneic engraftment after IUHCT in mice fetus.

Addressing the relation among transplanted cell dose, initial chimerism level and long-term maintenance of engraftment, Durkin et al. [83] showed that the dose of transplanted cells exhibited a strong correlation with both engraftment and the chimerism level. Initial chimerism (at 3 weeks of age) was not detectable in any of the recipients after infusion of 2×10^4 cells (10^8 cells/kg). Serial increases in cell dose resulted in a higher engraftment as all of the recipients of 2×10^6 cells (10^{10} cells/kg) were chimeric and with peak of chimerism level at 7.5%. Moreover, a threshold level of initial chimerism (>1.8%) was identified to predict a durable engraftment (through 12 months of age) without GVHD, whereas low initial chimerism (<1.8%) predicted a graft failure.

III.2. Induction of Tolerance in Large-animal fetuses

Large-animal models were also used in the IUHSCT. The ovine (sheep) model was the first large-animal model to demonstrate engraftment of syngenic, allogeneic or xenogeneic (human to sheep) cells after fetal transplantation. The composition of

the donor cell graft, the number of cells transplanted, and the ages of the fetal recipients have only been systematically studied in the ovine sheep model [84-89].

In the research of Schoeberlein A. et al. [90], the donor fetal liver stem cells were collected from sheep fetuses with an open or ultrasound-guided technique. These liver stem cells were labeled with PKH26 and then transplanted intraperitoneally into allogeneic and autologous fetal recipients at 48 to 64 days of gestation. Engraftment was determined by flow cytometry and real-time polymerase chain reaction 1 to 2 weeks after transplantation. Engraftment of donor cells was found in all surviving fetuses, with a level of $\leq 4.7\%$ in fetal liver, spleen, bone marrow, blood and thymus. Overall, there was no difference between allogeneic and autologous grafts. Authors concluded that differences in the MHC between donor and recipient seem not to have a major impact on stem cell engraftment in early gestation; MHC-independent donor/host competition might be responsible for low engraftment in immunocompetent recipients.

Tissue	Transplantation group	n	% Donor cells	p
Blood	Allogeneic	11	0.112	0.928
	Autologous	4	0.117	
Bone marrow	Allogeneic	11	0.560	0.174
	Autologous	4	0.160	
Liver	Allogeneic	11	0.233	0.041
	Autologous	4	0.647	
Spleen	Allogeneic	11	1.140	0.935
	Autologous	4	1.067	
Thymus	Allogeneic	11	0.395	0.396
	Autologous	4	0.053	

Table 2: Comparison of engraftment between allogeneic and autologous transplantation in sheep.

Short-term engraftment (1–2 weeks after birth) of allogeneic versus autologous transplantation in White Alpine sheep by FACS analysis (PKH26-labelling) [90, 91].

However, the competence of alloimmune in sheep fetus was confirmed by McCullagh P. [92] who performed skin autografts and allografts to fetal lambs with 53-55 days gestational age. At 38 days post-transplantation, all the autografts of skin were accepted with scarless healing and grew approximately twice-folds in each dimension (long and width); while all the allografts of skin were rejected. Histological examination of fetal skin allografts revealed that these were subject to lymphocytic invasion, evident as the entry of lymphatic vessels and extravasation of lymphocytes within the first week after placement. These manifestations of allograft reaction became evident at 2 weeks after skin-grafting and with significant graft rejection by 1 month after grafting. These results showed the existence of an immune barrier in fetal sheep which is similar to the results observed in fetal mice [82].

Canine was also used as large-animal model in the study of IUHSCT. Omori F. et al. transferred human HSCs into the yolk sacs of mid-gestation, pre-immune canine fetuses under ultrasound guidance and the results showed that all surviving dogs were chimeric without signs of GVHD. Human cells in blood declined from 38% to 0.05% between 10 and 44 weeks after birth. Corresponding numbers in bone marrow were from 20% to 0.05% [93].

Remarkably, in swine model, MHC-mismatched HSCT in fetal stage induced donor specific tolerance to solid organ which was transplanted postnatally. In study of Lee P. W. et al. [94], MHC-mismatched bone marrow cells were transplanted to 50 to 55 days gestation pig fetuses. Long-termed survived animals demonstrated multilineage peripheral blood hematopoietic chimerism. Donor matched kidney transplants were performed postnatally without immunosuppression and functioned for more than 100 days, with no evidence for rejection [95].

However, IUHSCT in nonhuman primates showed low levels of engraftment. Limited data from nonhuman primates described optimal transplantation conditions. Cowan M. J. et al. [96] studied the efficacy of T cell-depleted parental bone marrow as a source of HSC for transplantation into 44 days or 61 days gestation non-defective fetal rhesus monkeys (165-day total gestation). The qPCR assay for the rhesus Y chromosome was used to detect male donor cells in survival female

recipients. The overall engraftment rate was 73% with no difference in terms of gestational age at transplant. In six long-term engrafted females, donor cells were detected in the peripheral blood and bone marrow up to 3 years of age. In vitro MLR and cell-mediated lymphocytotoxicity (CML) studies between the recipient and donor cells indicate that tolerance was induced to donor cells. Individual and pooled erythroid and myeloid marrow colonies grown in methyl cellulose were collected and analyzed for donor origin by qPCR. In a fetal recipient studied at 35 days post-BMT, donor cells were detected in bone marrow and liver in both erythroid and myeloid lineages. In long-term engrafted animals, the amount of donor cells in marrows was <0.1%. These results indicated that T cell-depleted parental marrow can durably engraft in utero, while the degree of peripheral blood engraftment (percent of donor cells) in this non-defective primate model is very low.

Xenograft researches showed that multilineage engraftment of human peripheral blood stem cells (PBSCs) can be achieved in the fetal rhesus recipient. From study of Tarantal A. F. et al. [97], human peripheral blood stem cells were obtained from a human male donor and enriched for the CD34⁺ population with T-cell depletion (<0.03% CD3⁺ cells, 98% CD34⁺ cells) and without T-cell depletion (0.83% CD3⁺ cells, 95% CD34⁺ cells). Infusion doses were 10~20 x 10⁶ cells/fetus at among 50 to 56 gestational days. All recipients were showed with donor cell engraftment (in peripheral blood and bone marrow) by postnatal qPCR examination of the human Y chromosome. Flow cytometer analysis of human CD45⁺ cells in recipient marrow showed engraftment ranging from 0.1-1.7%, without evidence of GVHD in any of the animals.

More recently, the effects of infused T-cell number on engraftment and the level of chimerism after *in utero* transplantation were determined by Shields L. E. et al. [98] in nonhuman primate model. An average dose of 3.0 x 10⁹ cells/kg CD34⁺ allogeneic adult bone marrow cells, including a T-cell dose ranged from 2.6 x 10⁵ to 1.1 x 10⁸ cells/kg, were transplanted into female fetal recipients. Chimerism was determined in peripheral blood subsets and in progenitor cell populations by PCR. Chimerism was noted in 7/8 live-born animals. The level of chimerism in the

progenitor population was related to the fetal T-cell dose. At the lowest T-cell dose (2.6×10^5 cells/kg), no chimerism was detected. As the T-cell dose increased to $10^6 \sim 10^7$ cells/kg, the level of chimerism increased. With the T-cell dose increased to 1.1×10^8 cells/kg, fatal GVHD was observed. This study emphasized the importance of T cells in facilitating donor cell engraftment and in producing GVHD in fetal nonhuman primates. However, authors concluded that the levels of chimerism in peripheral blood were too low for therapeutic benefit (for immune and hematologic diseases of fetuses and newborns) and further studies are needed to test methods that are likely to enhance donor cell engraftment and peripheral blood levels of donor cells.

Under such background, Shields L. E. et al. [99] continued to study the effect of fetal immune suppression (at the time of transplantation) and postnatal donor cell infusion as adjunctive therapy for in utero hematopoietic stem cell transplantation in nonhuman primates. In this study, fetal *Macaca nemestrina* received haploidentical, marrow-derived, CD34⁺ enriched donor cells, with a combination of the corticosteroid betamethasone (0.9 mg/kg) and rabbit anti-human globulin (ATG; 50 mg/kg). Chimerism was determined by PCR from marrow, marrow progenitors, peripheral blood and mature peripheral blood progeny. After birth, the level of chimerism in the progenitor population was higher in the immune-suppressed animals than in controls (11.3% +/- 2.7% and 5.1% +/- 1.5%, respectively; $p = .057$) and remained significantly higher at age of ≥ 14 months. Peripheral blood chimerism, both at birth and at long-term, was similar in immune-suppressed and control animals. In the animals receiving postnatal donor cell infusions, there was an initial increase in progenitor chimerism; however, at 6-month follow-up, the level of chimerism fell back to the pre-infusion values. Authors concluded that although fetal immune suppression correlated to an increase in the level of progenitor and marrow chimerism, the total contribution to chimerism in the peripheral blood remained low. Moreover, the level of long-term chimerism was not improved with postnatal donor cell infusion.

All data observed in large-animal models demonstrate the technical feasibility of

in utero transplantation and confirm the potential to achieve stable engraftment after IUHSCT in an immunocompetent host. Implications from studies on large-animal models are as below. (1) Postnatal multilineage engraftment can be achieved after IUHSCT in large-animal fetuses without GVHD; however, the immune barrier to MHC-mismatched cell/skin grafts is also exist in the fetuses. (2) Under similar IUHSCT conditions, levels of chimerism in large-animal models (especially in nonhuman primates), either in lymphoid tissues or peripheral blood, are lower than in mice model, indicating a more intensive immune barrier in high-developed large-animal fetuses. (3) Although with a low chimerism level after IUHSCT in large-animal models, donor-specific tolerance to postnatal kidney transplant can be achieved. State of donor-specific tolerance can also be confirmed by in vitro analysis such as MLR and CML assay. (4) Persistent postnatal peripheral blood chimerism after IUHSCT is not necessary for donor-specific tolerance, while transient chimerism in peripheral blood and persistent chimerism in lymphoid tissues (thymus, bone marrow, liver, spleen, lymph nodes) might have more importance for donor-specific tolerance induction in postnatal stage. (5) Fetal immune suppression at the time of transplantation can improve the level of chimerism in the progenitor population but not in peripheral blood mature donor progeny. (6) MHC-independent donor/host competition is also responsible for low engraftment in immunocompetent recipients. This may indicate the importance of creating more available “niches” allowing a higher engraftment after an appropriate conditioning regimen.

III.3. IUHSCT in Human Fetuses

The success of IUHSCT in rodent and large-animal models, however, has not yet been realized in humans [100]. The only clear success has been obtained in fetuses affected by immunodeficiency syndromes such as bare lymphocyte syndrome (BLS) or other types of severe combined immunodeficiency (SCID) [101].

SCID is a genetic disorder characterized by the absence of functional T-lymphocytes, which results in a defective cellular or humoral response (due to either direct involvement with B lymphocytes or through improper B lymphocyte activation

due to non-functional T-helper cells) [102]. SCID is the result of an immune system so highly compromised that it is considered as almost absent. BLS can be regarded as a form of SCID in which lymphocyte precursor cells are improperly formed caused by mutations in certain genes of the MHC- I or MHC- II [103].

The earliest IUHSCT was performed in a fetus with BLS by Touraine J.L. et al. in 1989 [104] in Lyon, France. Subsequent to BLS, several studies have achieved success in treatment of SCID with IUHSCT [105-109].

In these cases either fetal liver, paternal BM, or maternal BM-derived CD34+ cells were transplanted between 16 and 26 weeks of gestation, and resulted in engraftment of donor cells at birth. SCID was also treated by SCT in postnatal stage with satisfied outcomes. In the study of Touraine J. L. et al. [110], 19 patients with SCID, including 17 infants and 2 fetuses, received fetal liver SCT. All patients were subjected to immunological investigations (3 of them were analyzed more extensively over a long period of time 21–34 years), especially on peripheral blood lymphocytes. Fourteen of the 19 patients had evidence of donor cell engraftment and developed an immunological reconstitution. The chimeric patients had donor-derived T lymphocytes which progressively demonstrated positive interactions with other host cells and they were shown to be tolerant toward both host and donor antigens. The latter tolerance relies upon clonal deletion from the T-cell repertoire, and it results from the contact between thymocytes of donor origin and dendritic cells or macrophages also deriving from donor stem cells. The former tolerance does not imply clonal deletion of T-cells with host reactivity. Numerous T-cells recognizing the allogeneic, host-type antigens are identified in these patients, but these cells are anergized, following interaction with epithelial cells of the host thymus. Authors concluded that induction of transplantation tolerance at the fetal or neonatal stage requires only minimal engraftment.

Attempts at using IUHSCT to treat diseases other than SCID have been unsuccessful, indicating that the host (fetal) alloreactive immune response is a critical factor in the barrier to donor cell engraftment. Alternatively, in human fetus, alloreactive immune response can begin to function even at 16 and 26 weeks of gestation (though with an extent of deficiency). Besides, donor cells engraftment

could be limited by the competitive disadvantage when cells are transplanted into an intact fetal host with a limited number of hematopoietic niches available for donor cells.

III.4. Conclusion

Experiences of IUHST in large-animal and human fetuses can present significant implications for tolerance induction in neonatal stage. In large-animal and human neonates, with an immune system which is much more developed than in the fetus, the immune barrier to donor cell engraftment must be more intensive to traverse through. Thus, neonatal tolerance induction without recipient conditioning and immunosuppressive therapy in Medawar's or other researchers' rodent models, evidently, is not reproducible in large-animal or human neonates for which the immune systems have been relatively well developed. Tolerance induction in "premature" neonatal large-animals and human would confront an immune barrier more similar to that of tolerance induction in fully mature, immunocompetent individuals, and should be very cautious to take neonatal tolerance induction in mice as a counterpart.

IV. Characterization of Premature Immune System in Human Neonates

Neonatal tolerance induction protocols, which have been proved to be effective in rodent species, as previous discussed, are not reproducible in large-animal models and human. Nevertheless, in large-animals and human, neonatal and infant immune system still demonstrates several unique features after solid organ or hematopoietic stem cell transplantation, as described below.

(1) Liver transplantation in infants younger than 90 days has similar graft and patient survival compared to those in older children and in adults; but these pediatric patients are more susceptible to tolerance induction and subsequent withdrawal of immunosuppression, indicating that T-cell immunity in infants is competent but in the meantime has more plasticity than in adults. [111-117].

(2) Recipients of umbilical cord blood cells experience less GVHD than recipients of HSCT from an adult donor [118], suggesting that T-cell immune function is lower in neonates than in adults.

(3) ABO-incompatible organ transplantation and B cell tolerance are achievable in human infants [79], showing that B cell immunity in infants is partially impaired, especially regarding polysaccharidic antigens.

In such circumstances, we can conclude that compared to adults, human neonates and infants may have more advantage of donor-specific tolerance induction in organ and HSC transplantations. Exploring of donor-specific tolerance induction protocol which is specific for neonates and very young infants with immature immune system may pave the way of specific immunosuppressive regimen. In advance of this exploration, a thorough examination to neonatal adaptive and innate immune is essential.

IV.1. Neonatal T-cell Immunity

T cells response play a crucial role in allograft rejection. Following allotransplantation, allogeneic donor antigens are recognized by the recipient's T cells by two distinct but not exclusive pathways: direct recognition and indirect recognition [119]. During direct recognition pathway, donor derived antigen presenting cells (APCs) expressing donor allogeneic-MHC presenting donor peptide, activate recipient T cells, which are mainly responsible for the acute rejection during the early post-transplant period. In contrast, the indirect recognition pathway is characterized by the stimulation of T cells by donor allogeneic peptides processed and presented on self-MHC expressed by recipient APCs. This pathway primarily contributes to acute rejection and the immunological processes involved in later-phase rejection.

Naïve T-cell activation requires at least two signals provided by APCs: one is antigen-dependent, TCR-mediated (signal-1) and the other one is antigen-independent and relies on costimulatory signal (signal-2). Recently, a new antigen-independent signal (signal-3) has been defined and it's characterized by the action of soluble mediators such as cytokines.

The interaction between APCs and activated T cells is considered to be a two-way dialog, which on one hand leads to APCs maturation, and on the other hand generate the fully activated effector T cells. Both quantitative and qualitative change in such interaction can alter the development effector T cells.

Several properties of human neonatal T cell immunity are characterized below.

(1) T-cell alloimmunity is largely competent [120, 121]. In response to allo-antigens of transplantation setting, human neonatal T cells are widely accepted to be immunocompetent whereas have altered thresholds of responsiveness. As a whole, human neonatal T cells showed normal *in vivo* responses to mitogen or allo-antigen stimulators, by both aspects of T-cell proliferation and cytokines (IL-4, IL-5, IL-10, and IFN- γ) production. However, naive T cells were also shown to require much longer periods of TCR triggering compared with mature effector T cells [122].

(2) Less memory T cells and more naïve T cells present in neonatal immune system [123]. Altered thresholds of T-cell responsiveness in neonates is partially due to intrinsic characters of neonatal T cells. Neonatal T cells exhibit intrinsic deficiencies

that may prevent them from becoming fully activated. Compared to peripheral blood T cells in adult, neonatal T cells have lower baseline expression of TCR/CD3 complex, but higher expression of CD45RA naïve phenotype, indicating that the cellular immune system of neonates is still antigen-inexperienced and consists of high percentage of naïve T cells and low percentage of memory T cells.

(3) Less cytotoxic T cells and more helper T cells. Compared to adults, T-lymphocytes in neonate are also observed to have higher percentage of helper T cells and lower percentage of cytotoxic T cells [124-128].

(4) Deviated T-cell immune to Th2 response. Although widely accepted as immunocompetent, neonatal T-cell immune is biased towards a Th2 response, as discussed in previous chapter. Diminished Th1 cell responses have been observed following certain infections and immunizations [50, 53, 58, 59, 129]. But compared to mice, the Th2 bias is less evident in human neonates as low levels of all cytokines can be detected [130].

(5) Neonates show hyporesponsiveness to viral pathogens. To several pathogens such as respiratory syncytial virus and human immunodeficiency virus 1, in neonates the hyporesponsiveness of CTL response was observed to be lower than adults. Low CTL response may be primarily due to immaturity of APC function or costimulatory molecule expression [123, 131, 132].

(6) Natural regulatory T cells (nT_{reg}) may be able to facilitate allograft tolerance induction in early life. The nT_{reg} have a thymic origin and have been prove to be essential in the establishment and maintenance of immunological tolerance. Human preterm infants cord blood contained a high proportion of $CD4^+CD25^+$ nT_{reg} that declined with gestational age [133]. Comparing to adult peripheral blood, cord blood nT_{reg} have higher expressions of naïve phenotypes such as CD45RA and T-cell receptor recombination excision circles (TREC). Cord blood nT_{reg} also express T_{reg} markers such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and forkhead box P3 (Foxp3). Thus, cord blood nT_{reg} are regarded as a functionally mature population with naïve phenotype. This population of cells, which are critical in maintaining homeostasis and preventing autoimmunity, might be essential in allograft specific

tolerance induction.

IV.2. Neonatal B-cell Immunity

Most peripheral B cells are produced in bone marrow, and referred to as B2 cells. A minor B-cell population, known as B1 cells (initially identified in mice, and very recently in humans), are found mainly in the pleural and peritoneal cavities, but also in the spleen. B1 cells express CD19, high levels of CD5 and produce low affinity natural antibody (mainly IgM) without activation by T_h . Regulatory B cell is a recently described subset which inhibits T-cell responses via the production of IL10. Regulatory B cells have recently been described in humans and are characterized by surface expression of CD5, CD1d, CD24, CD27 and CD38. There are also evidences that regulatory B cells act independently of IL-10 [134].

Recently, due to the practice of ABO-incompatible transplantation and the evidence suggesting that B cells play an important role in acute cellular rejection, as well as in acute and chronic antibody-mediated rejection, increasing attention has been paid to the role of B cells, plasma cells and the antibodies in the immune response to the allografts. On the other hand, B cells are also proved to be able to act as regulators of alloimmune response. Following paragraphs focus on the characters of B cell immune in human neonates compared to adults.

(1) Neonates and very young infants are able to produce IgM, IgG and IgA antibodies under antigen exposure, however, neonatal humoral response is often shown to have delayed onset, reach lower peak levels and have a shorter duration [77, 127, 135].

(2) In neonates, B cells comprise high percentage of naïve $IgM^+IgD^{low/-}$ B-cell subset. Neonatal B cell pool has higher composition of B1 cells which are not able to differentiate toward memory B cells [136-138].

(3) Before the age of two years, newborn infants are not able to respond to certain bacterial capsular polysaccharides, which create a window of susceptibility to infections at the time of disappearance of protective maternal antibodies. In human adults, complement component C3d can associate with pneumococcal PSs and then

bind to CD21 (cell surface complement receptor on B cells), and then by using CD19 as a signal-transducing moiety, the crosslinking of CD21 with the B-cell receptor can lead to the synergistic activation of B cells. In newborns, reduced levels of CD21 expression and low complement activity result in a lack of CD21/BCR synergy and contribute to defective B-cell activation [120, 139].

(4) Pediatric patients younger than the age of 2-year have low production of antibodies (isohemagglutinins) against blood-group antigens that are not completely expressed on their own tissues, render them the possibility to performed ABO-incompatible organ transplantation. After 2-year of age, although they normally do not have contact with incompatible blood transfusions, natural antibodies begin to increase significantly, probably because of contact of the immune system to analogous antigens on intestinal bacteria or presence of the antigens innutrition [140-143].

(5) Recently, more and more studies showed that neonatal B cells demonstrated immunoregulatory function [71, 144, 145]. By mice in vitro and in vivo model system, Wendy E. et al. [146] illustrated that neonatal B cells produced substantial amounts of the immunosuppressive cytokine IL-10 in response to a range of TLR agonists and suppressed production of IL-12 and up-regulation of costimulatory molecules by DCs in response to TLR agonists, whereas adult B cells did not. This suppression was IL-10 dependent. Neonatal B cells also demonstrated quantitative and qualitative differences relative to their adult counterparts, notably, an increased abundance of the CD5⁺ B-1a subpopulation. Finally, neonatal, but not adult, B cells suppressed Th1, but not Th2 alloimmune T cell responses, effectively skewing the response toward Th2, both during in vitro MLRs, and in vivo, following their injection into adult mice in models of alloimmune priming and allotransplantation. Thus, neonatal B cells may play an active role in modulating alloimmune responses.

IV.3. Neonatal Innate Immune

Innate immune system plays important role in protecting the newborns against infection given the limited exposure to foreign antigens *in utero* and the

previous-described defects in neonatal adaptive immune system [147]. Innate immune system involves a huge immunological network including numerous types of cells and cytokines; the following paragraphs focus on defects in neutrophils and antigen-presenting cells, as well as defective complement-dependent cytotoxicity (CDC) and antibody dependent cell-mediated cytotoxicity in human neonates.

IV.3.1. Neonatal neutrophils

Qualitative and quantitative defects were observed in neonatal neutrophils, as reviewed by O. Levy. Neonatal mammals have reduced progenitor cells of granulocytes and diminished precursor storage pool, thus may results in reduced neutrophil count in peripheral blood compared to adult individuals [147].

Such reduced neutrophil count is furthermore correlated with a high percentage of immature neutrophils within. Neonatal immature neutrophils show multiple functional defects which involve chemotaxis, rolling adhesion and transmigration [148, 149]. Immature neutrophils show lower expression of complement receptor 3 (CR3, also termed as CD11b/CD18) with reduced function in promoting phagocytosis [150]. Low expression of L-selectin was also observed in neonatal neutrophils [151].

IV.3.2. Neonatal APCs

APCs in human neonates mainly include dendritic cells (DCs), monocytes and macrophages. The defective APC functions have been well reviewed by P. A. Velilla et al. [131].

Due to the low frequency of DCs in peripheral blood, most studies of neonatal DCs have been carried out using in vitro monocyte-derived dendritic cells (MDDCs). Different from neonatal T cells, neonatal APCs are deficient in producing proinflammatory cytokines such as TNF- α , IL-1, IL-6 or IL-12, in response to bacterial lipopolysaccharide (LPS) or CD40 signaling [58, 152-155]. Human cord blood APCs also appear to be immature as they exhibit low or no basal expression of costimulatory molecule such as CD40, CD80 or CD86 [156, 157]. The intrinsic deficiencies of neonatal APCs function subsequently result in defective interaction between APCs and T cells, which could lead to secondary defects of adaptive T-cell responses.

Findings supporting defective function of cord blood (CB) T cells and neonatal APCs

1. Findings that support defective function of CB T cells

- Low baseline expression of TCR/CD3 complex and adhesion molecules
- Blunted up-regulation of CD40L expression
- Defects in the production of cytokines
- Limited CD8 cytotoxic activity

2. Findings that support defective function of CB monocytes and macrophages

- Low basal levels of expression of costimulatory molecules by monocytes
- Unresponsiveness to LPS and IFN- γ of both monocytes and macrophages
- Reduced capacity of monocytes to differentiate into DCs
- Altered IL-12 production by PBMCs
- Decreased phagocytic activity of CB macrophages

3. Findings that support defective function of CB dendritic cells

- Low basal expression of costimulatory molecules by CB-MDDCs, CB mDCs and CB pDCs
 - Altered maturation of CB-MDDCs, CB mDCs and CB pDCs in response to TLR or CD40 signaling
 - Defective production of cytokines by CB-MDDCs in response to TLR or CD40 signaling
 - Decreased ability of CB-MDDCs and CB pDCs to stimulate allogeneic responses
 - Reduced endocytic activity of CB-MDDCs
-

Table 3: Findings supporting defective function of cord blood (CB) T cells and neonatal APCs.

Abbreviations: CB, cord blood; APCs, antigen-presenting cells; TCR, T-cell receptors; CD40L, CD40 ligand; LPS, lipopolysaccharide; MDDC, monocyte-derived dendritic cells; mDCs, myeloid DCs; pDCs, plasmacytoid DCs [131].

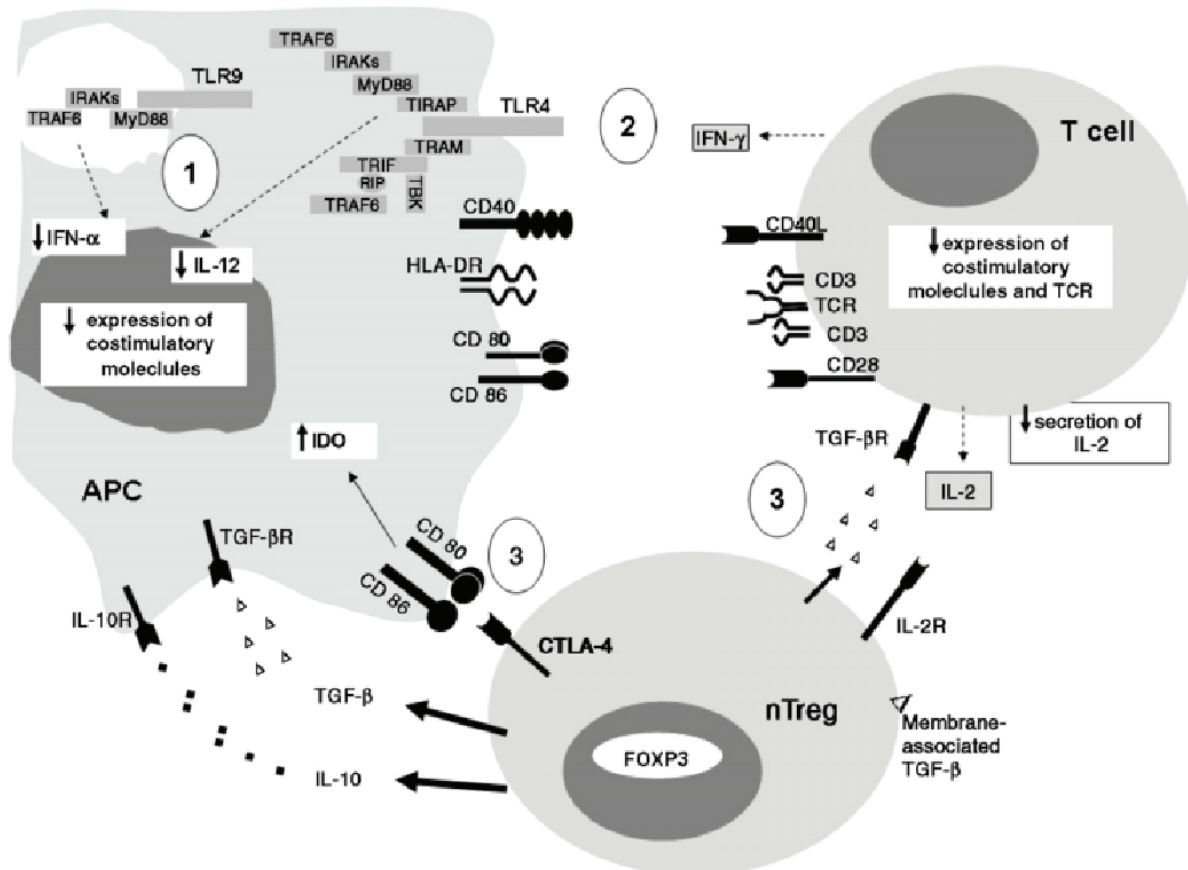


Figure 2: Potential mechanisms underlying dysfunction of neonatal dendritic cells.

Several mechanisms can be proposed to explain APC dysfunction in neonates (figure and legend from [131]):

(1) *Intrinsic immaturity of neonatal DCs.* Basal expression of costimulatory molecules is decreased in neonatal APCs. In addition, alterations in the TLR complex, affecting principally TLR4 and TLR9, have been described in neonatal APCs. This could lead to defects in up-regulation of costimulatory molecule expression, as well as cytokine secretion (IL-12, IFN- α) following stimulation with bacterial and/or viral products.

(2) *Defective interaction between neonatal APCs and T cells.* Defects in activation levels of neonatal T cells could lead to alterations in APC functions since T-cell-mediated signals play a major role in APC maturation/activation. Among the described T-cell defects are alterations in IFN- γ and IL-2 secretion in response to TCR-dependent stimulation, decreased expression of CD40L at basal condition and after stimulation, and decreased expression of the TCR complex.

(3) *Inhibition by nT_{reg} of both neonatal APCs and T cells.* Neonatal nT_{reg} can down-modulate the function of both APCs and T cells through direct and indirect mechanisms. Among these mechanisms

are interaction between the CTLA-4 molecule expressed by nT_{reg} and the CD80/CD86 molecules expressed by APCs, leading to increased IDO expression; secretion of immunosuppressive cytokines, IL-10 and TGF- β , and expression of membrane-associated TGF- β ; consumption of IL-2 by the high-affinity IL-2R expressed by nT_{reg} ; and finally, FOXP3-mediated mechanisms that have not yet been identified.

IV.3.3. Neonatal CDC

Neonatal CDC is deficient because in neonatal stage, complement levels and activity were significantly reduced when compared with adults.

Drew JH, et al. determined the whole complement activity (CH50), levels of some components of the classical (C1q, C4, C3) and alternative (factor B and properdin) pathways in 55 non-infected newborn infants. Results showed that normal newborn infants were lower than adults in all complement measurements; preterm infants being significantly lower than term infants. Absence of C3 split products indicated that the deficiencies were developmental and not due to activation of the complement system [158]. Wolach B, et al. compared the complement system's activities (CH50 and AP50) as well as its various components (C1q, r, s, C2-C9, Factor B, and properdin) in 60 preterm infants, 50 full-term newborn infants and 49 healthy adults. Results revealed that complement levels and activity were significantly reduced in preterm and full-term neonates when compared with adult levels, with the exception of C7 which was within the normal range in most infants [159]. Høgåsen AK, et al. measured native complement factors and complement activation products in 72 healthy neonates. The concentrations of complement components C4, C3 and factor B were lower in neonates compared with adults (factor B: 35.9%, C4: 45.1%, C3: 56.2% of adult values), which is in accordance with previous reports [160]. The concentration of C9 was even lower, only 10.8% of normal adult values in neonates. Fifteen percent of the neonates had C9 levels lower than 2% of adult values [161]. Moreover, in older children, Prellner K, et al. determined the concentrations of C1q, factor B, factor D and properdin in healthy children belonging to various age groups of 1 through 5 years of age. Results showed that all concentrations were found to be age-dependent. C1q

and properdin levels were lowest in the younger children [162].

IV.3.4. Neonatal ADCC-mediating cells

ADCC can be mediated by macrophages, neutrophils and natural killer (NK) cells. Besides defects in neutrophils, in newborn infants, previous studies have proved that the function of macrophages and NK cells mediated ADCC is defective when compared to adults. Due to the low proportion of macrophages and NK cells in peripheral blood, studies of neonatal ADCC were often realized by in vitro experiments using cord blood mononuclear cells (MNC). Assessed in the study of Shore SL, et al., MNC showed moderately reduced cytotoxic activity when compared with blood MNC from normal adults at the same effector cell: target cell ratio [163]. Similarly, Kohl S, et al. proved that human neonatal MNC had low ADCC compared with cells from adults in a chromium-release assay against Chang liver cells infected with herpes simplex virus (HSV) [164]. Reviewed by Velilla PA, et al., the findings that support defective function of cord blood monocytes and macrophages included: (1) low basal levels of expression of co-stimulatory molecules; (2) unresponsiveness to LPS and IFN- γ ; (3) decreased phagocytic activity; (4) altered IL-12 production [131]. It should be noted that IL-12 can greatly enhance ADCC activities of neonatal MNC [165]. Decreased production of IL-12 by cord blood MNC stimulated by *Staphylococcus aureus* Cowan (SAC) has been described [166]. Chelvarajan RL, et al. also found that lipopolysaccharide (LPS) failed to induce neonatal macrophages to produce interleukin (IL)-1 β and tumor necrosis factor alpha (TNF- α) mRNA and to secrete IL-1 β , IL-12, and TNF- α [153]. Since monocytes are the main producers of IL-12 in SAC-stimulated PBMC, these results also point towards defective activation of neonatal monocytes.

IV.4. Conclusion

In sum, currently identified differences between adult and neonatal immune systems may include:

(1) T cells are largely immunocompetent at birth, however, T cells of neonates are still antigen-inexperienced and consist of high percentage of naive helper T cells and

low percentage of memory T cells and cytotoxic T-cells [125, 128].

(2) Neonatal B cells have decreased ability to produce antibodies against bacterial polysaccharide antigens and reduced ability of ADCC. Thus, ABO-incompatible organ transplantation and B cell tolerance are achievable in human infants [79].

(3) The widely observed hypo-responsiveness to alloantigens or pathogens may be primarily due to immaturity of APCs function or co-stimulator molecules expression.

(4) Concentration and activity of serum complement components are reduced, resulting in defective CDC activity.

(5) Neonatal macrophages and monocytes are immature and with reduced phagocytic function, correlated to reduced ADCC activity.

(6) Neonatal neutrophils also demonstrate qualitative and quantitative defects.

V. Hematopoietic Stem Cells Transplantation and Tolerance

Induction in Neonates

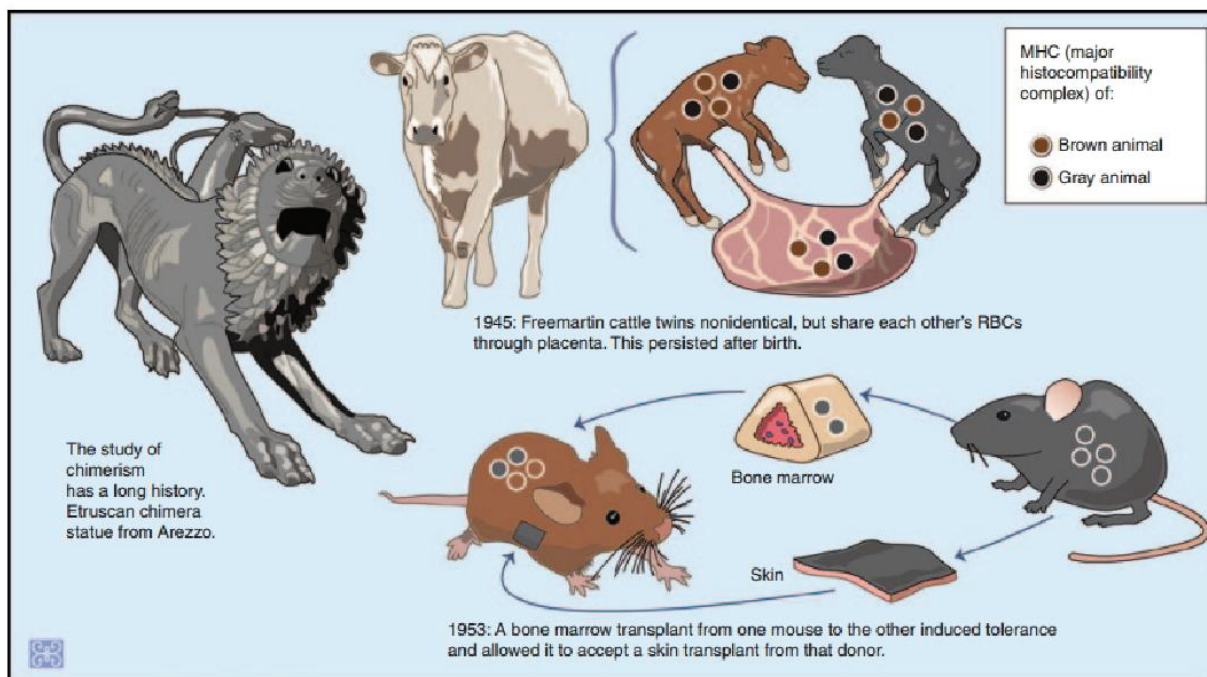


Figure 3: Discovery of chimerism and tolerance.

Figure from Leventhal, J., et al. [167].

Previous studies of IUHSTC elucidate that neonatal tolerance induction in rodent model through donor-derived bone marrow cells or splenic cells inoculation without any conditioning or immunosuppressive therapy is not reproducible in large-animal or human neonates; because in these recipients, although newborn ones, immune system is already competent enough to amount a powerful alloimmune response to reject allogeneic cells as in adult recipients. However, on the other hand, immune system of large-animal and human neonates still show an extent of “immaturity”, for example, relatively defective B cell immune and innate immune function, Th1 to Th2 immune deviation. Such immaturity makes neonatal recipients become a unique population in the settings of immunosuppressive therapy and tolerance induction after solid organ or HSC transplantation. Moreover, practices of liver transplant,

ABO-incompatible organ transplant and HSCT in newborn infants demonstrate that at least in these cases, newborn recipients are more susceptible to donor-specific tolerance induction than adult ones. An illuminating study was that immunosuppressive therapy in fetus by anti-thymocyte globulin could facilitate engraftment and improve chimerism level in lymphoid tissues after IUHSCT [99]. Implication of this study is that: in newborn human, whether donor-specific tolerance may be induced under less intensive conditioning therapy through HSCT, what kind of conditioning therapy, which level of engraftment/chimerism, could be enough for tolerance induction in neonates or very young infants? In order to clarify these questions, it's necessary to pursue the exploration of the evolving approaches transplant tolerance induction by hematopoietic stem cell-based therapies.

V.1. Nonmyeloablative Conditioning Regimens in Animal Models

After the finding of tolerance in neonatal mice by Medawar's team, subsequent studies revealed that tolerance can also be induced in adult mice. In 1955, Main J.M. and Prehn R.T. [168] demonstrated that under myeloablative conditioning, adult mice that received allogeneic bone marrow cells infusion were rescued from ablation and were also tolerant to donor skin grafts.

Success of tolerance induction was also extended from rodent model to large-animal models. In 1972, with a canine model, Rapaport, F.T. confirmed that induction of unresponsiveness to canine renal allografts could be achieved by total body irradiation (TBI) and bone marrow transplantation [169]. Thereafter, hematopoietic chimerism induces tolerance to organ transplants has been confirmed in all tested large-animal models [167].

In human patients, it has been concluded for long time that full tolerance has been developed in two circumstances only: (a) when the transplant recipient is not immunologically competent or mature and (b) when the transplant involves full replacement of host lymphocytes by donor lymphocytes, obtained by HSCT after myeloablative conditioning [110]. For the latter circumstance, although myeloablative conditioning was acceptable for the use of HSCT in hematologic malignancy, the

relevant risk of complications was unacceptable when applied to nonmalignant situations of solid organs and composite tissue allotransplantation.

Researchers therefore continue to pursue protocol of performing HSCT for tolerance induction with non-myeloablative conditioning (also called reduced conditioning or less intensive conditioning) that could be justified for use in organ-transplant recipients. Ildstad and Sachs firstly reconstituted the lethally irradiated (900 R whole body irradiation) adult mice with T-cell depleted bone marrow containing both recipient (5×10^6 syngeneic cells) and donor (1.5×10^7 allogeneic or 4×10^7 xenogeneic cells) components. As former study, this therapy led to long-term survival of the reconstituted animals and specific prolongation of subsequent skin grafts of donor type. Animals reconstituted in this fashion were fully reactive to third-party allografts and xenografts and did not appear to manifest signs of graft-versus-host disease [170].

Sharabi and Sachs continually evaluated the outcome of tolerance induction by unmanipulated fully MHC-disparate bone marrow transplant (1.5×10^7 cells) under nonlethal conditioning regimen (300 R whole body irradiation, 700 R thymic irradiation, anti-CD4 mAb and anti-CD8 mAb). The results demonstrated that stable mixed chimerism with donor-specific tolerance can be induced to skin graft across an MHC barrier after a nonlethal preparative regimen, without clinical GVHD and without the risk of aplasia [171].

Colson and Ildstad also reported that durable multilineage mixed allogeneic chimerism and donor-specific transplantation tolerance for skin and primarily cardiac vascularized allografts can be achieved across multiple histocompatibility barriers using a nonmyeloablative radiation-based regimen [172]. Similar nonmyeloablative radiation-based regimen (including 300 R whole body irradiation, 700 R thymic irradiation, anti-thymocyte globulin, CsA and splenectomy) had subsequently been extended successfully to the induction of tolerance to renal transplants in fully mismatched cynomolgus monkeys [173, 174].

In swine model, mixed chimerism across both minor and major histocompatibility barriers can be established using high doses of peripheral blood stem cells (200×10^8 cells/kg) in the absence of WBI [175]. In these studies, a critical finding was that animals with 1% peripheral blood donor chimerism showed just as much tolerance as those with 100% donor chimerism, suggesting that complete replacement of the recipient hematopoietic system with that of the donor is not a prerequisite to tolerance induction. It was also hypothesized that, although chimerism was required to induce tolerance, it was not necessary for its maintenance, and that immune regulation would be able to maintain the tolerant state after the loss of chimerism. Donor antigen from the kidney allograft was believed to directly contribute to the regulatory mechanism [176].

Nonmyeloablative conditioning regimens for stem cell transplantation					
Preclinical data					
Investigator	Model	MHC	Regimen	Chimerism	GVHD
<i>Sharabi</i>	Mouse	Mismatch	WBI (300 cGy) Anti-CD8 mAb, TI, CYA	Stable mixed 56%-84% donor PBL	None
<i>Pelot</i>	Mouse	Mismatch	CY, anti-CD4 and Anti-CD8 mAb, TI, CYA	Stable mixed (lymphoid>myeloid)	None
<i>Sykes</i>	Mouse	Mismatch	Anti-CD4, anti-CD8 mAb, TI, HD stem cells, CYA	Stable mixed	None
<i>Kawai</i>	Cynomologous Monkey	Mismatch	WBI (300 cGy), ATG, TI, CYA, splenectomy	Mixed; Max % donor cells 1.5% to 57.5%	None
<i>Sachs*</i> <i>Huang</i>	Miniature Swine	Mismatch Match	Anti-CD3, immunotoxin, TI, HD stem cells, CYA	Stable mixed (lymphoid>myeloid)	None
<i>Storb</i>	Dog	Match	WBI (200 cGy), post-transplant MMF, CYA	Stable mixed (4/5 dogs)	None

TI = thymic irradiation; WBI = whole body irradiation; mAb = monoclonal antibody; ATG = anti-thymocyte globulin; MMF = mycophenolate mofetil; CYA = cyclosporine; PBL = peripheral blood lymphocytes; HD = high dose.

* Personal communication.

Table 4: Nonmyeloablative conditioning regimens for stem cell transplantation.

Table from Spitzer [177].

V.2. Nonmyeloablative Conditioning Regimens in Clinical Kidney

Transplant

Tolerance induction protocol with nonmyeloablative conditioning subsequently

moved into clinical trial of kidney transplantation after it was justified in animal experiments. Following paragraphs focus on clinical protocols which have been performed in HLA-identical and HLA-mismatch renal transplants.

In the HLA-identical renal transplantation study from Scandling et al. [178-180] in Stanford University, sixteen patients conditioned with total lymphoid irradiation (TLI) and ATG were given kidney transplants and an injection of $10 \times 10^6/\text{kg}$ $\text{CD}34^+$ hematopoietic progenitor cells and $1 \times 10^6/\text{kg}$ $\text{CD}3^+$ T cells from HLA-matched donors. TLI was administered to recipients as 10 doses of 80 or 120 cGy each to the supradiaphragmatic lymph nodes, thymus, subdiaphragmatic lymph nodes, and spleen during the first 10 days post-transplant. $\text{CD}3^+$ T cells were given with HSC in order to facilitate the engraftment. Fifteen patients developed multilineage chimerism without GVHD, and eight with chimerism for at least 6 months were withdrawn from anti-rejection medications for 1-3 years (mean, 28 months) without subsequent rejection episodes. Four chimeric patients have just completed or are in the midst of drug withdrawal at the point of article publication, and four patients were not withdrawn due to return of underlying disease or rejection episodes. Blood cells from all patients showed early high ratios of $\text{CD}4^+\text{CD}25^+$ regulatory T cells and NKT cells versus conventional naive $\text{CD}4^+$ T cells, and those off drugs showed specific unresponsiveness to donor alloantigens. All 16 patients had excellent graft function at the last observation point with or without maintenance drugs. Authors concluded that TLI and ATG promoted the development of persistent chimerism and tolerance in a cohort of patients given kidney transplants and hematopoietic donor cell infusions.

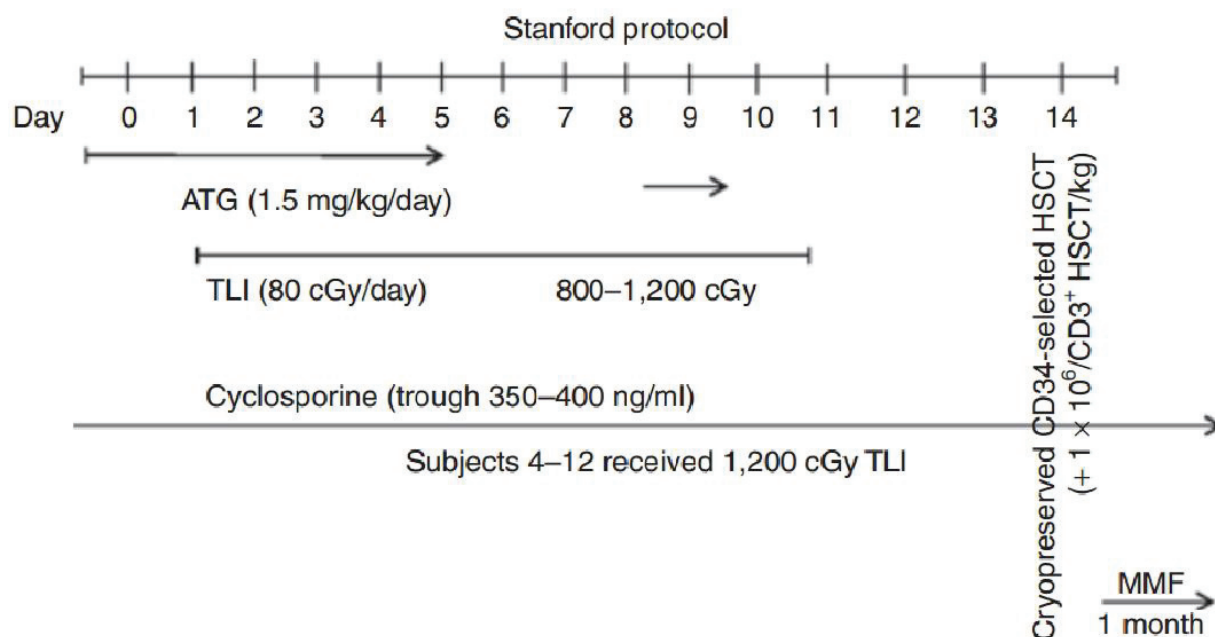


Figure 4: The Stanford protocol of nonmyeloablative conditioning therapy for tolerance induction for kidney transplantation.

The Stanford protocol included conditioning with ATG in combination with 800–1,200 cGy of fractionated total lymphoid irradiation. Cryopreserved CD34-selected G-CSF mobilized product supplemented with 1×10^6 CD3⁺ bone marrow cells per kilogram of the recipient's body weight was infused on day 14. ATG, antithymocyte globulin; G-CSF, granulocyte colony-stimulating factor; HSCT, hematopoietic stem cell transplantation; MMF, mycophenolate mofetil; TLI, total lymphoid irradiation [167].

Team of Massachusetts General Hospital also transferred kidney/HSCT tolerance induction protocol from animal experiments into clinical trials. For HLA-matched renal transplantation, in 1999, Spitzer et al. [181] treated a 55-year-old woman with end stage renal disease secondary to multiple myeloma with a combined HLA-matched bone marrow and renal transplant after conditioning with cyclophosphamide, ATG, and thymic irradiation. Outcomes showed early normalization of renal function, the absence of acute graft-versus-host disease, and the establishment of mixed lymphohematopoietic chimerism. CsA, which was the only posttransplant immunosuppressive therapy, was tapered and discontinued on day 73 posttransplant. No rejection episodes occurred, and renal function remains normal on

day 170 posttransplant (14 weeks after discontinuing cyclosporine). Later until 2006 [182], up to 6 patients with renal failure due to multiple myeloma received simultaneous kidney and BMT from HLA-identical sibling donors following similar nonmyeloablative conditioning with the first case treated in 1999, CsA was given for approximately 2 months posttransplant, followed by donor leukocyte infusions. All six patients accepted their kidney grafts long-term. Three patients lost detectable chimerism but accepted their kidney grafts off immunosuppression for 1.3 to more than 7 years (one such patient had strong anti-donor CTL responses in association with marrow rejection). Two patients achieved full donor chimerism, but resumed immunosuppression to treat graft-versus-host disease. Only one patient experienced rejection following CsA withdrawal. Authors concluded that combined kidney/BMT with nonmyeloablative conditioning can achieve renal allograft tolerance and excellent myeloma responses, even in the presence of donor marrow rejection and anti-donor alloresponses in vitro [167, 183].

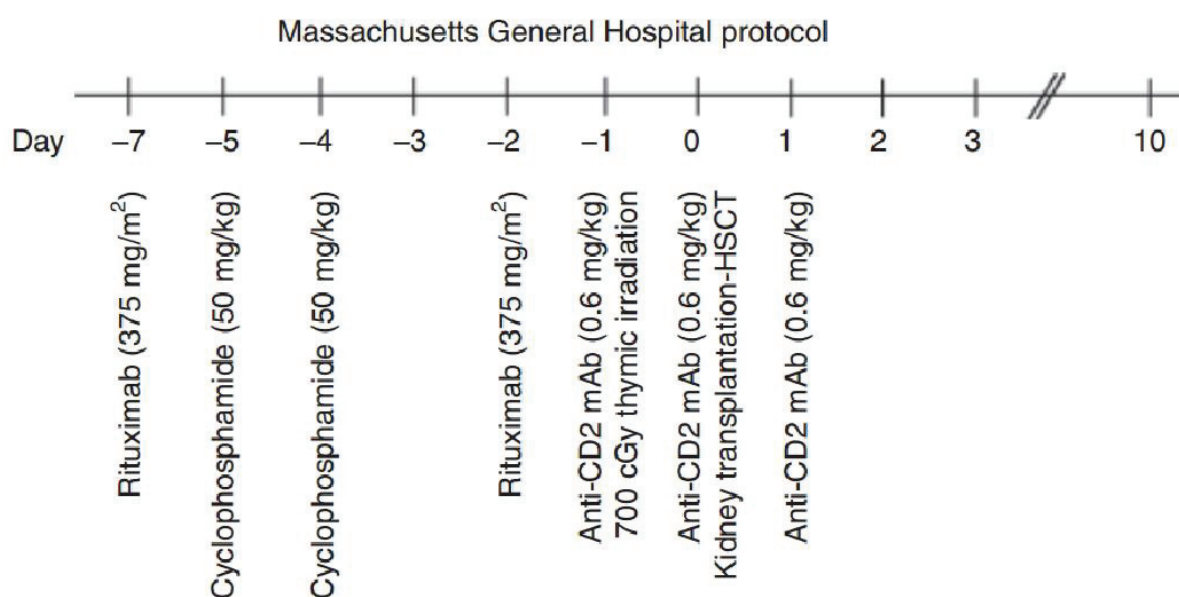


Figure 5: The Massachusetts General Hospital protocol of nonmyeloablative conditioning therapy for tolerance induction for kidney transplantation.

In the Massachusetts General Hospital (MGH) protocol, recipients were conditioned with a combination of thymic irradiation, rituximab, cyclophosphamide, and anti-CD2 monoclonal antibody (mAb) [167].

Subsequent clinical trial was conducted in HLA-mismatch renal transplantation [184, 185]. Beginning in 2002, a total of 10 patients (2 cohorts) received combined bone marrow and kidney transplants from HLA single-haplotype mismatched living related donors, with the use of a nonmyeloablative preparative regimen similar to HLA-matched renal transplantation, except in several cases anti-CD20 monoclonal antibody treatment was added to the recipient conditioning regimen to target B cells and humoral immunity. Transient chimerism and reversible capillary leak syndrome developed in all recipients. By recent report, Patient 1 remained well, without rejection for more than 10 years. Patient 2 also remained rejection-free for more than 9 years, although mycophenolate mofetil (MMF) was added after 7 years. Patient 3 developed irreversible humoral rejection, resulting in loss of the renal allograft. In Patient 4, Kidney-allograft function remained stable for more than 7 years, but MMF was initiated, since chronic humoral rejection was diagnosed at 5 years. In patient 5 chronic humoral rejection was also observed at 6.8 years post-transplant. In the second cohort, Patients 6, 7 and 9 successfully discontinued immunosuppression. Their condition remained stable, without evidence of cellular or humoral rejection for 3 to 4 years. Patient 8 developed acute cellular rejection 7 weeks after immunosuppression was discontinued and coincident with a viral infection, with subsequent graft loss. Patient 10 resumed dialysis after losing kidney function due to thrombotic microangiopathy 6 months after transplantation. In sum, the outcomes from HLA-mismatch kidney transplants are not as satisfied as those from HLA-match transplants: 3/10 patients experienced graft loss, 3/10 patients in the first cohort developed chronic humoral rejection and MMF was re-administrated in 2 of them, 3/10 patients in the second cohort remained stable but longer-term observation is necessary, 1/10 patient remained well and without rejection for more than 10 years [167].

V.3. Relatively Non-toxic Conditioning Regimens

As discussed above non-myeloablative protocols of BMT for tolerance induction were introduced in experimental studies. But conventionally, conditioning of the recipient still required TBI, thymic irradiation or cytotoxic drugs such as cyclophosphamide. The potential toxicity associated with such cytoreductive conditioning is a major reason preventing these protocols from routine clinical application. To increase the potential clinical acceptability of such regimens, it would be desirable to improve the success rate of such protocols and to achieve chimerism and tolerance avoiding cytoreductive conditioning.

Conception of lymphodepletion-based conditioning regimen was developed under such circumstance. In 1970, Gozzo J.J. and Monaco A.P. reported that allogenic, homozygous donor bone marrow cell infusion under lymphocyte-depleting condition with antilymphocyte serum significantly prolonged graft survival time in mice [186]. Later, Monaco et al. introduced similar conditioning and bone marrow transplantation protocol to human kidney transplantation [187]. One recipient was given anti-lymphocyte serum (ALS, first 14 days after renal transplant) and 11×10^9 donor bone marrow cells (at day 25 post-operation) along with conventional doses of prednisone and Azathioprin. The conventional immunosuppressive agents were tapered and renal function was normal 8 months after transplantation. Histological examination of the renal allograft showed only minimal evidence of rejection.

Nowadays, this lymphodepletion-based conditioning regimen has achieved success in HLA-identical renal transplantation in the clinical trial of Northwestern University which was initiated in 2008 [167, 188]. In this study, 20 cases of HLA-identical sibling donor-recipient pair renal transplants had been performed with this conditioning regimen. Lymphodepletion was induced by alemtuzumab (monoclonal antibody target CD52 which is the cell surface molecule of mature lymphocytes). Under lymphodepletion conditioning, 4 infusions of donor CD34-selected HSCs (cell doses range from 0.5×10^6 cells/kg to 4×10^6 cells/kg) were

performed postoperatively during the first 9 months after renal transplantation. Conventional immunosuppression was tapered and converted to sirolimus monotherapy. Only transient microchimerism was present, and it had disappeared at the 1-year follow-up. Immunosuppression is totally withdrawn by 24 months in subjects with stable renal function and normal protocol biopsies. At the time of this publication, patients in the study range from 2 to 52 months posttransplant and 50% of these recipients have been off immunosuppressive agents for more than 1 year. None of the 20 subjects has had deterioration from the optimal renal function that occurred in the early posttransplant period. Increased numbers of CD4⁺/CD25^{high}/FoxP3⁺ Treg were observed in the peripheral blood of these patients during lymphoid reconstitution, suggesting induction of an immunoregulatory state. This tolerance induction protocol excludes the need for permanent chimerism, but focus on a prolonged immunoregulatory environment which could be provided by donor HSC infusions in the setting of HLA-identical renal transplantation.

Besides the lymphodepletion-based conditioning protocol, concept of non-lymphocyte-depletion tolerance induction protocol, such as immunosuppression and costimulation blockade based regimen, is also developed nowadays. In mouse model, Blaha et al. introduced a tolerance induction protocol including unseparated BMCs infusion, anti-CD40L monoclonal antibody (blockade of CD40: CD154 costimulatory pathway) and CTLA4Ig (blockade of CD28: B7 costimulatory pathway) in addition to a short-term of immunosuppression (rapamycin, methylprednisolone, and mycophenolate mofetil for 4 weeks after bone marrow transplantation). This protocol led to long-term multilineage chimerism in 28/30 of the recipients and significant prolongation of skin graft survival [189].

In large-animal model, Lo et al. developed a non-lymphocyte-depletive, belatacept-based regimen without HSCT in rhesus monkeys [190]. Belatacept is a fusion protein composed of the Fc fragment of a human IgG1 immunoglobulin linked to the extracellular domain of CTLA-4, therefore can selectively block the process of T-cell activation. Studied rhesus monkeys undergoing MHC-mismatched renal allotransplants treated with belatacept and the mTOR inhibitor, sirolimus. To extend

prior work on costimulation blockade-resistant rejection, some animals also received CD28 blockade with alefacept (LFA3-Ig). Belatacept and sirolimus therapy successfully prevented rejection in all animals. Tolerance was not induced, as animals rejected after withdrawal of therapy, indicating that HSCT and engraftment may be critical to the success of such protocol.

Page et al. introduced a nondepleting protocol with combination of CD40 monoclonal antibody, CTLA4Ig and sirolimus, plus HSCT [191]. Results showed prolonged engraftment required the presence of all three agents during maintenance therapy, and resulted in graft acceptance for the duration of immunosuppressive treatment, with rejection resulting upon immunosuppression withdrawal. In this study, although complete withdrawal of immunosuppression was not achieved, notably, this protocol excluded the use of CNIs and steroid.

Recently, CD28 blockade has been successfully translated to the clinic with commercialized Belatacept. Translation of blockade of the CD154:CD40 pathway has been less successful, in large part due to thromboembolic complications associated with anti-CD154 antibodies. Translation of CD40 blockade has also been slow, in part due to the fact that synergy between CD40 blockade and CD28 blockade had not yet been demonstrated in either primate models or humans. So far, non-lymphocyte-depleted, immunosuppression and costimulation blockade based tolerance induction protocol has not yet come into clinical trial.

V.4. Conclusion

In summary, neonatal tolerance has been studied for more than 60 years and numerous investigations have been performed in order to transfer immune tolerance into clinical transplant setting. Now this research has come into an era where donor-specific tolerance is clearly within reach.

This chapter briefly summarized the experience of transplant tolerance induction by HSCT with nonmyeloablative conditioning therapy in animal models. Based on these preclinical studies, nowadays less-toxic nonmyeloablative tolerance induction protocols (which may include cytoreductive medication or irradiative therapy) have

been administered in clinical trials and outcomes were promising. However, the potential toxic effects associated with such cytoreductive and irradiative therapies constrict the routinely application of these nonmyeloablative conditioning protocols. Thus researchers turned to evaluate the potential of HSCT under relative non-toxic conditioning protocol which may require (1) lymphodepletive therapy by monoclonal or polyclonal antibodies or (2) costimulation blockade therapy by anti-CD40L monoclonal antibody or CTLA4Ig, as well as concomitant immunosuppressive therapy. The lymphodepletion-based tolerance induction protocols become more and more widely used in clinical transplant, while so far tolerance induction protocols with HSCT and costimulation blockade are still in the stage of preclinical studies.

To our project of tolerance induction to composite tissue allografts in neonates, if considering the vulnerability of these newborn patients, a lymphodepletion-based tolerance induction protocol is undoubtedly more applicable than a nonmyeloablative protocol which includes toxic cytoreductive medication or irradiation, or a costimulation blockade protocol which has not yet proved by clinical trials. This conclusion is a basic thinking in present project of tolerance induction in neonatal swines.

VI. Thymus and Tolerance Induction in Pediatric Transplantation

Thymus is a relatively large organ in a neonate or an infant. In contrast to adult recipient in whom the thymus has undergone a gradual involution, the existence of a potent thymus lets the very young recipient to be a different immune host during immunosuppressive therapy and tolerance induction.

VI.1. Thymic Emigrants and Peripheral Lymphocyte Constitution after T-cell Depletion

Peripheral T-cell reconstitution after T-cell depletion induced by ATG (or ALS, CD52 antibody, anti-CD3 antibody, et al.) can involve both thymus-dependent pathway (thymic emigration of T lymphocytes into peripheral blood and tissues) and thymus-independent pathway (homeostasis-driven proliferation of peripheral T cells) [192].

It has been confirmed that thymic emigrants take an important role in peripheral T cell homeostasis [193]. In normal adults, T-cell homeostasis appears to be maintained through thymus-independent pathway and the homeostasis-driven proliferation mechanism is generally competent to maintain peripheral normal T-cell number, function and adequate TCR repertoire diversity. However, in case of T-cell depletion, thymic emigrants take an important role in the full restoration of T-cell homeostasis. In such setting, patients with deficient thymic function can result in prolonged reductions in T-cell count and incomplete restoration of T-cell homeostasis [194-197].

Nevertheless, the respond of thymus in reconstitution of the peripheral T-cell after depletion therapy remains relatively unclear. Some researchers argued that thymus fails to respond rapidly and vary the export rate or composition of the emigrants in response to changes in the composition or size of the peripheral T-cell pool. The rate of emigration remained a product of thymus mass, with 1–2% of total thymocytes

exported daily, regardless of change in peripheral circumstance (no matter the mice with normal T-cell pool, or mice in which the size of the T-cell pool was increased by 80%, or depleted to <30% of normal). Thus the authors contend that thymic T cell export is internally regulated by as yet undefined mechanisms [198-200].

The activity of thymus is age-related. During fetal and neonatal life, thymus is the primary site of T lymphopoiesis. In human, thymus reaches its maximum size (about 25 cm³) within the first 12 months of life, and thereafter undergoes a gradual involution, so the volume of true thymic tissue reduces progressively throughout of life. The reduction rate of true thymic tissue is approximately 3% per year from one year of age until middle age; thereafter it slows down to less than 1% per year [201, 202]. In this regard, considering the huge size of thymocytes (approximately 10¹¹ cells) and the potent thymic activity (20-25% thymocytes are produced by cell division each day) of newborn infants [201], it's no doubt that thymic emigrant mechanism can play a more important role in the T cells restoration after profound lymphocyte depletion, compared to the adults who have smaller size of thymocytes. Even the rate of thymocyte emigration remained not changed as 1–2% of total thymocytes; each day recent thymic emigrants can amount to approximately 1~2×10⁹ T cells which is a comparable number to the total peripheral blood T cells in neonates. Thus, if the function of thymus is not impaired in the conditioning therapy and no interference of other immunosuppressive maintaining therapy after ATG infusion, it can be imagined that the full restoration of peripheral T cells could be completed rapidly.

So far, there is no direct evidence to prove this hypothesis in human neonates. But the implication of thymus-dependent pathway was demonstrated in the clinical setting of thymic function detection after bone marrow transplantation, which involved older children [203]. In these setting, the recovery of CD4⁺ T-cell number and total peripheral T-cell number was achieved in a relatively rapid phase in children, and such rapid recovery was accompanied by higher number of CD45RA⁺ T cells. Otherwise, in children undergoing partial thymectomy during cardiac surgery, levels of recent thymic emigrants was also proved to be decreased [204].

Besides thymic emigration, lymphopenia driven proliferation is the other

important mechanism to maintain peripheral T cell homeostasis. Lymphopenia driven proliferation is different to antigen-driven proliferation. It doesn't depend on foreign antigen stimulus but requires the interaction between TCR and MHC molecules loaded with self-peptides [205, 206].

Homeostasis-driven proliferation after T-cell depletion is associated with changes in the ratios of (1) CD4+ to CD8+ T cells and (2) naive to memory T cells.

It has been shown that the regeneration of CD4+ T cells is dependent on the thymic emigration; otherwise the regeneration of CD8+ T cells occurs through thymus independent mechanism. In adults, because of involution of thymus and subsequent poor recent thymic emigration, T cell repopulation will largely depend on the proliferation of residual peripheral T cells, so a persistent low level of CD4+ T cells is often documented, in company with over-regeneration of CD8+ T cells and reversed CD4+/CD8+ ratio [207]. The distribution of MHC-I and MHC-II molecules can also contribute to the reversed CD4+/ CD8+ ratio. The proliferation of CD4+ and CD8+ T cells respectively relate to the interaction of TCR with MHC-II and MHC-I molecules. The higher expression of MHC-I in the periphery may result in the over-generation of CD8+ T cells [205, 206]. Homeostasis-driven proliferation also results in changes in the ratio of naive to memory T cells. During homeostasis-driven proliferation, naive T cells have been shown to acquire the cell surface markers and functional properties of antigen-induced memory T cells, resulting in an over-proliferation of memory T cells [205, 208, 209]. The over-proliferation of CD8+ T cells and memory T cells, however, may result in acute cellular rejection and become a barrier to the induction of tolerance after transplantation [78, 210].

In neonates, the consequence may be different. In addition to vigorous thymocyte exportation as a resource of CD4+ T cells, naïve recent thymic emigrants can also proliferate rapidly driven by lymphopenia as memory T cells [211]. As a result, it can be hypothesized that the ratio of CD4+ to CD8+ T cells and also the ratio of naive to memory T cells may not be modulated so significantly in the neonates as in the adults.

Direct proof of this hypothesis has not been demonstrated in neonates or in very young infants of human. However, indirect evidence can come from older children

who had undergone chemotherapy for cancers [212, 213]. After lymphocyte depletion by chemotherapy, in contrast to adults, T-cell reconstitution showed a different profile in children: the recovery of CD4⁺ T cell number was achieved and accompanied by higher percentage of naïve-type (CD45RA⁺CD45RO⁻) CD4⁺ T cell. Moreover, these pediatric patients showed an enlargement of thymus which is termed “thymic rebound”, indicating that thymic function might have been activated by lymphocyte depletion in childhood.

VI.2. The Significance of Thymus in Donor-specific Tolerance Induction in Pediatric Transplantation

Thymus plays a major role in the development of self-tolerance, which has been proved since the earlier finding from Miller in the beginning of 1960s [214, 215]. Thymocytes undergo both positive and negative selection, resulting in the development of T cell repertoire with a broad range of reactivity to foreign antigens but without reactivity to self-antigens. Thymus also has significance in peripheral tolerance, because thymus is the source of natural regulatory T cells that inhibit autoreactivity of T-cell clones that may escape negative selection [216]. Thymus was shown to be critical in the induction of donor-specific tolerance [217]. Proper donor antigen presentation in the thymus after cellular or solid organ transplantation has been shown to induce tolerance to allografts. Here we focus on several studies which showed that thymectomy could result in acute cellular rejection or failure of tolerance induction.

In partially inbred miniature swine organ transplantation model, with recombinant swine lymphocyte antigen (SLA)^{gg} (class I^c/II^d) animals as kidney donors, and SLA^{dd} (class I^d/II^d) animals as recipients to achieve a 2-haplotype class I mismatch, Yamada et al. examined the role of the thymus during the induction of tolerance by performing a complete thymectomy in recipients 21 days before renal transplantation [218]. Results of this study show significant different outcomes between thymectomized and

non-thymectomized animals. Thymectomized swine developed acute cellular rejection characterized by a T cell infiltration, tubulitis, endothelialitis and glomerulitis, besides an anti-donor CTL reactivity demonstrated in vitro. However, non-thymectomized and sham-thymectomized animals had a mild T cell infiltration and no anti-donor CTL response in vitro. Author concluded that the thymus is required for rapid and stable induction of tolerance. Continual study showed that the requirement for thymic function in the induction of rapid and stable tolerance is greatest during the first 8 days post-transplant and then diminishes over the next 2 weeks. Thymectomy performed at 21 days post-transplant did not affect the course of tolerance induction, indicating that thymic function is not required for the maintenance of tolerance.

Studies of renal transplantation also demonstrated that the graft-to-thymus pathway plays an important role in tolerance induction to renal allografts. But such graft-to-thymus pathway appears to be relatively deficient in recipients of cardiac grafts and lung grafts [219, 220].

Strategies to increase donor antigen migration to the host thymus, such as donor-derived HSC infusion might therefore play especially important role in the tolerance induction to heart and lung allografts.

Besides, in rat MHC mismatched vascularized composite allotransplantation, the role of host thymus in tolerance induction was also evaluated.

In summary, implication from these studies may permit the development of tolerance induction strategies, especially for pediatric transplant recipients with potent thymic function.

In human neonates, partial or total thymectomy has been frequently performed with cardiac surgery for correcting congenital cardiac defect, in order to enable better surgical access. In these cardiac surgeries, surgical access to the heart and great vessels is obstructed by the thymus, which occupies a large space within newborns and is therefore removed during the intervention. Most of these cardiac surgeries do not involve in cardiac transplantation.

In this setting of thymectomy, thymus has been regarded as dispensable during

childhood and adulthood. However, current available evidences have showed that thymectomy in neonatal stage affects the peripheral T-cell population both in the short and long term and results in premature immunosenescence. Sauce et al. [221] reported that young adults who undergone thymectomy during early childhood exhibited an altered T-cell compartment: the absolute CD4+ and CD8+ T cell counts were decreased and these T cell populations showed substantial loss of naive cells and accumulation of oligoclonal memory cells. A subgroup of these young patients (22 years old) exhibited a particularly altered T cell profile that is usually seen in elderly individuals (more than 75 years old) with lower T cell numbers, lower naive/memory T cell ratios, and lower T cell diversity. Authors therefore highlighted the importance of the thymus in maintaining the integrity of T cell immunity during adult life. Prelog et al. reported lower total counts of peripheral blood CD4+CD45RA+CD62L+ naive T cells, as well as decreased numbers of T cell receptor excision circles (TRECs) in these patients who received thymectomy in childhood. Similar results were reported also by other researchers [204, 222-228]. However, these studies were not cardiac transplantation involved and therefore the question of whether donor-specific hyporesponsiveness and tolerance induction may be impaired by thymectomy was not addressed as a routine.

One issue from Ogle et al. [229] did involve in cardiac transplantation together with thymectomy in infancy, however, the question of tolerance induction was not addressed either. Authors mainly focused on the profound defects in the T cell compartment cause by thymectomy, which might be an urgent concern because in this setting the thymectomy was performed in conjunction with T-cell depletion, resulting in 10,000-fold less of T cell diversity in these subjects with no evidence of thymic function when comparing to the healthy controls.

VI.3. Thymic Manipulations as Approaches for Donor-specific

Tolerance Induction

Based on the results from previous experimental and clinical studies aimed at

harnessing the potential of central tolerance in humans, there are two manipulations involving in the thymus: donor thymic tissue transplantation and intrathymic injection of donor antigen.

VI.3.1. Donor Thymic Tissue Transplantation

Thymic tissue transplantation has a long history. Since 1960s when the bone marrow transplantation has not yet achieved essential success, many attempts have been made in order to reconstitute the immunological capacity in the infants with lymphopenic immunological deficiency by implanting fetal or infantile tissues and/or adult blood or bone-marrow [230]. In 1969, De Koning reported a case of transplantation of fetal thymus and bone-marrow cells in a 5-month-old infant with lymphopenic immunological deficiency and the clinical outcome was excellent [231]. However, thymus transplantation herein was in the scope of bone marrow transplant but not aimed at donor-specific tolerance induction.

Early thymus transplantation for this aim was performed in mice. Waer et al. reported the induction of transplantation tolerance in mice across major histocompatibility barrier by using allogeneic thymus transplantation and total lymphoid irradiation [232]. Hoffmann et al. studied the role of thymic medullary epithelium in tolerance induction by transplanting the third and fourth branchial clefts of donor embryos to athymic nude mice [233]. The grafts differentiated into tissue that morphologically resembled normal thymus. The T-cell immune of the athymic nude mice was reconstituted and donor-specific tolerance was observed.

So far, inducing donor-specific tolerance by thymic tissue transplantation across allogeneic and xenogeneic barriers has been achieved in immunocompetent mice after thymectomy, followed by lethal whole body irradiation [234, 235], total lymphoid irradiation [232] or T cell and NK cell depletion with monoclonal antibodies [236].

Both allogeneic and xenogeneic thymic tissue grafts in mice have been proved to be competent to support normal T-cell development [237-239].

In a pig-to-mouse xenogeneic transplant model involving host thymectomy, T and NK cell depletion; the recovery of T-cell repertoire in tolerant recipients was partially due to intrathymic deletion of both donor-reactive and host-reactive recipient

thymocytes developing in the xenogeneic thymic graft [236, 240, 241].

Non-vascularized thymic tissue allograft was subsequently extended to large-animal preclinical model. In 1999, Haller et al. reported thymic transplantation across an MHC class I barrier in miniature swine [242]. In this study, 4 recipients were involved, 2 were thymectomized before thymic tissue transplantation and 2 others remained euthymic. Donor thymic tissue was harvested from SLA class I-mismatched juvenile pigs and placed into 3 different sites on the recipients: sternocephalicus muscle, omentum, and kidney capsule. All recipients received 12 days of CsA started on the day of transplantation (because it had been demonstrated in previous study that Class I-mismatched kidney grafts could be uniformly accepted in euthymic pigs when transplantation is followed by a 12-day course of CsA). Results were striking because allogeneic thymic engraftment could only be achieved in euthymic recipients but not in thymectomized miniature swines. Both euthymic recipients showed good graft development with evidence of thymopoiesis, and also demonstrated donor-specific T cell hyporesponsiveness, as measured by MLR and cell-mediated lympholysis. Although there was presence of high levels of anti-donor specific cytotoxic IgG, authors observed that the thymic grafts continued to develop. From this study, authors concluded that thymic transplantation could serve as part of a regimen to induce donor-specific tolerance to xenogeneic organ grafts.

However, in another report from the same research center in Massachusetts General Hospital in Boston [243, 244], it was also concluded that after non-vascularized thymic tissue was autotransplanted, the ischemic period in the first 1-3 weeks leads to temporary loss of the autologous thymic graft structure, which is reconstituted over the following 5-7 weeks. The same report also argued that this initial ischemic period might be of much greater consequence to the results after allogeneic thymic transplants, because the damaged tissue could result in sensitization before thymic reconstitution can occur. Moreover, so far the revascularization procedure from recipient into an allogeneic transplant has not yet under essential study.

Further studies of thymic tissue transplantation continued to be conducted in the

same center. Creation of the "thymoheart" allograft was reported by Lambrigts et al. in 1998, by implantation of autologous thymus into the heart 60-90 days prior to organ procurement [245]. Histological study of the thymic autografts at organ procurement revealed viable thymus with preservation of normal thymic architecture. However, this study did not investigate subsequent changes in the thymic tissue or the function of the thymic grafts.

The establishment of a "thymokidney" by thymic autografting to the renal subcapsular space was reported by Yamada et al. in 1999 [244]. In this study, eight juvenile swine received autologous thymic grafts under the renal capsule. Thymic tissue was obtained through a partial (n=6) or complete (n=2) thymectomy. Two of the partially thymectomized animals received irradiated (1000 cGy) as well as non-irradiated autologous thymic grafts. Results showed that growth of transplanted thymic tissue was achieved in all thymic graft recipients. No difference was seen between partially and completely thymectomized animals. By POD 60, the thymic grafts exhibited normal macroscopic and microscopic structure, and normal thymocyte composition. Irradiated thymic tissue displayed a similar pattern of development, but growth was markedly delayed. To evaluate thymic function of the thymic graft, a composite thymokidney was transplanted into a previously thymectomized miniature swine which had few circulating CD4-single positive T cells and had lost MLR reactivity. From day 30 to day 150 post-transplant, the number of CD4+/CD45RA+ cells in this recipient increased steadily, indicating that the thymus of the composite thymokidney allograft was functional. In addition, MLR assays demonstrated that the recipient recovered immunocompetence.

Based on this achievement, Yamada et al. subsequently stepped forward into the research of allotransplantation of the composite thymokidney into thymectomized miniature pigs [246]. Recipients were thymectomized 3 weeks before transplant. 3 groups recipients received respectively 1) a kidney allograft alone, 2) a composite allogeneic thymokidney (kidney with vascularized autologous thymic tissue under its capsule), or 3) separate kidney and thymic grafts from the same donor. All recipients received a 12-day course of cyclosporine as previous study. Results showed that

thymectomized animals receiving a kidney allograft alone or receiving separate thymic and kidney grafts had unstable renal function due to severe rejection with the persistence of anti-donor cytotoxic T cell reactivity. Class I-mismatched kidney transplants between the same donors and recipients could be accepted in euthymic pigs with 12-day course of CsA, as mentioned in previous study [247]. In contrast, recipients of composite thymokidney grafts had stable renal function with no evidence of rejection histologically and donor-specific unresponsiveness. At day 14 post-transplant, the thymic tissue in the thymokidney was observed to contained recipient-type dendritic cells. At day 60 post-transplant, recipient-type class I positive thymocytes appeared in the thymic medulla, indicating the recovery of thymopoiesis between recipient T cell progenitor and donor thymic tissue. As a result, peripheral T cells were both recipient and donor MHC-restricted. This study has demonstrated that the presence of vascularized-donor thymic tissue could induce rapid and stable tolerance to class I-disparate kidney allografts in thymectomized recipients. However, in this study, authors had preferred to used thymectomized recipients but not euthymic ones might because thymectomized recipients allowed easier examination of the recovery of thymopoiesis in thymokidney; in order to better mimic the situation of clinical kidney transplant and to achieve application of this protocol into clinical trial, allotransplantation of thymokidney still required to be performed in recipient with intact thymus.

A report from Menard et al. in 2004 exactly addressed at this question [248]. In this study, 3 groups of miniature pigs were established according to different transplantations. Each group treated with the same 12 days of cyclosporine. Different transplants and respective survival times were listed as below. Group 1: euthymic recipients received MHC-matched, minor antigen-mismatched thymohearts (72-194 days) or un-manipulated control hearts (42-64 days). Group 2: euthymic recipients received MHC class I-mismatched thymohearts (64-191 days) or unmanipulated control hearts (30-55 days). Group 3: thymectomized recipients received MHC class I-mismatched thymohearts (41-70 days) or unmanipulated control hearts (8-27 days). Cellular and humoral functional assays, as well as skin grafting confirmed the

presence of donor-specific hyporesponsiveness in long-term thymoheart allografts recipients. Thus, it can be concluded from this study that transfer of vascularized, functional donor thymic tissue to the euthymic host may also provide the induction of tolerance.

However, as concluded by authors, although thymoheart transplantation may be useful for swine-to-human heart xenograft, it is not applicable to cadaveric human cardiac allotransplantation given the time needed to create the composite organ. This difficulty could be resolved by two different solutions. Firstly, LaMattina et al. developed a microsurgical technique for vascularized thymic lobe transplantation [249, 250]. After transplanted, allografts showed intact cortical and medullary structure post-transplantation, without evidence of rejection or ischemia. Recipient thymocytes repopulated the donor cortical thymus by 30 days post-transplantation thus thymopoiesis and tolerance induction across fully MHC-mismatched barriers were achieved.

Secondly, for heart transplantation, Johnston et al. developed a novel technique by which the donor heart and en-bloc thymus grafts were prepared allowing the preservation of the entire arterial supply and venous drainage of the right thymic lobe [251]. This technique therefore permitted donor thymus transplantation that could have utility in human heart transplantation.

Donor-thymus co-transplantation with solid organ for tolerance induction, although shown to be effective in small and large-animal model, so far it has never been performed with clinical organ transplantation. Nowadays, thymus transplantation only serves as treatment for pediatric patients with profound primary immune deficiency due to primary athymia and the resulting lack of functional T cells, such as DiGeorge syndrome. The main complication of which should be taken care is GVHD [252].

VI.3.2. Intrathymic Injection of Donor Antigen

Earlier study of central tolerance induction by intrathymic injection of donor antigen was reported by Remuzzi et al. in 1991 [253, 254]. In this study, isolated glomeruli from Brown-Norway (BN) rat kidney were inoculated into the thymus of

MHC-mismatched Lewis rats pretreated with CsA for 2 days and subcutaneous dexamethasone at inoculation. Ten days later the kidney from the BN rats were transplanted to the intrathymic injected Lewis rat. Donor-specific unresponsiveness allowed the renal allograft to survive indefinitely without further immunosuppression.

Intrathymic injection of donor antigen also served as a treatment for autoimmune diseases. In 1992, Posselt et al. reported the injection of the beta cell antigens into the thymus of neonatal diabetes-prone BioBreeding rat, in which the diabetes resulted from the destruction of pancreatic islet by autoreactive T lymphocytes recognizing beta cell-specific antigens, successfully prevented diabetes and insulinitis in the native pancreas [255].

Many other studies in rodent models also showed that intrathymic injection of donor antigen in various forms, such as donor spleen cells, dendritic cells or MHC allopeptides, could induce donor-specific tolerance to organ allografts [256-259].

While intrathymic injection of donor antigen has been demonstrated to be very effective in rodents, successful studies in large-animals have not been reported.

However, different to thymic tissue co-transplantation, intrathymic injection has been applied to humans in a clinical trial of pediatric heart transplantation, reported by Remuzzi et al. in 1995 [260]. Thirty-seven children (median age of 7.4 years) entered the study. Fourteen patients received intrathymic inoculation of 8×10^7 cells/kg of unmodified donor bone marrow cells prior to sternal closure; 23 patients for whom marrow could not be harvested acted as controls. All received standard tacrolimus-based immunosuppression without induction therapy. Freedom from acute cellular rejection and the number of rejection episodes were not different between the study group and controls in the first 6 months after transplant. However, there was greater freedom from late acute cellular rejection (beyond 1 year) in the study group. Of 13 survivors in the study group, there were only 2 episodes of late acute cellular rejection, in contrast to 20 episodes in the 22 surviving controls. However, serial MLR using irradiated donor and 3rd party splenocytes showed no evidence of increased hyporesponsiveness in intrathymic injection group. This clinical trial indicated that intrathymic injection with donor bone marrow is feasible and safe in the setting of

pediatric heart transplantation. However, the outcomes was not promising enough to allow this approach to be widely performed with other organ transplantations such as renal transplant, considering that except heart transplant, other organ transplantations don't share the same surgical access with thymic injection and intrathymic injection would be either too invasive by open-chest intervention or almost impossible to achieve right orientation. Other disadvantages may include: (1) intrathymic injection can't be performed in adult patient in whom the thymus has undergone involution; (2) intrathymic injection can neither eliminate the donor-reactive T-lymphocytes pre-existed nor prevent their expansion in peripheral blood.

VI.4. Conclusion

Thymus is a potent lymphoid organ in neonatal stage. This makes neonates to be unique individuals in transplant setting. Active recent thymic emigration might bring in some characteristic outcomes when they receive a lymphodepletive therapy, such as the difficulty to achieve and maintain maximum lymphocyte depletion by routine doses of lymphodepletive agents administered in adult patients; the reconstitution of peripheral T-cell pool may rely more on recent thymic emigration and less on homeostasis-driven lymphocyte proliferation. On the other hand, the existence of a functional thymus may also improve the outcomes of central tolerance induction by intra-thymic depletion of alloreactive T cells, as it has been frequently proved that thymus play an indispensable role in preclinical studies of donor-specific tolerance induction.

However, manipulating thymus by surgical interventions such as donor thymic tissue transplantation for tolerance induction has not been performed in clinical transplant settings, considering the risk of GVHD. Besides, the application of intra-thymic infusion of donor-derived cells is restricted by difficulties in precise positioning of thymus with ultrasonic equipment, thus has only been performed in the pediatric heart/lung transplants which share the similar surgical pathway. However, the tolerance induction outcomes were not promising enough, thus prevent this approach to be widely performed. Therefore, surgical thymic manipulation is not

considered to be an available approach in our project of tolerance induction to VCA in neonates.

VII. Vascularized Bone Marrow Transplantation and Tolerance

Induction

Different from solid organ transplantation, VCA such as limb allograft comprises not only the structural components (bones, muscles, skin, etc), but also immunological component: bone marrow cells. This makes limb transplantation to be a unique model of vascularized bone marrow transplantation (VBMT). Limb transplantation can spontaneously create a status of hematopoietic chimerism, even if the recipient hasn't received any myeloablative or non-myeloablative conditioning therapy prior to transplantation.

The viable bone marrow component containing in the bone allograft, if it has not been rejected, will continually provide donor immunocompetent cells into the recipient immune system. Thus, the establishment of long-term chimerism can be achieved, which may subsequently play important role in the induction of donor-specific tolerance. Bone marrow compartment in limb allograft represents the main source of donor hematopoietic stem cells, which are pluripotent and capable to differentiate into T and B lymphocytes, neutrophils, monocytes, tissue macrophages, and dendritic cells. These immunological cells may express more MHC molecules, thus might be relatively more allogeneic than other cells of the structural components, such as bone and muscle (but except the skin) and are more susceptible to rejection in immunocompetent host. Even they can escape from rejection with immunosuppressive therapy, these cells may either facilitate graft acceptance or be potentially lethal by inducing GVHD. Therefore, essential questions involved in this approach are probably as below.

(1) Whether the bone marrow component contained in the bone allograft will be rejected?

(2) Whether this bone marrow component can create a long-term, multilineage chimerism and subsequently induce tolerance?

(3) Whether VBMT will cause GVHD?

In order to answer these questions, a review on the previous research of VBMT is necessary.

The concept of VBMT evolved in the late 1970s and early 1980s from experiments involving composite tissue allotransplantation of rat hind limb [261]. An early conception of chimerism induced by VBMT was coming from the report of Hawitt et al. in 1986 [262]. In serial researches of MHC-mismatched vascularized hind limb allografts between Lewis rats and LBN rats (Lewis x Brown Norway hybrid). The Lewis recipients received CsA treatment in order to induce indefinite hind limb allografts acceptance. Long-term-surviving (206-701 days) Lewis rat recipients were tested for donor-host lymphoid chimerism. Histological examination of long-term-surviving hind limb allografts demonstrated normal-appearing bone marrow in the donor limb. Lymphocytes isolated from long-term CTA host peripheral blood and/or spleen showed 19.7% of donor LBN mononuclear cells. Thus, it appeared that VBMT could result in high level of chimerism in peripheral blood and spleen. The presence of chimerism may have contributed to the long-term CTA survival.

In rat model, Lukomska et al. reported that VBMT in an orthotopic hind limb graft brought about complete repopulation of bone marrow cavities in lethally irradiated syngeneic recipients within 10 days [263]. Authors then compared the capacity of immunological reconstitution induced by VBMT versus cellular bone marrow transplantation (CBMT). Intravenous infusion of an equivalent volume of BMC suspension was evidently less effective in reconstitution of lymphoid tissue.

Indeed, VBMT, in comparison with CBMT, has the advantage of providing a syngeneic microenvironment and immediate engraftment of both mature and progenitor hemopoietic cells at the time of transplantation. The presence of the stromal microenvironment is one of the key differences between VBMT and CBMT. In VBMT, hematopoietic stem cells are transplanted within their natural microenvironment, thus do not require host conditioning and permits donor cell engraftment. The immunocompetent role of the stroma incorporated in VBM was also discussed. Other than providing trophic support for cellular replication, the stromal

environment has important regulatory roles in lymphocyte functions and transplantation immunology [264].

However, on the other hand, GVHD caused by VBMT was also documented in rat model. Ramsamooj et al. reported that after LBN rats received hind limb allografts from Lewis donors, characteristics of graft-versus-host disease were observed [265]. But authors also confirmed that VBMT has a lower incidence of GVHD compared with other methods of bone marrow transplantation and represents an alternative to conventional bone marrow transplantation.

Although VBMT was proved to be effective in establishment of chimerism in rodent model and resulted in long-term acceptance of the composite tissue allograft as previous discussed, such outcomes are not so promising in large-animal model. Bourget et al. reported that transient early peripheral chimerism was observed after performing musculoskeletal allograft without skin component in MHC-matched, minor-antigen-mismatched miniature swines with only a 12-day course of cyclosporine, but this chimerism became undetectable by postoperative day 19 in the CsA-treated group and by day 13 in the non-CsA-treated group [266].

Mathes et al. reported that in a swine model, CTAs from donor swine were heterotopically transplanted into six MHC-matched, minor-antigen-mismatched recipients, and a 12-day course of CsA was given. Results showed that animals receiving cyclosporine demonstrated permanent tolerance to their allografts, however, without the presence of long-term peripheral donor cell chimerism. In all grafts donor bone marrow cells were present only at the time of transplantation and during the immediate postoperative period. By 48 weeks, donor cells were no longer detectable within the marrow space of the allograft. No evidence of donor cell engraftment was found in recipient animals. Furthermore, in long-term animals, the recipient's bone marrow cells and lymphocytes replaced the donor marrow space of the graft. This study indicated that although VCA structural components such as bones, muscles and skin survived after transplantation, bone marrow component might be more difficult to escape rejection [267].

Furthermore, the healing and rejection patterns of bones and associated bone

marrow tissues were investigated in nonhuman primate model. In study of Mundinger et al. fibular vascularized composite tissue allotransplantations were performed between mismatched cynomolgus macaques [268]. Recipients were treated with either tacrolimus monotherapy or tacrolimus plus co-stimulatory blockade with a novel anti-CD28 antibody. Results showed nonhuman primate fibular vascularized composite tissue allotransplantation model showed early skin loss. Short-tandem repeated genotypic analysis revealed that donor marrow had been completely replaced by recipient marrow. Histological examination confirmed the presence of viable cortical and marrow elements. Biomechanical analysis supported donor-recipient bony union. This VCA study might require an optimized immunosuppressive regimen to improve the acceptance of allografts; however, it confirmed that donor marrow can be completely replaced by recipient marrow after rejection.

In human hand transplantation, VBMT's potentials of inducing donor-specific tolerance or GVHD were reported by Granger et al. from Louisville in 2002 after followed two hand transplant recipients more than one year [269]. Results showed that donor-specific hyporesponsiveness did not develop clinically or in mixed lymphocyte reaction. Donor chimerism was not detectable using flow cytometry. Microchimerism was observed at the level of detection (approximately 1:75,000 cells) in some of the early post-transplantation specimens and was undetectable thereafter. Thus, under the traditional immunosuppressive regimen in these 2 subjects of hand transplantation, the CTA with VBMT did not provide the immunologic benefit of tolerance induction nor cause GVHD.

Similar results were also reported by VCA research team in Lyon after long-term follow-up in four bilateral hand-grafted patients (10, 7, 3 and 2 years of follow-up, respectively). Although authors proposed that hematopoietic stem cells containing in the transplanted bones might be involved in the long-term acceptance of the graft; however, chimerism in the peripheral blood of our hand transplanted patients was never detectable [270].

Although no long-term peripheral blood chimerism was achieved in large-animal

model, the beneficial effect of VBMT accomplished with VCA is still proposed by a majority of researchers, because in rodent models the outcomes of VBMT were really very promising. Moreover, previous studies of VBMT on large-animals or human have not evaluated the level of chimerism exist in recipient lymphoid tissues, in which tolerance induction effects of chimerism might be more significant than in circulating peripheral blood.

In a recent study of VBMT in nonhuman primates, the data supported the immunomodulatory role of VBM in composite tissue allograft on reducing immunosuppressive regimens while providing improved acceptance. Barth et al. developed a model to evaluate the benefit of concomitant VBM in VCA [268]. In this study, facial segment VCA with VBM treated only with FK506 and MMF demonstrated prolonged rejection-free survival, compared to VCA without VBM which demonstrated early rejection episodes and graft loss. Sporadic engraftment of donor cells was observed in recipient lymph nodes, spleen and bone marrow; however, tolerance was not induced, as acute and chronic rejection and graft loss occurred after discontinuation of immunosuppression.

Although GVHD was demonstrated in rodent VBMT, it was never observed in large-animal models under traditional immunosuppressive regimen: obviously, in these setting of VBMT, the first concern is rejection and replacement of donor bone marrow by the recipient, but not GVHD.

Beside of rejection, detectable chimerism was observed in rodents but not in the human recipients who received one or two hands transplants could also be explained by the property and function changes of the bone marrow in human long bones. Generally, there are two types of bone marrow: red marrow, which mainly consists of hematopoietic tissue; and yellow marrow, which is mainly made up of fat cells. Red marrow is found primarily in the flat bones, such as the hip bone, breast bone, cranium, ribs, vertebrae and shoulder blades, and in the cancellous material at the epiphyseal ends of long bones such as the femur and humerus. Yellow marrow is found in the medullary cavity, the hollow interior of the middle portion of long bones. At birth, bone marrow is red marrow containing plenty of hematopoietic stem cells. With

age, more and more of red marrow is converted to the yellow type, thus, hematopoietic stem cells might be rare in the bone marrow which is incorporated with hand allograft. This may partially explain why after hand transplantation in adult patients (allografts often came from aged donors), donor chimerism was never detectable.

In conclusion, hand transplantation in adult is unnecessarily combined with VBMT; while in pediatric patients, hand transplantation could take the advantage of VBMT, because in this period, the bone marrow component is still hematopoietic competent red marrow and capable to establish chimerism in recipient which would consequently facilitate tolerance induction.

VIII. Tolerance Induction to Vascularized Composite Allograft

Vascularized transplantation of body structures composed of multiple tissues derived from ectoderm and mesoderm, such as upper or lower extremities, face, knee, larynx and abdomen wall, is termed as vascularized composite allograft (VCA), also termed as composite tissue allograft (CTA).

Composite tissue allograft including skin component, although never gained essential success until late 20th century, was since long time the initial goal of transplantation. Up to the end of World War II, Peter Medawar had tried to perform skin allograft to reconstruct the defect of many British soldiers who suffered from severe burn deformities. However, this attempt and most of the subsequent explorations on CTA were not successful because of the violent rejection after transplant. This failure resulted in a perception that the antigenicity of skin and associated structures was insurmountable, which hampered the further research on CTA until better immunosuppressive agents, such as CsA and FK506 became available.

The major milestone of CTA was the first successful hand transplantation which was performed by CTA research team of Lyon (France) in 1998. Since then, CTA has achieved rapid development. Until 2010, more than 60 cases of unilateral or bilateral upper extremities transplant, 20 cases of partial or full face transplant, as well as several cases of transplantations of other body structures such as larynx, knee, abdomen wall, et al. were reported by the international registry on hand and composite tissue transplantation.

Providing optimized immunosuppressive regimen, proper rehabilitation and careful follow-up, outcomes of hand and face allotransplant are generally satisfied. However, hand and face allotransplant are still regarded as a domain with great difficulties and frequent controversies, mainly due to these following reasons.

(1) Less significant clinical outcome in contrast to solid organ transplantation, as

VCA is not a life-saving therapy but life-improving therapy.

(2) More intensive immunosuppression at acute rejection phase post-transplant to prevent acute rejection or to reverse the acute-rejection episode, because of the strong antigenicity of skin.

(3) More difficult to minimize this intensive immunosuppressive regimen in later-phase post-transplant, which results in more immunosuppression-associated complications, such as hypertension, opportunistic infection, risk of malignancy, new onset hyperglycemia, and renal impairment.

(4) Life-long immunosuppressive therapy and careful follow-up, improve the cost of maintaining the allograft, which become another big barrier to perform VCA, especially in those countries with less-developed social security system.

Thus, in order to be justified, VCA should have greater desire of donor-specific tolerance induction, or more practically speaking at present moment, inducing donor-specific hyporesponsiveness in order to minimize the immunosuppressive regimen.

Following paragraphs are devoted to pursue the development in the research of tolerance induction, as well as immunosuppression conversion and minimization in the setting of VCA.

VIII.1. Tolerance Induction of VCA by Chimerism Creation through HSCT

Researches of tolerance induction to VCA are following similar research in solid organ transplantation. Considering the difficulty to perform microsurgery on mice with too small vessels, most of the experimental researches were conducted in rats, canines, swines and nonhuman primates. Here we focus on the researches of VCA in large-animal models and in human which aimed at creation of chimerism through HSCT, in order to obtain more practicable information for future clinical study.

Although long term acceptance of MHC-matched, minor antigen mismatched porcine hind-limb composite allograft (without skin component) without HSCT or stable chimerism was reported by Lee et al. in 2001, tolerance induction to

MHC-mismatched VCA in large-animal models was demonstrated to be a large difficulty [271]. Early study of tolerance induction to skin-bearing VCA across MHC barrier was reported by Hettiaratchy et al. in 2004. Seven miniature swine recipients received transient T-cell depletion by a single intravenous injection (on day -1) of 0.05 mg/kg pCD3-CRM9 which is an immunotoxin that transiently depletes T cells, with normal levels being regained within 2 to 3 days. A total dose of MHC-mismatched 7.5×10^8 cells/Kg BMC or 15×10^9 cells/Kg cytokine mobilized peripheral blood mononuclear cells (CM-PBMC) containing peripheral blood stem cells (PBSC) mobilized by pig stem cell factor and IL-3 was transplanted on day 0 and 2. Heterotopic limb transplantation was performed on day 3. Oral CsA was administered from day -1 to day 30. Chimerism in the peripheral blood and lympho-hematopoietic organs was assessed by flow cytometry. Results showed that all seven experimental animals accepted the musculoskeletal elements but rejected the skin of the allografts (occurred between day 35 and 50). All but one of the animals displayed donor-specific unresponsiveness in vitro. The animals that received CM-PBMC showed chimerism but had clinical evidence of GVHD. None of the animals that received BMC showed stable chimerism and none developed GVHD. As discussed by authors, the lack of stable macrochimerism in the animals received BMC might be due to the lower absolute cell numbers infused (7.5×10^8 cells/Kg of BMC vs. 15×10^9 cells/Kg of CM-PBMC) or the lower T-cell count versus (10% in BMC vs. 40-50% in CM-PBMC) because T-cell can facilitate the engraftment. However, stable macrochimerism might not be the prerequisite for tolerance induction and high level of donor-derived lymphocyte present in recipient may cause GVHD which is not acceptable as VCA is not a life-saving therapy. Moreover, what can be assumed is that clinical composite allografts can only be cadaveric source and not suitable for the regimen using cytokine mobilized stem cells [272] on alive donors.

The induction of tolerance of vascularized skin allograft was not successful until 2009 when Horner et al. reported their research conducted in miniature swines [273]. In this study, recipients received a conditioning regimen including 100 cGy total body irradiation (TBI), T-cell depletion by CD3-immunotoxin, before transplantation of

15×10^9 cells/Kg of CM-PBMC and a 45-day course treatment of CsA. Both conventional skin allografts and primarily vascularized skin flaps were transplanted respectively in different groups. Results showed that HSC engraftment and stable mixed chimerism was achieved in all of the 4 animals received HSCT, but 2 of them which received conventional skin allografts eventually rejected the grafts at day 55 and day 64; while 2 others which received vascularized skin flaps, one of them died of an unrelated infection at day 46 without evidence of rejection macroscopically or histologically, the other achieved long-term (>300 days) tolerance to vascularized skin flap and a conventional skin donor graft subsequently applied to this tolerant animal was not rejected and did not trigger rejection to the vascularized skin flap. However, this study had not mentioned the observation of GVHD. This is the first time skin tolerance had been achieved across a MHC barrier in a large-animal model and this experiment confirmed again that non-vascularized skin allograft is more allogeneic than vascularized skin flap.

Leto Barone et al. evaluated the tolerance induction protocol of VCA by inducing mixed chimerism through BMT across a full MHC barrier [274]. This study was reported in 2011 with some un-completed results. Under similar conditioning and immunosuppressive regimen reported by Horner et al. in 2009, including low dose TBI, T-cell depletion and CsA as mentioned above, a vascularized myocutaneous flap was transplanted with BMC infusion. Chimerism was assessed by flow cytometry. Only 2 experimental animals were reported with results up to post-transplantation day (PTD) 43. In the first recipient no detectable chimerism was observed. Rejection crisis of skin component occurred on PTD8 but recovered to 100% viability by PTD13 and all components of the flap appeared viable up to PTD43. Upon cessation of CsA, the epidermis sloughed by PTD45, and was followed by loss of all components of the flap by PTD71. In the second recipient, myeloid chimerism was observed at different time-points. All components of the flap are accepted up to PTD43 in this animal when this report was given. However, subsequent results of additional time-points were not available from the authors.

Another study with similar source of HSC from Wachtman et al. in 2011 might

have well demonstrated the outcome missed in former study [275]. Miniature swine recipients were conditioned by TBI (100cGy) and thymic irradiation (700cGy) for cytoablation before infusion of different doses of unmodified bone marrow cells (15, 30 and 60×10^6 cells/Kg) and transplant of hind-limb VCA in the same day, followed by 30-day course of FK506. Detected by PCR, macrochimerism was observed in all recipients, with significant higher level in the animal received highest dose of BMC (60×10^6 cells/Kg). Clinical outcomes showed indefinite prolonged survival of musculoskeletal component while skin component was rejected about 3 weeks after ceasing of FK506 (day 50, 52, 53). In this study, very low higher dose of BMC than 60×10^6 cells/Kg might be required in order to improve the outcomes of chimerism and tolerance, considering that in unmodified BMC the numbers of CD34⁺ hematopoietic stem cells and CD3⁺ T cells are very low.

Thus, in previous studies, MHC-mismatched VCA conducted in miniature swines has not yet achieved tolerance in any large-animal model by chimerism creation through unmodified bone marrow cells infusion, even the recipients were conditioned with sublethal TBI and thymic irradiation.

In Canine model, Mathes et al. reported in 2011 the success of tolerance induction to dog leukocyte antigen (DLA) identical VCA by establishing mixed-chimerism under a nonmyeloablative conditioning regimen [276]. The conditioning/immunosuppressive regimen included a sublethal dose (1-2 Gy) TBI before transplantation of DLA-identical BMC ($2.8-9.2 \times 10^8$ cells/Kg) from a respective littermate, followed by a short course (approximately 30 days) of immunosuppressors comprised of CsA (except one recipient which received sirolimus) and MMF. Vascularized myocutaneous rectus abdominus flap from marrow donors were performed after a median of 36 months (range, 4-54 months) after HCT and immunosuppression was not given simultaneously. Results demonstrated that mixed-chimerism was established and maintained in all BMT recipients, which led to the prolonged acceptance of VCA for 52-90 weeks (in contrast to 18-29 days in non-chimeric control recipients). Besides, in mixed chimeras, elevated levels of regulatory T cells were found in skin and muscle of VCA compared with normal

tissues; increased numbers of regulatory T cells were also observed in draining lymph nodes of VCA compared to non-draining lymph nodes.

So far, except several researches on VBMT, research of HSCT-based protocol for tolerance induction of VCA has not yet reported in nonhuman primate model. However, in human face and upper-extremity transplantation, donor BMC-based treatment has already been applied.

In the first human face allograft [3], donor bone marrow cells were cryopreserved and thawed immediately before administration on day 4 and 11 post-transplant. The total numbers of nucleated hematopoietic cells infused were 1.6×10^8 cells/Kg on day 4, and 1.8×10^8 cells/Kg on day 11. The graft contained 2×10^4 cells/Kg and 4×10^4 cells/Kg colony-forming-unit granulomacrophage cells, 0.12×10^6 cells/Kg and 0.12×10^6 cells/Kg CD34⁺ cells, and 2.7×10^6 cells/Kg and 4.1×10^6 cells/Kg CD3⁺ cells on days 4 and 11, respectively. The conditioning regimen included only T-cell depletion by ATG (1.25 mg/kg/day, from day 0 to day 10), the conventional triple-maintenance therapy comprised of tacrolimus, MMF and prednisone. Chimerism was assessed once a week by microsatellites and quantitative PCR on total blood and CD3⁺ and CD56⁺ cells, and monthly on bone marrow total cells and CD34⁺ cells. Results showed no development of chimerism: it was never detectable in peripheral blood; the only positive result was from BMC 2 months after transplantation when real-time quantitative PCR showed 0.1% of donor cells among the CD34⁺ enriched BMC. No GVHD was observed [277].

Moreover, concluded by authors, in this case donor BMC infusion did not influence the patient's immunological profile in 5 years follow-up. The maintenance therapy is based on prednisone (5 mg/day), sirolimus (targeted trough level: 6-12 ng/mL) and MMF (1500 mg/day). As analysis from authors, the absence of durable donor chimerism and tolerance might be due to poor hematopoietic stem cell quality or an insufficient conditioning regimen [6].

Although donor cells infusion did not showed beneficial outcomes in the first face allograft, further study with similar protocol conducted in human upper-extremity allograft has led to minimization of immunosuppression in the recipients, as reported

by researchers from Pittsburgh [278]. This donor BMC-based treatment protocol (referred as “Pittsburgh protocol”) so far has been performed in 5 recipients who received bilateral or unilateral hand/forearm transplantation. Patients’ preparation included lymphocyte-depletion induced by 30 mg alemtuzumab (anti-CD52 monoclonal antibody) plus 250 mg prednisone and maintained by tacrolimus monotherapy with target trough levels of 10 to 15 ng/mL (first month), 8 to 10 ng/mL (2-3 months), 5 to 10 ng/mL (4-12 months), and 3 to 7 ng/mL thereafter. Unmodified donor BMC with a dose of $5-10 \times 10^8$ cells/Kg were given on day 14. Within infused BMC, CD3⁺ cell dose was $3.7 \pm 3.1 \times 10^7$ /kg (cell viability $71 \pm 28\%$), CD34⁺ cell dose was $5.4 \pm 3.1 \times 10^6$ /kg (cell viability $98 \pm 2\%$), containing $15.5 \pm 7.5\%$ of CD34⁺CD90⁺CD117^{dim} hematopoietic stem cells. Besides, $0.04 \pm 0.04\%$ of CD45⁻CD73⁺CD105⁺ mesenchymal stromal cells was detected in the total BMC. Peripheral blood chimerism was evaluated by short tandem repeat (STR) analysis. In this study, doses of BMC and CD34⁺ cells were much higher than which was administrated in face transplant mentioned above, however, STR analysis showed no evidence of donor cells present in peripheral blood. Skin rejections were infrequent and reversible. Latest protocol skin biopsies showed minimal or absent perivascular cellular infiltrates (Banff grade 0-I) and were C4d negative. Authors recommended that this BM cell-based treatment protocol is safe and well tolerated, and allows using low-dose tacrolimus monotherapy in upper-extremity transplant.

Summarily, so far, for donor-specific tolerance induction of organ transplantation in large-animals or human, donor HSC infusion is the most promising approach. Although still requiring TBI to improve HSC engraftment and to enhance outcome of tolerance to skin component, as far as we know, donor HSC infusion is only strategy which has achieved tolerance to MHC-mismatched vascularized skin allograft in large-animal model. However, nowadays it has not yet led to tolerance induction to HLA-mismatched VCA in human. Future study on this protocol may be optimized by the modulation of HSCT dose and components, proper improvement of the conditioning therapy, and addition of effective facilitating cells, etc.

Table 5: Summary of previous studies on tolerance induction to VCA through bone marrow cells infusion.

Animals/ Human	Author	Year/Location	VCA Type/ Day	MHC- mismatched	Conditioning, Immunosuppression	BMC or (cytokine mobilized)/CM- (N/A, not available)	CD34+ component	CD3+ component	BMC infusion time	Flesh/ Frozen BMC	Chimerism Outcome PB and/or Lymph tissues (LT)	Detection Method	Clinical Tolerance Outcome of VCA	GVHD
Miniature swine	Heitarachy et al	2004/ Boston	Heterotopic limb transplantation n/Day 3	Yes	T-depletion, CsA (-1 to 30)	BMC 7.5x10 ⁸ cells/Kg	N/A	10%	Day 0, 2	Flesh macrochimerism in PB and LT	No macrochimerism in PB and LT	FCM	Musculoskeletal component: accepted; Skin component: rejected	No
Miniature swine	Heitarachy et al	2004/ Boston	Heterotopic limb transplantation n/Day 3	Yes	T-depletion, CsA (-1 to 30)	CM-PBMC 15x10 ⁹ cells/Kg	N/A	40-50%	Day 0, 2	Flesh macrochimerism in PB and LT	Stable macrochimerism in PB and LT	FCM	Musculoskeletal component: accepted; Skin component: rejected	Yes
Miniature swine	Homer et al	2009/ Boston	Vascularized skin flap/ Non- vascularized skin graft	Yes	TBI (100cGy), T- depletion, CsA (45 days)	CM-PBMC 15x10 ⁹ cells/Kg	N/A	40-50%	N/A	Flesh	Stable macrochimerism in PB and LT	FCM	Vascularized skin flap: accepted; Non- vascularized skin graft: rejected	N/A
Miniature swine	Leito Barone et al	2011/ Boston	Vascularized myocutaneous flap	Yes	TBI (100cGy), T- depletion, CsA (45 days)	BMC dose N/A	N/A	10%	N/A	Flesh	Fig1: No Fig2: Yes	FCM	Fig1: rejected Fig2: accepted at day 43 (still under CsA), more results N/A	N/A
Miniature swine	Wachman et al	2011/ Pittsburg	Vascularized skeletal- myocutaneous flap/ Day 0	Yes	TBI (100cGy), T1 (700cGy), FK506 (30 days)	BMC, 15- 60x10 ⁶ cells/Kg	N/A	10%	Day 0	Flesh	Macrochimerism was established in all animals after BM cell infusion.	STR	Musculoskeletal component: accepted; Skin component: rejected (day 50, 52, 53)	N/A

Animals/ Human	Author	Year/loca tion	VCA type/ Day	MHC- mismatched	Conditioning, Immunosuppression	BMC or (cytokine mobilized)CM- PBMC dose	CD34+ component (N/A, not available)	CD3+ component	BMC infusion time	Fresh/ Frozen BMC	Chimerism Outcome PB and/or Lymph tissues (LT)	Detection Method	Clinical Tolerance Outcome of VCA	GVHD
Canine	Mathes et al	2011/Seattle	Myocutaneous s rectus abdominus flap/ 4-54 months after HCT (No IS)	No (DLA- identical)	TBI (100-200cGy), T- depletion, CsA (except one with Sirolimus)+MMF (about 30 days)	BMC: 2.8-9.2 ×10 ⁸ cells/Kg	N/A	N/A	Day 0	Fresh	mixed-chimerism	STR	prolonged acceptance of VCA for 52-90 weeks (mg/day)	N/A
Non-human primate	No report													
Human	Devauchelle et al	2006/ Lyon	First human face allograft/ Day 0	1/6 HLA- mismatche (1.25 mg/kg/day day 0-10), FK506+MMF+ HLA antigens)	T-depletion by ATG (1.25 mg/kg/day day 0-10), FK506+MMF+ Prednisone	1.6×10 ⁸ cells/Kg on day 4, and 1.8×10 ⁸ cells/Kg on day 11	0.12×10 ⁶ cells/Kg and 0.12×10 ⁶ cells/Kg	2.7×10 ⁶ cells/Kg and 4.1×10 ⁶ cells/Kg	Day 4, 11	Frozen	Not detectable in PB, 0.1% in CD34+ enriched BMC at 2 months, never detectable again	STR	No tolerance. Now maintained by prednisone (5 mg/day), sirolimus (targeted tough level 6-12 ng/mL) and MMF (1500 mg/day)	No
Human	Schneeburger et al	2013/ Pittsburg	5 recipients of hand transplant/ Day 0	5/6, 5/6, 3/6, 2/6, and 5/6 mismatched for 5 recipients	T-depletion by 30 mg alemtuzumab, tacrolimus monotherapy (tapered)	5-10×10 ⁸ cells/Kg on day 11	5.4 ± 3.1×10 ⁶ cells/Kg	3.7 ± 3.1×10 ⁷ cells/Kg	Day 14	Frozen	Not detectable in PB	STR	Minimization of IS tapered FK506 monotherapy	No

Table 5. Summary of previous studies on tolerance induction to VCA through bone marrow cells infusion.

Original table.

VIII.2. Tolerance Induction of VCA and Costimulation Blockade

The studies above of establishing chimerism through HSCT to induce tolerance of VCA, involved in a lymphocyte-depletion conditioning regimen, no matter by anti-CD3 immunotoxin or by TBI and thymic irradiation, otherwise, a costimulation-blockade based, non-lymphodepletion protocol was also reported. Wachtman et al. confirmed the beneficial effect of CTLA4-Ig in the VCA tolerance induction [275]. Miniature swines receiving MHC-mismatched hind-limb VCA on day 0 was administered with 20 mg/kg CTLA4-Ig on day 0, 2, 4 and 6, in addition to a 30-day course of FK506, no cytoreductive conditioning regimen or donor cells infusion were given to the recipients. The recipients demonstrated indefinite prolonged muscle survival (>150 days post-transplant); the skin component also had remarkably prolonged survival: one animal rejected at day 128, while the other two animals survived indefinitely (>150 days post-transplant). Skin and muscle histology in all long-term surviving animals were normal. These results are striking, demonstrating that at least in hind-limb VCA in swine model, co-stimulatory blockade protocol is superior to the lymphodepletion-base donor HSC infusion protocol. Although further study on the underlying mechanisms is required; from the aspect of tolerance induction through chimerism, a possible explanation might from the VBMT concept. The bone marrow component in the VCA might have profited from the co-stimulatory blockade protocol and therefore microchimerism had been developed to regulate the recipient alloimmune.

VIII.3. Split Tolerance of Composite Tissue Allografts: the Roles of Skin

Dendritic Cells and Regulatory T Cells

The discovery of split tolerance might initially be due to the research of Lee et al. who sought to induce tolerance to VCA in by performing musculoskeletal allograft without skin component in miniature swine [271]. In this study, authors attempted to achieve tolerance by matching MHC antigens between donor and recipient pigs with

only a 12-day course of cyclosporine. CsA-treated, MHC-mismatched allografts were rejected in less than 6 weeks. Non-CsA-treated, MHC-matched, minor antigen mismatched allografts were rejected between 9 and 12 weeks; while CsA-treated, similarly matched allografts showed no evidence of rejection until sacrifice between 25 and 47 weeks.

Subsequent study reported that transient early peripheral chimerism was observed, which might have contributed to the tolerance of allografts [266, 267]. In these early researches, the CTA did not contain skin. In further study [279], in order to determine whether tolerance to limb allografts with a vascularized skin component could be achieved in this MHC-matched model, hind limb grafts harvested with a 100 cm² cutaneous paddle were transplanted. Frozen donor skin grafts were applied to all animals that survived to postoperative day 100, in order to test for sensitization. Results showed that skin survival was prolonged to between 30 and 65 days in five animals, only one animal demonstrated tolerance to the skin for more than 180 days. All the 6 treated pigs were tolerant to their musculoskeletal allografts at the time of necropsy (>100 days) regardless of the status of the epidermis. Frozen donor skin grafts demonstrated accelerated rejection (<10 days) in three of the animals and led to simultaneous rejection of both the skin graft and the epidermis of the composite allograft in the long-term tolerant animal. However, the rejection of the skin grafts did not break tolerance to the musculoskeletal component in any of the animals. This research confirmed the great obstacle of achieving skin tolerance and was a demonstration of “split tolerance,” the simultaneous rejection of one tissue, while another tissue from the same donor is accepted. Thus, the central role of tolerance induction to VCA in future study would be skin- targeting and a thorough study to the characters of skin alloimmune is required. In the following paragraphs, the roles of skin dendritic cells (DC) and regulatory T cells (Treg) in skin transplant tolerance induction will be discussed.

VIII.3.1. Role of Skin DCs in Rejection and Acceptance of Skin Allograft

Skin DCs may have dedicated to the intensity of alloreaction to the skin

component. Skin DCs are a heterogeneous family of APCs residing in the skin. They critically determine whether immunity or tolerance will be generated in the skin. At least 2 types of myeloid DCs have been indentified in the two distinct epidermal and dermal layers of the human skin, including epidermal Langerhans cells and dermal DCs [280].

Langerhans cells are migratory DCs located in the basal and supra-basal layers of the epidermis and represented 2-4% of the total epidermal cell population. Besides expression of CD1 and MHC-II molecules which differentiate them from surrounding keratinocytes and melanocytes, skin LCs express the C-type lectin-specific marker Langerin (CD207) which devote to the formation of cytoplasmic Birbeck granules [281].

Dermal DCs (dDCs) have been less characterized because of lack of specific makers. They share several membrane antigens such as CD45 and MHC-II molecules with dermal macrophages and were first identified by their allostimulatory property and expression of CD1 [282].

Although it's widely accepted that human skin LCs and the dDC subsets must have played important roles in priming naïve T cell response and regulating the balance between immunity and peripheral tolerance in skin, the detailed capacities of these different DC subsets have been little studied.

DCs of donor origin residing in the skin component of VCA migrate to regional lymph nodes post-transplantation and then prime naïve T cells, referred as direct recognition pathway, should be important to initiation and regulation of skin-target alloimmune [283].

Besides, host DCs that acquire allograft skin antigens and presents them to naïve T cells, named as in direct recognition pathway, also participate in these alloimmune response. However, the detailed contributions of donor and host-derived DCs to rejection, especially to further immune regulation, are unclear [284].

Most researches on functions of epidermal LCs and dermal DCs were conducted in rodent model with non-vascularized skin graft, whereas the mechanisms involved in the rejection or acceptance of vascularized and non-vascularized skin may be

different [285].

Vascularized skin flap had been shown to be less antigenic than non-vascularized skin graft, as demonstrated by Horner et al. in a swine VCA model, recipients which were tolerant to vascularized skin flap could reject the non-vascularized skin graft from the same donor [273].

In following paragraphs we mainly discuss the immunological events which may have involved in the split tolerance of VCA in large-animals and human.

In the split tolerance VCA model, the failure to achieve tolerance to the epidermal component of the VCA should be first due to donor skin origin LCs which are able to migrate to draining lymph node and effectively present donor antigens as well as costimulation signals [279].

By an organ culture system, Larsen et al. showed that within 24 hr post-transplantation, mouse skin graft LCs increased in size and expression of MHC-II molecules; by 24 hr, LCs could migrate to epidermal-dermal junction; by 3 days, migratory LCs formed cords in dermal lymphatics before leaving the skin [286].

Reported by Furio et al. in an *ex vivo* research [282], human migratory LCs showed far more activated phenotype than CD1c⁺ dDCs and demonstrated distinct expression of new molecules of the B7 family; compared to CD1c⁺ dDCs, LCs also showed lower programmed cell death 1 ligand 1 (PD-L1) and higher inducible co-stimulator ligand (ICOS-L) expression; therefore, LCs showed higher allostimulatory property than CD1c⁺ dDCs and were significantly more efficient at inducing allogeneic naive CD4⁺ T cells to secrete both Th1 and Th2 cytokines. Notably, the allogeneic T-cell response activated by both LCs and CD1c⁺ dDCs can be almost completely blocked by CTLA4-Ig adding into the mixed lymphocyte culture system, thus showing the importance of CD80/CD86 signaling in the skin DC-induced naive T-cell proliferation.

These results of skin LCs on the one hand correspond to the intensive skin antigenicity compared to other tissues in VCA and non-vascularized skin allograft shows even more antigenicity than vascularized skin flap might because in the procedure of non-vascularized allograft, skin cells experience more “dangerous

events” such as more surgical injury (especially for split thickness skin allograft) and longer ischemia time (revascularization of skin graft may require about 1 week depending on skin thickness), thus epidermal LCs and dDCs would show more activated phenotype and express more MHC-II molecules and co-stimulatory molecules. On the other hand may give explanation to former VCA study conducted in swine which showing that administration of CTLA4-Ig could result in long-term acceptance of skin proportion of VCA, indicating that co-stimulatory blockade might play important role in preventing skin allograft rejection and in tolerance induction to skin component in VCA [275].

For clinical VCA, the commercialization and application of Belatacept, which is a fusion protein composed of the Fc fragment of a human IgG1 immunoglobulin linked to the extracellular domain of CTLA-4, provide the possibility of performing co-stimulatory blockade therapy in human hand and face transplantation. However, so far such therapy has not been included in the immunosuppressive regimens clinical VCA, which is probably due to the wide application of induction therapy based on lymphocyte depletion in order to prevent early phase rejection of VCA, whereas the synergy of lymphocyte depletion and costimulation blockade still remains controversial; further study on effects of costimulation blockade in VCA may be necessary [287, 288].

Besides the capacity of activating alloimmune, the skin DCs were also shown to have tolerogenic function, for they have critical role to mediate and maintain peripheral tolerance. In steady state, LCs/dDCs uptake and transfer innocuous environmental proteins and self-antigen from cells underwent programmed death to draining lymph nodes for presentation. As naïve T cells do not circulate through skin but can only scan migratory DCs presenting tolerogenic antigens in the lympho nodes, these functions of skin migratory DCs in steady state determine self-tolerance in the skin [280, 289].

However, which DC subsets have participated in this procedure and the detailed mechanism of the action between steady state migratory DCs and T cells still require further study. Initially, LCs was considered to critical for the epidermal antigen

presentation and the maintenance of tolerance in epidermis, however, the newly discovered Langerin⁺ dDCs have made this obscure theory to be more complicated, because in most researches of LC function, the LC population was identified by the marker of Langerin, but now the Langerin⁺ dDCs are also demonstrated to be able to uptake and transport keratinocyte antigens to regional lymph nodes. However, there is no report showing that dDC markers can be observed in the epidermis. Possible mechanisms are: (1) DDC may stick their cellular protrusions into the epidermis to capture antigens without entering by the whole cell body; (2) alternatively, steady state migratory LC may transfer antigen to dDC in the dermis before entry into the lymphatic vessels; (3) or, epidermal cell-associated antigens are delivered to the dermis without involvement of LC. All of these possible scenarios require to be clarified [289].

In human hand transplantation, punch-biopsy specimens were taken from the skin of the allografts from day 0 to 10 year after transplantation. Immunohistochemistry study showed that the LCs present in the skin of the allografts remained of donor-origin up to 10 years post-transplant, although an occasional LC of recipient's origin was found in the epidermis of 1 patient shortly after an episode of acute rejection in the first post-transplant year. Authors also observed a LC in mitosis within the epidermis 8 years postgraft. These results strongly suggest that in human VCA, epidermal LCs are able to self-regenerate and the replacement of LDs by recipient-derived bone marrow precursor occurs very slow or probably never happen [290, 291]. Authors also suggested that the maintenance of donor epidermal LC in the epidermis of VCA might have contributed to the prevention of frequent acute rejection episodes as well as chronic rejection in VCA, as it's noticeable that VCA is relatively resistant to chronic rejection [6].

However, as discussed in previous paragraph, the newly discovered Langerin⁺ dDCs are also believed to participate in the maintenance of tolerance to epidermis. In the setting of VCA, future study is require to evaluate the exact roles of LCs and migratory dDCs in the in rejection or acceptance of allografted skin.

VIII.3.2. Role of Skin Regulatory T Cells in Tolerance Induction of VCA

Regulatory T cells (T_{reg}) are characterized by their property of suppressing excessive effector T-cell responses and mediating central and peripheral tolerance to self-antigen. In allotransplantation among various species, T_{reg} have been confirmed to associate with prolonged allograft survival and tolerance induction. T_{reg} are primarily characterized by expressions of cell surface markers CD4, CD25 and transcription factor Foxp3, as such identification is widely applied to experimental or clinical studies.

Skin is much more widely regarded as a structural organ as bones or skeletal muscles than as an immune effector organ composed of numerous, high-differentiated immunocompetent cells with a great variety. Bos et al. defined the skin as “skin immune system” because essentially, the primary function of the skin is to protect the body by innate or adaptive immune responses [292-294].

Recent researches indicated that the skin of an adult human contains approximately 20×10^9 resident T cells (which include various T_h , T_c and T_{reg} subsets) with memory T-cell phenotype ($CD45RO^+CLA^+CCR4^+$) [295].

This memory T-cell number in the skin is twice than the total T-cell number in the peripheral blood. Most previous studies of skin immune focused on the capacity of skin DCs to migrate through afferent lymphatics into draining lymph nodes where they stimulate naïve T cells, as discussed in previous paragraphs. However, skin DCs can also readily present antigen to skin resident memory T cells, a response that can occur immediately after pathogen invasion of skin and requiring no cellular recruitment or migration from blood [296, 297]. In such process, skin resident T_{reg} play important role in maintaining the immune homeostasis within the skin.

In normal human skin, the resident T_{reg} represent between 5 to 10% of total resident T cells [298, 299]. Under normal steady state, these skin T_{reg} have been shown to be important for mediating peripheral tolerance, however, in the setting of skin allograft, no matter vascularized or non-vascularized, the role of these T_{reg} with donor-origin in alloimmune remains unknown. It's also totally unclear whether recipient-derived T_{reg} can migrate to allografted skin and regulate the alloimmune in

long accepted VCA. In following paragraphs, some VCA study concerning skin Tregs will be cited and analyzed.

In swine VCA, Kuo et al. examined the T_{reg} in grafted skin by performing immunohistochemical staining of CD25+ on skin tissue section. Results showed that significant increase of CD25+ T cells in the subcutaneous and dermis layers was associated with long-term acceptance of VCA [300].

However, the limitation of this examination is that CD25 is a relatively non-specific marker for Treg; although relatively less identified in swine, CD25 has been constitutively expressed by a large proportion of resting memory T cells [301]. Thus, it should be cautious to conclude these CD25⁺ cells as regulatory T cells.

In canine model of VCA [276], CD3⁺ T cells were isolated from muscle and skin of the allografts, and then qRT-PCR methods were used to the expression of Foxp3. Results showed that CD3⁺ T cells obtained from muscle and skin biopsies taken from vascularized composite allografts on mixed chimeric recipients had increased levels of FoxP3 compared to normal muscle and skin. Foxp3 is a more specific marker than CD25 for T_{reg}; however, in human it is also expressed by recently activated conventional T-cells, although such expression of Foxp3⁺ in canine is unknown, this examination of comparing Foxp3 expression in CD3⁺ T cells from allografted skin and normal skin, unfortunately, was not able to exclude the possibility that increasing of Foxp3 expression in allografted skin than normal skin, was due to more recently activated conventional T-cells but not T_{reg} in the skin allograft. More examinations such as expression of perforin mRNA on these T cells should be included in order to confirm the results.

Indeed, in more recently study from non-human primate model of VCA, Brazio et al. performed Foxp3 staining on allograft biopsies from non-human primate received heterotopic partial facial segment allotransplantation by Immunohistochemistry [302]. Graft skin T_{reg} values were compared with graft outcomes and with histological grade from concurrent biopsies. Results showed that animals which experienced rejection did not have significantly different graft skin T_{reg} values from those that did not. Notably, biopsy specimens with grade I or II rejection were more likely to contain T_{reg}.

Authors suggested that present T_{reg} examination may have limited prognostic value with standard immunosuppressive protocols used in VCA.

In a human bilateral hand transplantation recipient, skin biopsies were performed in up to 6 years' follow-up [303]. Both Immunohistochemistry staining and qRT-PCR were used for examination of skin T_{reg}. Foxp3 staining was found positive on the initial biopsy and appeared to increase with time; this result was supported by the measurement of FoxP3 mRNA expression in the latter fresh biopsy without any culture, which showed higher levels than that of blood, together with no expression of perforin mRNA, but increased expression of TGF- β and IL-10. No FoxP3 mRNA expression was found in the contralateral leg, because of the absence of T cell infiltrate. This study well confirmed the Treg presence in the skin of well accepted VCA, with the positive FoxP3 detection and negative perforin mRNA expression.

In sum, skin T_{reg} may be an important modulator of alloimmunity in skin allograft, but nowadays the conclusions from T_{reg} examination in the skin of VCA remain controversial and may not be regarded as an effective biomarker or predictor for tolerance induction. Future study should on the one hand improve the technique protocol of tissue T_{reg} examination in order to better detect and characterize T_{reg} in allografted skin, and on the other hand re-determine the role of T_{reg} markers detection as a prediction of tolerance and subsequent minimization of immunosuppression.

VIII.4. Conclusion

In this present chapter, we focus on the studies of tolerance induction to VCA in large-animal models and human patients. Among this investigations, mixed chimerism creation by HSCT with a nonmyeloablative (by cytoreductive or irradiative therapy) or a lymphodepletive (by ATG or AST) conditioning protocol is frequently administered. Moreover, the HSCT plus lymphodepletive conditioning protocol has been translated into clinical face and upper limb transplantations; although in these setting, neither macrochimerism nor microchimerism was detectable, some patients were reported to require relatively low doses of immunosuppressive medication to prevent acute rejection. Tolerance induction to VCA by co-stimulatory blockade (by CTLA4-Ig)

protocol has been evaluated in swine model and the beneficial effect was confirmed, but such protocol has not been performed in clinical VCA yet.

Split tolerance is a unique feature discovered in the studies of VCA tolerance induction and this should be due to the high antigenicity of epidermis. Future study of VCA tolerance induction may have to pay more attention on the role of skin DCs (including epidermal LC and dermal DCs) and T_{reg}. Topical immunosuppressive therapy might serve as a promising solution to the difficulty of split tolerance.

Part II: Experimental Studies

I. Composite tissue allotransplantation in newborns: A swine model

I.1. Background

Vascularized composite allograft (VCA) is an alternative management for congenital limb or face defects in newborn infants; however, an effective tolerance induction protocol to composite allografts is mandatory to justify this high-risk intervention in these vulnerable neonatal patients. Establishment of preclinical VCA model in neonates is the precondition for tolerance induction of VCA in neonates.

VCA models have been established in small rodents, mainly in rats [304-306], mouse [307, 308] and rabbit [309] VCA models were also reported; or in large-animals, such as swines [310-313], canines [276, 314] and non-human primates [315-317]. However, none of them is performed on neonatal animals.

Advantages of performing VCA in rodents such as in rats may include: more inbred animal strains to control MHC-mismatch parameters and experiment results are more reproductive; more availabilities to specific antibodies or other specific reagents thus suitable for thorough mechanism study. On the other hand, the disadvantages of rat VCA model may contain: neonatal rats are too small for microsurgery thus are not possible to be used in our present study; moreover, because of large variability in immunogenetics between rodent and human species [318, 319], results from rat VCA model may not be able to translated into clinical study [13].

Therefore, a large-animal model is necessary for our present research. Compared to canines and non-human primates, swines can serve as an excellent model for neonatal VCA study. (1) Canines and non-human primates are well known to have altricial newborns. These altricial newborns are difficult to be separated from their mothers thus it limits the experimental intervention such as drugs administration

and blood sampling [320]. In contrast, swines have more precocial newborns, so are free for management. (2) Swines have physiological and immunologic similarities to humans and have been proved to be a useful model for the studies of organ transplantation or VCA, as well as for the studies on development of adaptive immune system [320]. (3) Anatomically and physiologically, Porcine skin is very similar to human skin [321], thus may be an proper model for VCA of which the skin is one of the most important components. (4) Miniature swine is the only large-animal which can provide several SLA-defined inbred strains for MHC-mismatching transplantation researches [322]. (5) Other advantages of swine model are unlimited availability and size similarity to human beings [323]. Under such consideration, we concluded that swine is a suitable model for establishment of neonatal VCA model. However, the disadvantages of swine model are also evident, such as lymphocyte subsets are less clearly characterized, as well as less availability to specific antibodies and reagents compared to rodent model. These disadvantages may be potential barriers to future profound exploration.

We then decided to perform our study on piglets with age of 1~2 weeks as this stage is in the critical “window phase” of immunological development in neonatal swines [320], in order to evaluate whether neonatal, premature immune system may provide any privilege for donor-specific tolerance induction.

I.2. Publication

Composite Tissue Allotransplantation in Newborns: A Swine Model

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Abstract

Background: Management of congenital limb aplasia or facial malformations could be improved by composite tissue allotransplantation (CTA), a technique that has never been performed in newborns. For this, however, the induction of donor-specific tolerance would be mandatory, as long-term immunosuppression is not acceptable in this non-lifesaving procedure. Induction of tolerance has been shown to be possible in a newborn CTA rat model but has never been tested in large-animal models. Our goals were to establish a model of CTA in newborn swine to see if tolerance could be obtained without immunosuppression and to assess rejection or tolerance properties via clinical and histologic examinations.

Materials and methods: We applied a CTA heterotopic knee swine model. We performed two series of surgical procedures: Series 1 was 20 autografts in 6-day-old (1e10) 2,544 kg (1,140e4,060 kg) piglets; Series 2 was 10 allografts without immunosuppression between outbred animals aged 7.8 d (6e10) and weighing 2,770 kg (2,200e3,550 kg).

Results: In Series 1, six early deaths and two cases of vascular failure were observed. In Series 2, no spontaneous deaths were observed and all piglets presented clinical and histologic rejection.

Conclusions: Our findings strongly suggest that newborn immunologic status is not sufficient for the development of tolerance in large-animals without immunologic intervention. Complications and animal death after transplantation correlate with age and weight. Low rates for both vascular failure and postoperative death permit the use of this model in piglets weighing over 2 kg and aged more than 6 d for research on newborn CTA.

Keywords: Composite tissue allotransplantation; Newborn; Swine model; Tolerance; Facial malformation; Limb aplasia.

1. Introduction

Composite tissue allotransplantation (CTA) has never been performed in newborns (although it should be noted that an isotransplantation of one hand was successfully performed between identical twins in 2000).

CTA could improve the management of congenital limb aplasia (particularly upper limb transverse aplasia) or that of facial malformations such as Treacher-Collins syndrome, hemifacial microsomia, and some vascular anomalies [4, 7, 8].

However, CTA is a non-lifesaving procedure and thus the requisite long-term immunosuppression would not be acceptable, as it may lead to delayed growth and an increased incidence of tumors and infections [324-326]. Consequently, inducing specific tolerance would be mandatory in this framework.

In the neonatal period, specific tolerance has been induced without immunosuppression in a rat model [327]. Authors have demonstrated the existence of a short period of immunologic immaturity that could facilitate the induction of tolerance to CTA [327, 328], but such an approach has never been tested in newborn large-animals or humans. As they were for CTA in adults [311, 329-334], preclinical large-animal models for tolerance research will be needed to make neonatal CTA a clinical reality [335, 336].

Our goal for the present study was to obtain a reliable model of CTA in newborn swine, a species having an immune system similar to that of humans [336]. Our aims were to test the possibility of obtaining specific tolerance to CTA without immunosuppression in newborn swine and to assess transplantation rejection or tolerance properties via clinical and histologic examinations.

We thus performed heterotopic transplantations of vascularized osteo-chondro-myocutaneous knee flap in newborn swine, as previously done in adult swine [326, 330].

2. Methods

2.1. Experimental protocol/ethical considerations

Our study was carried out in the research department of the veterinary school of Lyon (VetAgro Sup Lyon).

The animals were purchased from an ordinary pig farm. Swine race for autotransplantation and for recipients in allotransplantation was a crossbreed of 50% Large White and 50% Landrace. Swine race for donors in allotransplantation was a crossbreed of (25% Large White + 25% Piétrain) + 50% Duroc.

One postpartum sow for every 10 piglets (natural or adopted) was transported to the veterinary school to provide normal maternal feeding.

All experimental phases were submitted to and approved by the regional ethics committee for animal experimentation. Animal care followed guidelines published by the National Institutes of Health (USA). Endpoints resulting in animal euthanasia were defined as follows: untreatable pain, severe sepsis, weight loss exceeding 20%, lack of feeding over 48 h, graft necrosis. For all other cases, the piglets were sacrificed randomly between postoperative days (POD) 4 and 14 in autografts, or at the appearance of necrosis in allografts.

All surgical procedures were performed by the same senior operator.

We performed two series of transplantations. In Series 1, 20 autografts were performed in piglets with an average age of 6 d (range 1-10 d) and an average weight of 2,544 kg (range 1,140-4,060 kg). In Series 2, 10 allografts were performed between outbred ABO-compatible animals, without immunosuppression. Average recipient age and weight were 7.8 d (range 6-10 d) and 2.77 kg (range 2.20-3.55 kg) respectively. Average donor age was 8.9 d (range 8-12 d).

2.2. General anesthesia

Intramuscular premedication by ketamine (10 mg/kg) and xylazine (2 mg/kg) was administered 10 min before induction. General anesthesia was induced with 5% isoflurane by facial mask and maintained with 2% isoflurane by orotracheal intubation. An ear vein was catheterized to administer a continuous perfusion of 5% glucose polyionic solution. Analgesia was provided by one or more boluses of intravenous morphine 0.1-0.3 mg/kg.

Continuous pulse oximetry, capnography, and rectal temperature were monitored.

2.3. Surgical technique

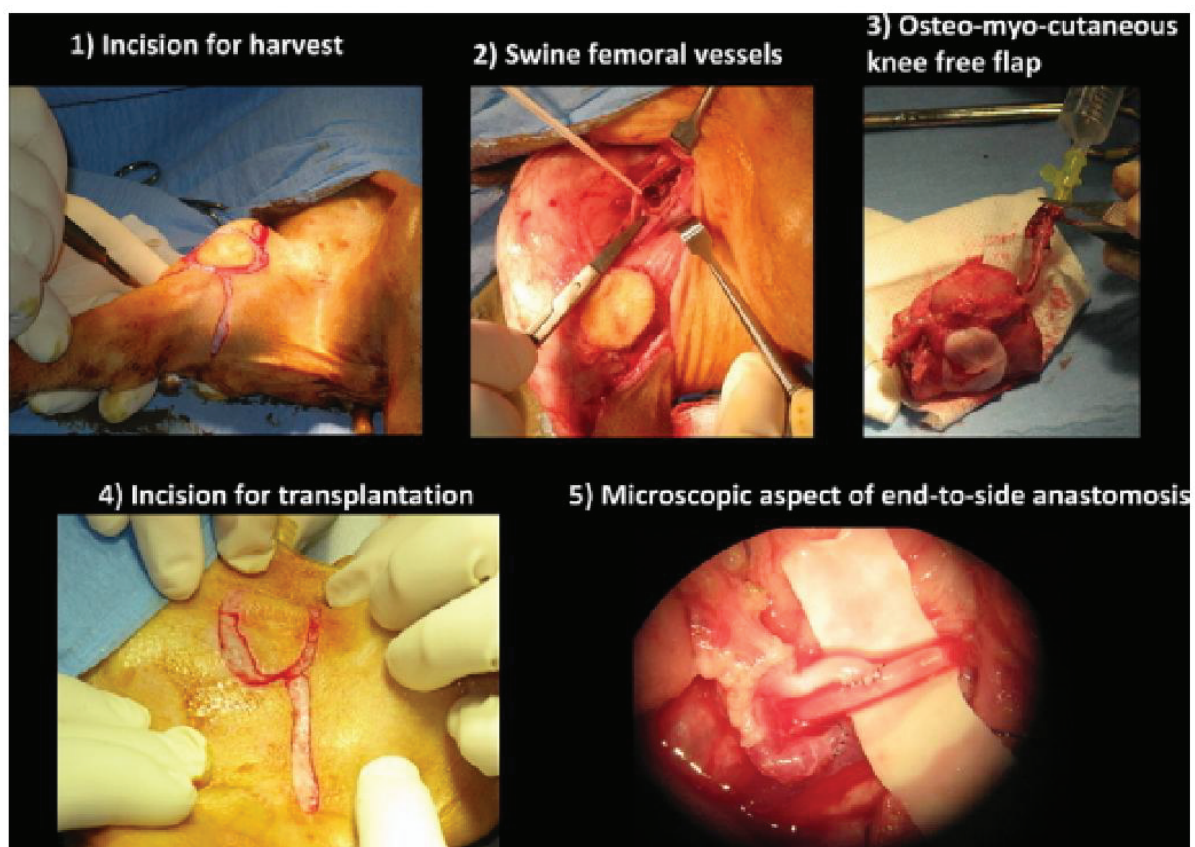
Surgical technique consisted of 1) harvest of an osteochondro-myocutaneous free flap of right knee (containing distal femur, proximal tibia and fibula, knee articulation, periarticular muscles and tendons, and medial skin of the knee), vascularized by femoral vessels; and 2) heterotopic transplantation in a subcutaneous inguinal pocket and lateroterminal anastomosis on left femoral vessels.

Installation and draping were the same for both the harvest and transplantation procedures: in dorsal decubitus, the lower part of the body was prepared, draped, and installed on a lead hand with back limbs in abduction.

2.3.1. Harvest

A 4-cm circle was drawn on the medial skin of the knee, centered on the intersection of the femorotibial articulation and femoral superficial vessels. This formed the cutaneous paddle of the flap.

This incision was extended proximally toward the groin, then into the inguinal fold; distally, it continued around the leg for amputation (Fig. 1).



CTA-Figure 1: Surgical procedure.

Panel 1: Incision for harvest on right hind limb. A 4 cm circle on the medial skin of the knee forms the cutaneous paddle of the flap. This incision is extended proximally toward the groin, then into the inguinal fold; distally, it continues around the leg for amputation. **Panel 2:** Femoral vessels are prepared from the origin of the deep femoral vessels to the distal division. **Panel 3:** Osteo-myo-cutaneous knee flap is rinsed by IGL1 solution through femoral artery. **Panel 4:** Incision for transplantation on left groin and inguinal fold with a circle on lateral edge. **Panel 5:** Microscopic aspect of end-to-side anastomosis after clamp removal.

The distal ligation of the cutaneous branch of the femoral superficial vessels was then performed. Femoral vessels were prepared from the origin of the deep femoral vessels to the distal division, i.e., a length of about 12 mm, corresponding to the length of the flap vessels.

Thigh and leg muscles were sectioned to preserve only periarticular muscular tissue along with its insertions. A Liston forceps was used to cut the tibia and fibula, then the femur. Finally, the femoral vessels were clamped.

For the cases of autotransplantation, vessels were tied proximally and cut distally to clamp. The flap was then rinsed by artery with 20 mL of heparin solution (100 UI/mL). The femur was shortened and covered by its periosteum. Lateral muscles were closed by continuous suture with absorbable 3/0 thread. Skin was closed with separated stitches of absorbable 4/0 thread (Fig. 2).



CTA-Figure 2: Autograft.

Left panel: Incision for harvest. **Central panel:** Closure of harvest site, aspect of the free flap and lodge in inguinal region for transplantation. **Right panel:** Closure of transplantation site with the flap cutaneous paddle and tattoo for identification of the animal.

For the cases of allotransplantation, the flap was left on the donor until the recipient femoral vessels were ready for grafting. After separation, the flap was rinsed by artery with IGL1 solution and the donor was sacrificed by overdose of pentobarbital.

2.3.2. Transplantation

The incision followed the inguinal fold and was completed laterally by a circular skin excision 3 cm in diameter. A pouch was developed by digital dissection and some hemostasis points between the abdominal wall, skin, and thigh region.

Femoral vessels were prepared as for harvest but with a shorter length (about 8 mm). The femoral nerve was carefully preserved. The graft was posed on lodge and sutured by three or four stitches to the abdominal and femoral aponeuroses with the graft vessels perpendicular to the recipient vessels and the skin paddle in the lateral cutaneous defect.

Using the operating microscope, after double-clamping the recipient vein, venotomy was performed using a 30-degree ophthalmic scalpel and microsurgical scissors. Extreme proximal and distal points were placed. A continuous suture was

performed on the medial edge, and four or five interrupted points were used for the lateral edge.

The same technique was used for the artery. Arterial pulsatility was appreciated and the patency of the recipient vein tested. Closure was performed with separated points of absorbable 4/0 thread: edge to edge on the medial part of the incision and donor to recipient for the cutaneous paddle (Fig. 3).



CTA-Figure 3: Aspect of cutaneous paddle in allograft.

Left panel: Post-operative day 1, normal skin. **Right panel:** Complete rejection and necrosis of cutaneous paddle.

A patch of fentanyl 12mg/h was placed on the thorax. Antibiotic prophylaxis was cephalexin 30 mg/kg intraoperatively and amoxicillin 15 mg/kg/d for 7 d postoperatively.

Subcutaneous injections of morphine 0.1 mg/kg were administered in the presence of pain. Postoperatively, 1.5 mg/kg of enoxaparin was administered daily until POD 5 for Series 1.

Immediate postoperative and daily examinations were focused on general conditions, weight, feeding, temperature, and graft status (color, bleeding after

pricking, capillary pulse). Daily photographs of grafts were taken at dressing renewal.

For both series, the following parameters were recorded: operating time, ischemia time, operated animal survival, graft survival / vascular failure, beginning of cyanosis, other complications.

For Series 2, late graft survival and date of rejection were also registered.

2.4. Histology

At exitus, skin, muscle, cartilage and bone samples from grafts and surrounding recipient tissues were fixed in neutral buffered 10% formalin, cut to 4µm thick and stained using hematoxylin and eosin (HE). For Series 2, one or several skin biopsies were performed before animal sacrifice. A histological scoring system was adapted based on grading in a swine composite tissue allograft model [337, 338] and evaluation of allograft rejection in human allotransplantation [310, 339]. Important features were:

- In the epidermis: thickness, presence of exocytosis and spongiosis, keratinocyte apoptosis sometimes with satellitosis and basal cell vacuolization.
- In the dermis: inflammatory cell infiltrate (density, composition and localization), presence of hemorrhage, vascular change (dilatation, necrosis, thrombosis, vasculitis), adnexal (hair follicle, sweat gland) alteration, dermal edema.

Grades are detailed in Table 1.

Table 1 – Histologic grading of rejection.			
Grade	Meaning	Aspect of epidermis	Aspect of dermis
0	No rejection	Normal	Normal or some perivascular infiltrates
I	Mild rejection	Normal	Small mononuclear perivascular cuff in superficial or mid dermis
II	Moderate rejection	Normal or moderate exocytosis and/or spongiosis	Moderately dense infiltrate predominantly lymphocytic without fibrinous thrombi
III	Marked rejection	Exocytosis and/or spongiosis and scattered apoptotic keratinocytes	Fibrinous thrombi and/or dense infiltrate with lymphocytes, histiocytes, and mast cells; vasodilation and edema
IV	Severe rejection	Numerous apoptotic keratinocytes, lymphocytic exocytosis, basal cell vacuolization, dermal-epidermal separation, and/or diffuse coagulative necrosis	Large aggregates of inflammatory cells with multifocal fibrinous thrombi, edema, necrosis, hemorrhage, hair follicle and sweat gland alteration

CTA-Table 1: Histologic grading of rejection.

3. Results

Results are summarized in Table 2.

Table 2 – Summary of results.		
Series	Autografts	Allografts
Number of procedures	20	10
Age (d)	6 (1–10)	7.8 (6–10) ^b
Weight (kg)	2,544 (1,140–4,060)	2,770 (2,220–3,550) ^b
Operating time	3 h 55 min (3 h 15 min – 5 h 8 min)	4 h 8 min (3 h 12 min – 5 h 20 min)
Ischemia time	2 h 17 min (1 h 44 min – 2 h 55 min)	1 h 48 min (1 h 5 min – 3 h 15 min)
Rate of spontaneous exitus ^a	45%	0%
Date of spontaneous exitus ^a	POD 1–6	
Rate of cyanosis at spontaneous exitus or euthanasia	11%	0%
Rate of random euthanasia	50%	0%
Rate of euthanasia for graft necrosis	5%	100%
Date of euthanasia for graft necrosis (POD)	1	See Table 3
Rate of scar complications	30%	10%

^aSpontaneous exitus or euthanasia for bad condition.
^bRecipient data.

CTA-Table 2: Summary of results.

3.1. Series 1 (20 autografts), Table 3

Table 3 – Mode of exitus in autografts in piglets under or over 2 kg.				
	POD 1-2*	POD 3-6*	Random euthanasia POD 4-14**	Euthanasia for graft necrosis
Number of piglets <2kg	5	1	1	0
Number of piglets >2kg	1	2	9	1

*Spontaneous exitus or euthanasia for bad condition
 ** Good condition of animal and graft

Mode of Exitus	Number of piglets <2kg	Number of piglets >2kg
POD 1-2*	5	1
POD 3-6*	1	2
random euthanasia POD 4-14**	1	9
euthanasia for graft necrosis	0	1

CTA-Table 3: Mode of exitus in autografts in piglets under or over 2 kg.

Exitus occurred on POD 1 or 2 for six animals (33%). Autopsies suggested surgical and anesthetic trauma as cause of death, due to the length of the procedure (around 4 h) in very young subjects, associated with blood loss, hypothermia, and metabolic distress (high catabolic activity and lack of glucose and lipid stock). In one animal that died on POD 2, graft cyanosis had been observed since POD 1.

Two exitus due to hypovolemic shock caused by hemorrhage at the transplantation site were observed on POD 1 and POD 6. Exitus occurred on POD 3-6 for two animals (17%) following sepsis or stump scar necrosis.

One animal was sacrificed because of graft necrosis on POD 2.

Ten animals survived until sacrifice, which was randomized to POD 4, 7, or 14, with a normal aspect of the graft paddle.

Observed complications were four stump scar problems (one necrosis, three sepses) and two graft scar dehiscences.

3.2. Series 2 (10 allografts), Table 4

No spontaneous exitus or vascular failure was observed.

In nine animals, a nascent cyanosis was observed between POD 3 and 5 followed by complete rejection between POD 4 and 8. One animal presented a nascent hyperemia on POD 5 and complete necrotic rejection on POD 80. All animals were sacrificed after complete rejection. No wound healing complications were observed.

Student t was used to compare weight, age, and operating time in survivor versus early-death animals in Series 1. Significant differences were observed for weight ($P=.009$) and age ($P=.017$) but not for operating or ischemia times ($P>.05$).

Table 4 – Results of allografts.

Animal	1c	2c	3c	4c	5c	6c	7c	8c	9c	10c
Recipient age (d)	6	8	8	9	9	6	6	8	8	10
Weight (kg)	2,235	3,471	2,833	2,539	2,781	2,812	2,207	2,709	2,600	3,549
Operating time	4 h 45 min	3 h 50 min	3 h 20 min	3 h 15 min	5 h 20 min	4 h 10 min	3 h 35 min	4 h 48 min	3 h 25 min	4 h 55 min
Ischemia time	1 h 5 min	1 h 30 min	1 h 17 min	1 h 20 min	3 h 15 min	1 h 25 min	1 h 15 min	2 h 3 min	1 h 24 min	2 h 9 min
Date of beginning of hyperemia (POD)	1					5	5	2	2	
Date of beginning of cyanosis (POD)	7	4	4	4	4	70	2	5	11	1
POD first biopsies	7	5	5	4	4	8, 11	8	6, 9	8, 11	4
Rejection grading at first biopsies	III	IV	III	III	III	II	IV	II	III	III
POD other biopsies	8					16, 29, 36, 43, 50, 57				
Rejection grading at other biopsies	IV					I-II				
Date of exitus (POD)	10	7	7	6	6	80	10	14	14	7
Rejection grading at euthanasia	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV

CTA-Table 4: Results of allografts.

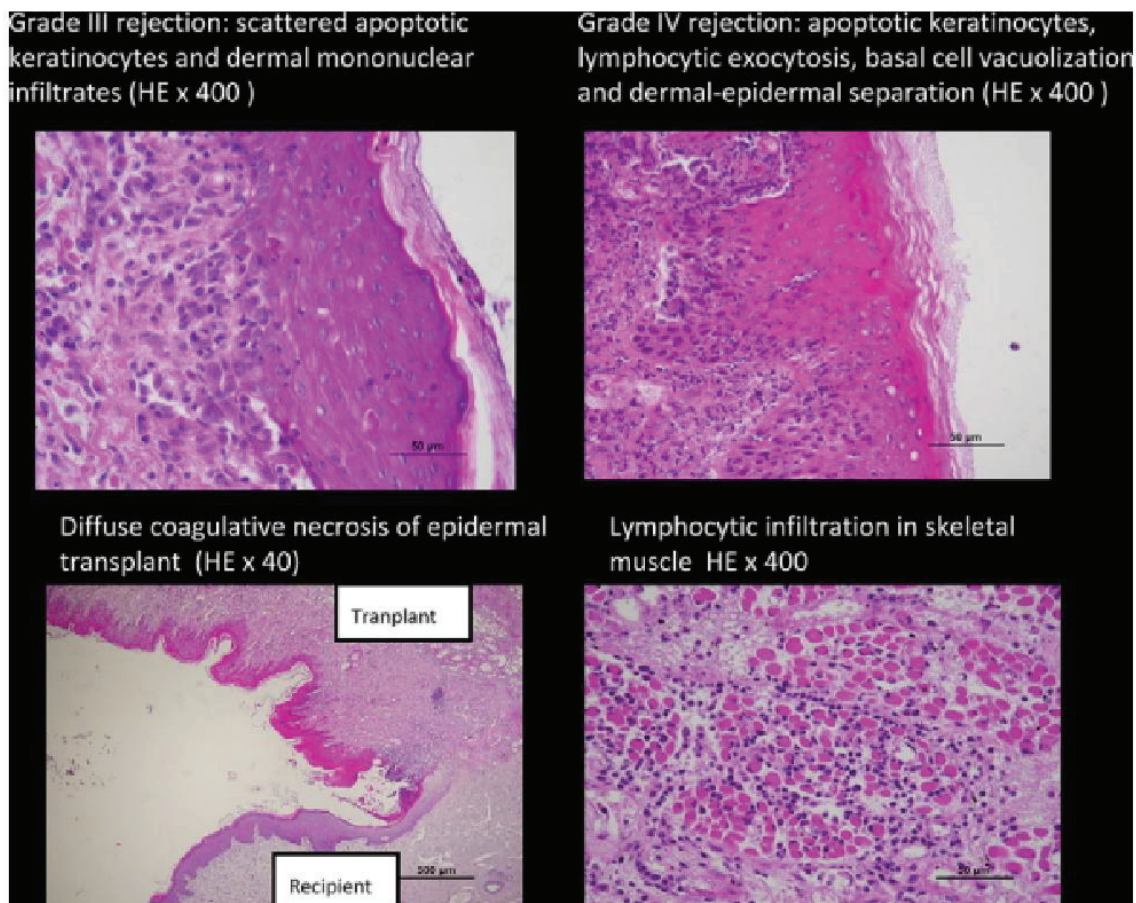
3.3. Histology

3.3.1. Autograft

Excluding piglets with graft loss secondary to ischemia caused by vascular complications, there were no epidermal lesions (Fig. 5) except at the suture location (acanthosis and crusts). The dermis showed marked vasodilation and edema in animals that died prematurely. In animals euthanized at POD 4 to 14, edema and vasodilation decreased and there was granulation tissue between the autograft and surrounding tissue.

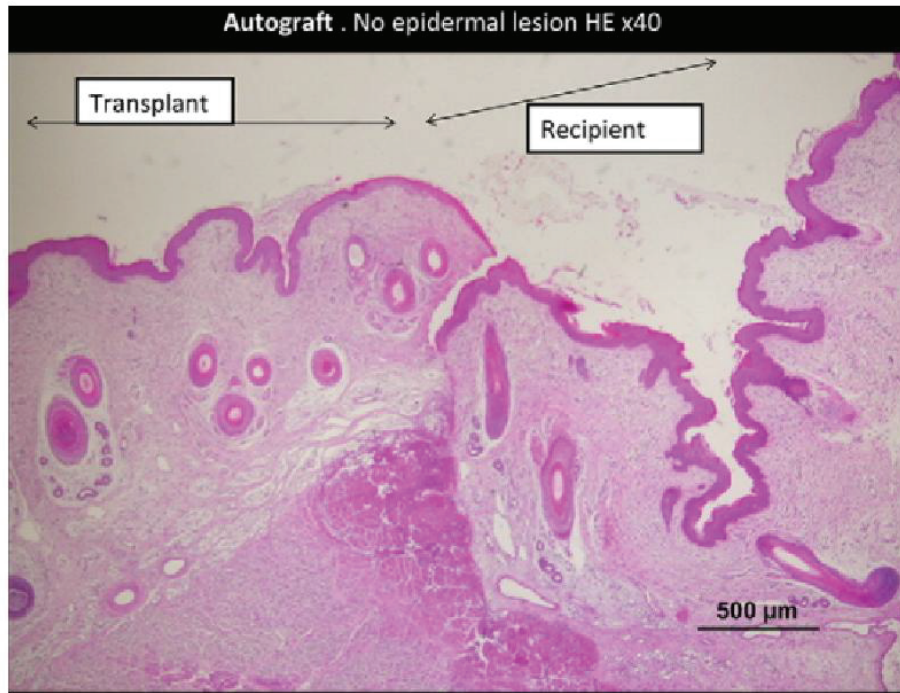
3.3.2. Allograft (Table 4)

Skin biopsies and terminal skin samples were graded and the results summarized in Table 4. Except for the animal that rejected at POD 80 (Fig. 6), biopsies performed between POD 4 and 11 were most often grade III and IV. Skeletal muscle and connective tissue in transplants showed multifocal perivascular and diffuse mononuclear infiltrates, predominantly lymphocytic (Fig. 4), sometimes associated with edema and necrosis foci. No lesions were found in cartilage.



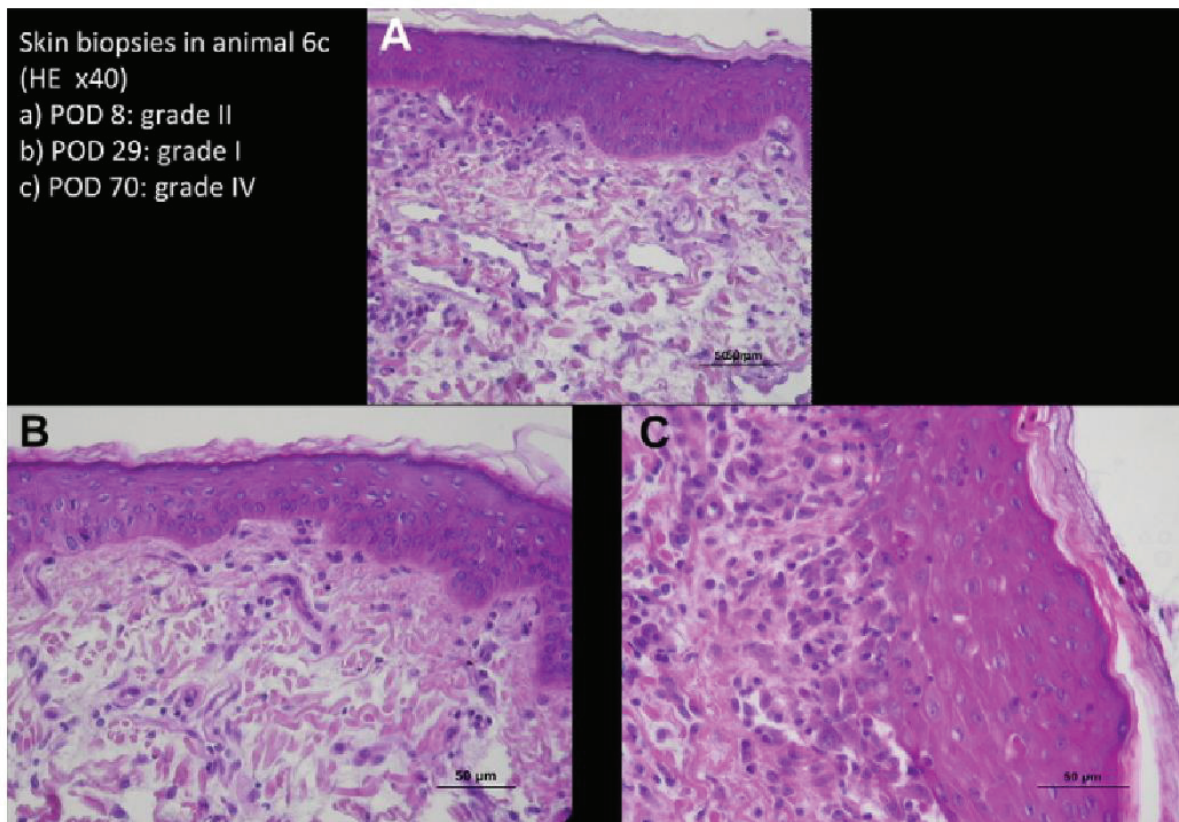
CTA-Figure 4: Histology of rejection.

Upper left panel: Grade III rejection. Scattered apoptotic keratinocytes and dermal mononuclear infiltrates (HE X 400). **Upper right panel:** Grade IV rejection. Apoptotic keratinocytes, lymphocytic exocytosis, basal cell vacuolization and dermal-epidermal separation (HE X 400). **Lower left panel:** Diffuse coagulative necrosis of epidermal transplant (HE X 40). **Lower right panel:** Lymphocytic infiltration in skeletal muscle (HE X 400).



CTA-Figure 5: Histology of autograft.

Normal skin, no epidermal lesion (HE X 40).



CTA-Figure 6. Skin biopsies in animal 6c.

Panel A: POD 8, grade II rejection. **Panel B:** POD 29, grade I rejection. **Panel C:** POD 70, grade IV rejection (HE X 40).

4. Discussion

The procedure described in the present article is adapted to swine weighing over 2 kg and aged 6 d or more.

In Series 1, five early deaths may be explained by low weight and age, as these two factors were predominant in early-death piglets ($P=.009$ and $P=.017$). Operating time was not associated with early death ($P>.05$).

Consequently, we performed allotransplantation experiments in “stronger” animals (weight >2 kg and age >5 d). In these, no postoperative death was observed.

Vascular failure was 15% in Series 1, with the bias of 5/10 early deaths. It was 0% in Series 2.

Surgical procedure duration was longer in the autografts because both harvest and transplantation times were supported by the same animal.

Early death in the autotransplantation series may be explained more precisely by surgical and anesthetic “injury” due to this long procedure (around 4 h) in very young subjects, associated with blood loss, hypothermia, and lack of glucose and lipid stocks.

We observed a longer ischemia time in allotransplantation compared to autotransplantation ($P=.002$), due to the longer recipient site preparation time for the former. This duration could be decreased if two teams worked simultaneously. A learning curve may also explain this result.

Some adjustments from the original description and technical points merit discussion:

- Although end-to-end anastomosis was proposed by Lee et al. in their first description [329], we always performed end-to-side anastomosis on veins and arteries to respect rheological principles and avoid vessel loops.

- The skin surface needs to be large enough for biopsy; this implies a diameter of at least 4 cm.

- Cases of suture dehiscence or infection were observed in Series 1, where rapid absorbable thread was used to close skin. In Series 2, normal absorbable thread was used and no scar complications were observed. We thus recommend this kind of thread.

- Perfusion fluid should be IGL1 or another organ conservation fluid to decrease lesions due to ischemia. - Flap hemostasis must be carefully performed before separation.

- In cases of autotransplantation, the femoral stump on the donor site must be significantly shortened to avoid scar complications.

- Postoperative heparin treatment resulted in some bleeding from the operation site; in the later series, we used only intraoperative heparin bolus and observed only one early thrombosis.

Furthermore, some aspects of femoral region anatomy in swine need to be stressed. The femoral vessels are easily found at their emergence from under the inguinal ligament, covered by femoral fascia and skin. In their central part, they are covered by a long and thin pelvic-tibial muscle, which is vascularized by femoral vessel branches. A few millimeters from their emergence under the inguinal ligament, the femoral vessels split into a deep and a superficial branch.

At the distal third of the femur, the femoral superficial vessels split into two branches: an antero-medial, myocutaneous branch that is clearly visible under the medial skin of the knee; and a postero-lateral branch, which becomes popliteal.

The inguinal skin is rich in its lateral part and forms a plica from flank to thigh.

From a clinical and histologic perspective, in our study, as in previous studies [311, 332, 333], we observed that the skin was the first tissue component of CTA to demonstrate visible indications of acute rejection, because of its special intrinsic immunologic features (high concentration of Langerhans and other dendritic antigen-presenting cells). Grading levels tend to differentiate severity by addition of new lesions at each level [339]. Up to grade II, lesions are unspecific and found in a wide range of disorders. More specific lesions of acute skin rejection are interface dermatitis with apoptotic keratinocytes (with or without satellitosis), lymphocytic

exocytosis, vasculitis, and multiple fibrinous thrombi.

Except for in the sixth pig of Series 2 (6c, Table 4), CTA flaps demonstrated complete rejection (grade IV) between 5 and 14 d after implantation. This is consistent with results of previous studies in adult swine with no immunosuppression [326]. Nine of the 10 piglets in our study rejected the graft in a time frame similar to or faster than that of adult pigs [POD 4-8 in our series, POD 9-14 in adults [330]]. This strongly suggests that newborn immunologic status is not sufficient for the development of tolerance without immunologic intervention.

The delayed rejection of Pig 6c (POD 80) was probably due to incidental related swine leukocyte antigen, but we have no results for swine leukocyte antigen typing.

5. Conclusion

Low rates of vascular failure and postoperative death in allografts permit the use of this model for research in newborn CTA.

This model may be applied in research focused on specific tolerance of recipient versus donor CTA antigens by immunologic manipulations, such as short-term immunosuppression and donor bone marrow stem cell transplantation concomitant to CTA. In these cases, genetically defined animals held in a controlled environment should be used.

II. The Pharmacokinetics and Acute Renal Effects of Oral Cyclosporine in Newborn Swines

II.1. Background

In our previous study of VCA model with neonatal swines, partial hind-limb composite allografts were rapidly rejected as immunosuppressive therapy was absent, indicating that in neonatal swines, alloimmunity is largely competent. Therefore, proper immunosuppression is necessary for allograft survival and tolerance induction.

Cyclosporine A (CsA) is currently the predominant immunosuppressive drug for pediatric transplantation, even in very young infants [340]. CsA is also widely administered in young pigs in experimental transplant studies. We thus explored the feasibility of CsA's application in neonatal pigs for immunosuppressive therapy [340].

CsA is a cyclic polypeptide immunosuppressant agent consisting of 11 amino acids. It is produced as a metabolite by the fungus species *Beauveria nivea*. CsA belongs to the family of immunophilin-binding drug and can bind to cyclophilin. The CsA-cyclophilin complex can subsequently bind to the calcium/calmodulin-dependent phosphatase, calcineurin; thus, can lead to inhibition of activation and nuclear translocation of the nuclear factor of activated-T cells (NF-AT), and then inhibit IL-2 gene transcription. Interruption of IL-2 production will result in the inhibition of the initial events of T-cell activation (G0- and G1- phase of T-cell proliferation cycle), hence the suppression of T-cell immune [341].

In animals and in human patients, cyclosporine is a potent immunosuppressive agent that prolongs survival of allogeneic transplants involving skin, kidney, liver, heart, pancreas, bone marrow, small intestine, and lung.

However, in transplant patients, the use of CsA is mainly limited by its renal toxicity, which is characterized by a reversible, dose-dependant reduction of the renal blood flow (RBF) and the glomerular filtration rate (GFR), manifested clinically by an increase in serum creatinine (Cr) and blood urea nitrogen (BUN) [342].

CsA pharmacokinetics and nephrotoxicity have been studied in juvenile pigs with

about 6 months of age [342, 343]. However, in breast-feeding neonatal pigs (< 4 weeks of age) which may have a different characters on drug absorbance and metabolism, the pharmacokinetic profiles and acute renal effects of CsA are not clear.

Under such background, we performed the present study of pharmacokinetics and acute renal effects of oral cyclosporine in newborn swines in order to give implications to our future transplantation study.

II.2. Publication

Oral Cyclosporine A in Neonatal Swines for Transplantation Studies

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Abstract

The purpose of this study is to define the optimal dose of oral cyclosporine A (CsA) in newborn swines for transplantation studies, and to describe its pharmacokinetics and acute renal effects in short-term administration. Thirteen piglets were randomized into 4 groups: 1 control and 3 groups with CsA administration at 4, 8, and 12 mg/kg/day for 15 days (d). Blood were collected on d0, 2, 4, 9 and 14 to determine CsA trough concentrations, creatinine (Cr) and blood urea nitrogen (BUN) serum concentrations. On d14, blood was collected every hour from 1 to 10 hours (h) after CsA administration to evaluate the area under the curve (AUC). Kidneys were removed for histological analysis. We observed a stabilization of CsA trough concentrations from d4 to d14. On d14, respectively in Groups 4, 8 and 12 mg/kg/d, CsA trough concentrations were 687 ± 7 , 1200 ± 77 and 2211 ± 1030 ng/ml; AUC_{0-10h} were 6721 ± 51 ng·h/ml, 13431 ± 988 ng·h/ml and 28264 ± 9430 ng·h/ml. Cr concentrations were not significantly different among 4 groups; BUN concentrations of the 3 treated groups were significantly different from control group. CsA was well tolerated; neither acute adverse event nor renal histological abnormality was observed. In conclusion, a 15-d course of CsA treatment ranged from 4 to 12 mg/kg/d is safe for breast-feeding pigs which need much lower CsA dose than adult pigs to reach comparable drug exposure. For immunosuppressive therapy in these pigs, we recommend a CsA dose of 4 mg/kg/d to achieve blood concentration of 400-800 ng/ml.

Keywords: cyclosporine A, pharmacokinetics, acute renal effects, neonates, swine

1. Introduction

Neonate stage is an immune “window phase” susceptible for induction of transplantation tolerance. In mice, it has been known for decades that the injection of allogeneic spleen cells during neonate period under certain conditions can result in neonatal tolerance and chimerism without any conditioning regimen [14, 75]. Although the relevant immunological mechanisms were not precisely understood, neonatal tolerance could be regarded as the result of immature and plastic immune system [68, 110, 344]. However, neonatal tolerance induction protocols which have been proved to be effective in rodent species are rarely, if ever, as effective as in large-animals and human which are born with almost entirely functional T cell immune system [13, 78].

Nevertheless, in large-animals and human, neonatal immune system still demonstrates several immature features: (i) the cellular immune system of neonates is still antigen-inexperienced and consists of high percentage of naïve helper T cells and low percentage of memory T cells and cytotoxic T-cells [125, 128]; (ii) ABO-incompatible organ transplantation and B cell tolerance are achievable in human infants [79]; (iii) neonatal innate immune cells including neutrophils, dendritic cells, macrophages and NK cells, are qualitative or quantitative incompetent [120, 131, 153, 345]; (iv) recipients of umbilical cord blood cells experience less graft-versus-host disease than recipients of hematopoietic stem cell transplantation (HSCT) from an adult donor [118]. In such circumstances, exploring of central tolerance induction protocol in neonate large-animal might give essential indications to transplantation program in very young human recipient [13].

Hand or face vascularized composite allograft (VCA) is a potential therapy for severe congenital upper extremity or craniofacial defects in newborn infants; however, an effective tolerance induction protocol to composite allografts is mandatory to justify this high-risk intervention in these vulnerable neonatal patients [7, 8]. Swine is an excellent model for transplantation immunology research program because of its similarities to human on genetics and body size [346]. In a previous study, we have established a vascularized composite tissue allograft model in non-weaned newborn pigs [347]. Besides, preclinical model of lung allotransplantation conducted in neonatal swines was also reported [348].

However, knowledge of immunosuppressive therapy and relevant side-effects in newborn pigs remains unknown. Cyclosporine A (CsA) is currently the predominant immunosuppressive drug for pediatric transplantation, even in very young infants [340]. However, the use of CsA is mainly limited by its nephrotoxicity, which is characterized by a reversible, dose-dependant reduction of the renal blood flow (RBF) and the glomerular filtration rate (GFR), manifested clinically by an increase in serum creatinine (Cr) and blood urea nitrogen (BUN) [342]. Swine serves as a suitable animal model in pharmacological and toxicological studies of CsA due to its close organ structure [343] and activity of the drug-metabolizing cytochrome P450 3A enzyme system to human analogs [349, 350]. In human and pig neonates (< 4 weeks of age), the pharmacokinetic profiles and acute renal effects of CsA still remain unknown.

In this present study, our principal objective was to describe the pharmacokinetics and acute renal effects of oral microemulsion CsA in a newborn swine model, in order to define the appropriate dose and monitoring strategy of CsA for immunosuppressive treatment for VCA in newborn piglets. This study may also help other preclinical transplantation studies such as ABO-incompatible heart, lung or heart-lung transplantations in neonatal swines to perform immunosuppressive therapy with CsA.

2. Material and Methods

2.1. Animals

Thirteen newborn domestic piglets with a patrilineal pedigree of Large White and a matrilineal pedigree of Youli, the same age of 5 days (d), weights ranged from 1.39 to 4.06 kg, 11 males and 2 females, were housed together with the sow under the standard procedure for neonatal pigs in the Institute Claude Bourgelat, animal research center of the veterinary school of Lyon (VetAgro Sup-Campus Vétérinaire de Lyon). The sow was fed with standard pig diet. Newborn piglets were breastfed exclusively and unrestrictedly. All animal experimentations were approved by the Ethical Committee of VetAgro Sup (Number 2012/1257) and conformed to the European Guideline 86/609/EEC of 1986, for the care and use of animals for scientific purposes.

2.2. Groups Design

Considering that the difference in body weight might cause a change in drug distribution, piglets were weighed at d0 and classified into 3 classes according to their body weights. Then, we randomly allocated piglets according to CsA doses: 0, 4, 8 and 12 mg/kg/d during 15 d, the weight was balanced within groups (Table 1).

Table 1: Distribution of piglets

Weight(kg)		Group 0 (0mg/kg/d)	Group 1 (4mg/kg/d)	Group 2 (8mg/kg/d)	Group 3 (12mg/kg/d)
A: 3.55 to 4.06 (n=3)	A		4,06	3,61	3,55
B: 1.84 to 2.57(n=6)	B B'	2,26 2,05	2,57	2,25 1,84	1,85
C: 1.39 to 1.75 (n=4)	C C'	1,66 1,75	1,39		1,51

Table 1: Distribution of piglets was according to body weights and CsA doses.

Body weights on day (d) 0 were balanced among 4 groups with CsA doses of 0, 4, 8, and 12 mg/kg/d.

CsA-Table 1: Distribution of piglets.

2.3. Drug Administration

Cyclosporine (Neoral® 100mg/ml, oral solution) was purchased from Novartis Pharma S.A.S. The piglets were weighed at every 9:00 AM and CsA doses were calculated according to their weights. Appropriate doses of CsA solution were diluted with 0.5 ml of normal saline solution in a 1 ml syringe and released directly into deep buccal cavity of the piglets to make sure cyclosporine was swallowed down. CsA was administrated orally, divided by two half-doses at 9:30 AM and 5:30 PM. On the last d (d14), only a single half-dose of CsA was administrated at 9:30 AM.

2.4. Blood Sampling and Analysis

The blood samples were drawn from the axillary vein of the anterior limbs. Collected samples were not more than 1% of circulating blood volume according to the practice guide to the administration of substances and removal of blood published by Diehl et al. [351].

CsA blood concentrations were measured just before drug administrations in the morning of d0, 2, 4, 9 and 14, to determine the changes of the trough concentrations. By d14, to determine the area under the curve (AUC), blood samples were collected every hour from 1 to 10 hours (h) after CsA administration. In addition, at random timing spare samples were also performed from d7 to d12 in order to complete the information on pharmacokinetic profiles. CsA whole blood concentrations were measured by Biomnis (Lyon, France) using the antibody-conjugated magnetic immunoassay (ACMIA) method on the Dimension® RxL Max® Integrated Chemistry System (Siemens Healthcare Diagnostics S.A.S., Saint Denis, France). The coefficient of variation (CV) for this measurement was $\pm 5\%$. In parallel, blood samples at d0, 2, 4, 7, 9, 11 and 14 were used to evaluate renal function based on the serum Cr and BUN concentrations measured with a KONELAB KL20 ISEND automatic analyzer (Thermo Clinical Labsystems, Vantaa, Finland).

2.5. Kidney Histopathology

By d 15, euthanasia was performed by heart injection of 5ml pentobarbital Sodium (Dolethal, Vétoquinol, Paris, France). Kidneys were removed and histological analysis was performed using a scoring system as previously described [343].

2.6. Statistical Analysis

CsA-AUC_{0-10h} was computed using the linear trapezoidal rule. The impact of CsA doses on the body-weight increase, CsA trough concentrations, Cr and BUN was tested through an analysis of variance (ANOVA) with an interactive model including days as a numeric variable. To assess the proportionality of CsA dose on trough concentrations and AUC of CsA at d14, a Pearson correlation test has been performed. This test was also used to determine the correlation between the increase of body weight and Cr, and the correlation between the increase of BUN and Cr. The significant threshold was set at 5% for all tests. Statistics has been carried out using R statistical program (version 2.15.2).

3. Results

3.1. Clinical Side Effects

CsA was tolerated by the piglets in different groups without severe adverse events. Hirsutism, inflation in testicles and mammary glands were observed in all treated piglets since the second week of treatment. No sign of dermatitis was observed. All piglets survived except the smallest piglet (Pig 1C) which presented osteoarthritis of the ankle and very slow body-weight growth was euthanized during experiment on d9. Bone biopsy showed severe infection and necrosis in the anterior left ankle joint. Among the other piglets, a very significant increase ($p<0.001$) in body weight was observed with growing rates between 108% and 159% from the initial body weight, with a median rate of 137% on d14. There was no significant difference of body weight increase among the 3 CsA groups of piglets whatever the dose was (4, 8, and 12 mg/kg/d), or between treated groups and the control group.

3.2. CsA Trough Concentrations

In our experiment, the CsA blood concentration began to be detected after 2 d of drug administration.

The Figure 1-A showed the results of CsA trough concentrations on d2, 4, 9 and 14. We observed a significant higher CsA trough concentrations at d 14 compared to d 2 ($p=0.019$) in 7 out of 8 cases with an increase ranging from 7% to 106%. Stabilization of CsA trough concentrations was observed since d4 in all cases except piglet 3C. On d14, trough concentrations were significantly different within the 3 CsA doses groups ($p=0.04$) and were correlated ($r=0.75$) to the CsA doses (Table 2).

CsA-Figure 1: Pharmacokinetic profiles of oral CsA in neonatal swines.

(A) CsA trough concentrations in 3 groups of 8 piglets on d2, 4, 9 and 14. Stabilization of CsA trough levels were observed since d4 to d14 ($p=0.18$). (B) CsA blood concentration versus time curves (C-T curves) in 3 groups of 8 piglets monitored for 10 h on d14. The C-T curves were flat. No classic period containing an obvious, single absorption peak and a subsequent consecutive clearance trough was observed.

3.3. CsA Concentration versus Time Curves and AUCs

CsA concentrations were detected from 0 to 10h on d14. The Figure 1-B shows the results of CsA concentration versus time curves (C-T curves) on d14. AUC_{0-10h} were significantly different within the 3 CsA doses groups ($p=0.04$) and they were also correlated ($r=0.86$) to the CsA doses (Table 2).

The C-T curves were unusual, flat with no peak or time-to peak (T_{max}) observed. There was no classic period containing an obvious, single absorption peak and a subsequent consecutive clearance phase in these piglets; instead, multi-waved curves can be observed in most of them.

Table 2: CsA Pharmacokinetics in neonate piglets

CsA levels		1A	1B	2A	2B	2B'	3A	3B	3C
Trough concentrations (ng/mL)	Day 2	522	437	591	1373	941	733	1696	1914
	Day 4	597	903	619	1522	977	920	1493	2436
	Day 9	563	1039	1396	1352	1590	1355	1891	3046
	Day 14	692	682	1215	1269	1117	1434	1821	3379
Cmax (ng/mL)	Day 14	736	752	1484	1364	1689	2307	3123	4310
AUCs (ng·h/mL)	0 - 4h	2707	2721	5195	5251	5265	7846	11315	15460
	0 - 10h	6757	6685	13780	12317	14198	18589	28773	37429

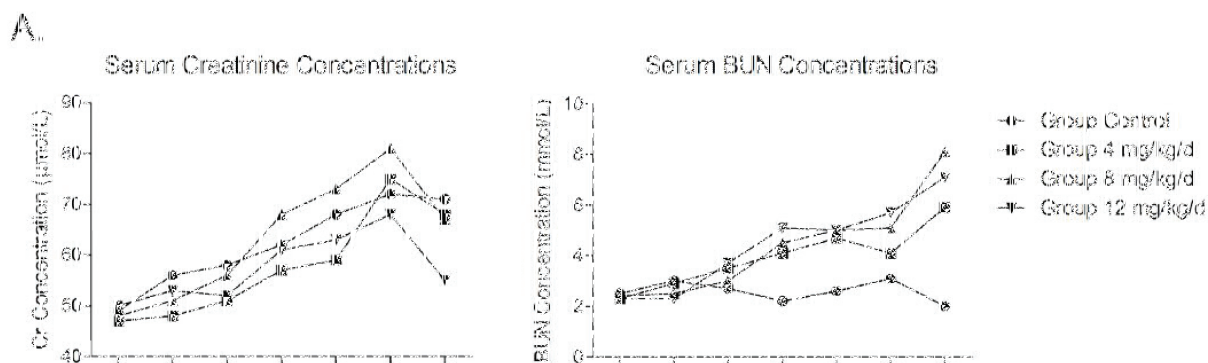
Table 2: CsA pharmacokinetics results in neonatal piglets (data was expressed in Mean \pm SD). The trough levels and AUC_{0-10h} on D14 were correlated ($r=0.75$ in trough levels and $r=0.86$ in AUC_{0-10h}) with the CsA doses among 3 groups.

CsA-Table 2: CsA pharmacokinetics in neonatal piglets.

3.4. Acute Renal Effects

The serum Cr and BUN concentrations on d0, 2, 4, 7, 9, 11 and 14 are shown respectively in Figure 2-A. At d0, the baselines of serum Cr before drug administration were between 40 to 50 $\mu\text{mol/L}$. During the experiment, the values of Cr increased significantly from d0 to d14 ($p < 0.001$) except in Fig 1C. The increase of Cr concentrations was equivalent among these groups ($p = 0.15$). In all animals, the increases of serum Cr concentrations appeared to be correlated with the body weight increase ($r = 0.84$, $p = 0.0002$). The baselines of BUN in most of the pigs were less than 3.0 mmol/L. In control group, serum BUN concentrations remained stable through the experiment. Compared to control group, significant increase of serum BUN concentrations in CsA-treated groups were observed from d4 and the values continued to increase during the 15-d course of experiment ($p < 0.00001$). On d14, the mean values of serum BUN in CsA-treated groups were approximately 3~4 folds as that of control group. Thus, the ratio of BUN/Cr increased during CsA treatment. Among 3 CsA-treated groups, the increases of serum BUN were not dose-dependent; however, from d7 to d14, higher values of BUN were consistently observed in Groups 8 and 12 mg/kg/d compared to Group 4 mg/kg/d.

In kidney histological analysis (Figure 2-B), we did not observe any renal histological abnormality such as glomerulosclerosis, arteriolar hyalinosis, interstitial fibrosis or tubular atrophy after 15 d of CsA treatment.



CsA-Figure 2: Nephrotoxicity of oral CsA in neonatal swines.

(A) Means of the serum creatinine (Cr) and blood urea nitrogen (BUN) concentrations in 4 groups on d0, 2, 4, 9 and 14. The increasing of Cr level were equivalent among 4 groups ($p=0.035$). BUN level increasing of 3 CsA groups was significantly different ($p<0.00001$) from the control group with a stable BUN level. **(B)** Kiney histological examination of all groups (stained with haematoxylin and eosin, X100) showed no abnormal changes such as glomerulosclerosis, arteriolar hyalinosis, interstitial fibrosis or tubular atrophy.

4. Discussion

Our study described the different pharmacokinetic profiles and renal effects of oral CsA in newborn pigs. It is interesting to compare our results to those obtained from adult or juvenile pigs. It has been described by Frey et al. [352] that in order to obtain the same blood concentration of CsA, adult pigs required about 2-folds intravenous dose and about 4-folds oral dose of CsA than adult patients, because of higher volume of distribution and lower bioavailability of CsA in pigs than in human patients. This conclusion was confirmed by the study of Cibulskyte et al. [342]; in juvenile pigs, 5 d of CsA treatment at 30 mg/kg/d was necessary to reach a trough concentration of 475 ng/ml, a peak concentration of 914 ng/ml and an $AUC_{0-11.5h}$ level of 6275 ng·h/ml. Our results in newborn pigs showed oral CsA of 4 mg/kg/d was necessary to reach a trough concentration at 400-800 ng/ml, with an AUC_{0-10h} level of d14 at 6721 ng·h/ml. Thus, to reach a comparable CsA trough level and AUC, newborn pig might require much lower CsA dose than juvenile pigs.

The pharmacokinetic profiles of CsA in newborn pigs were also different from the results observed in juvenile pigs [342] or in pediatric patients [353] where the distribution and clearance of CsA are well described by a 2-compartment model. In neonatal pigs, the C-T curves on d14 appeared flat and we did not observe any peak. For this reason, the AUC_{0-10h} was highly correlated with the trough levels and we had difficulties to analyze other pharmacokinetic parameters (CsA absorption time, T_{max} and half-life).

These pharmacokinetic profiles should not be caused by CsA accumulation after administration of a high dose during a long period of time because CsA trough concentrations in the 3 groups were highly relevant and proportional to CsA doses. Moreover, a stabilization of CsA trough concentrations since d4 was achieved. In addition, at random timing spare samples were performed from d7 to d12 that confirmed this CsA level stabilization.

Probable explanations of these special pharmacokinetic profiles include: (a) newborn pigs with breast-feeding may have improved CsA absorption compared to juvenile/adult pigs with formula feeding (CsA mixed with food) [354, 355]; (b) lipid profile of breast-feeding

newborn pigs may have changed the distribution of CsA and improved CsA blood concentration [356, 357]; (c) newborn pigs on unrestricted breast-feeding may have altered absorption and clearance phases of CsA.

Renal impairment is a well-known complication of CsA treatment in human patients. In contrast, CsA nephrotoxicity was not evident in swines. In juvenile pigs after 5 d of CsA treatment at a dose of 30 mg/kg/d [342], significant increase of serum Cr was observed, but values remained within normal range; besides, decrease of glomerular filtration rate in the same group was revealed as non-significant.

In our study, acute renal effect was evaluated only by serum Cr and BUN concentrations. RBF and GFR were not assessed because of the difficulty to perform these examinations and to compare these parameters in piglets with high body weight variability [342]. In all piglets, the values of Cr increased significantly from d0 to d14 with no significant difference within the 4 groups. In parallel, a very significant growth of body weight was also observed in all piglets except Pig 1C. In newborn pigs, the increase of serum Cr may probably be dominated by muscle growth and did not reflect an acute renal toxicity of CsA. We thus concluded that CsA treatment had no impact on body weight growth and serum Cr increase. On the other hand, BUN increased significantly in all CsA treated piglets compared to control group of which BUN level kept stable. No evidence showed that higher dose of CsA resulted in higher BUN levels, corresponding to the results from human patients [358]. CsA causes more pronounced increase in serum BUN than in the Cr concentration, this is a common finding in clinical and experimental studies [359, 360]. These results suggest that cyclosporine may have selectively reduced urea clearance. However, underlying mechanisms of this disproportionate BUN increase are relatively not clear. Laskow et al. explained this phenomenon by decrease in GFR and more significantly, CsA causes increase in urea reabsorption [361].

In addition, we did not observe any abnormal change in the renal histological examination after 15 d of CsA treatment, it might be due to the non-significant CsA nephrotoxicity, or was perhaps because of the short-term drug exposure. Similarly, Dean et al. [362] who have treated pigs with high dose of CsA at 100-140 mg/kg/d given orally for 2 weeks to reach CsA trough concentration of 1500 ng/mL, did not observe any changes in

renal histology; the serum Cr concentrations were not adversely affected in spite of the high dosage. Cybulskyte et al. administered juvenile pigs with orally CsA at 30 mg/kg/d for 4 weeks but no abnormal changes were found in renal histological examination [342].

Thus, oral CsA treatment range from 4 to 12 mg/kg/d for a 15-d course is safe for newborn pigs in spite of very high CsA blood concentrations were reached. Nevertheless, mild and compensable decreases in RBF and GFR probably existed and need to be confirmed in future study.

Another main purpose of this present study was to define the appropriate dose and monitoring strategy of CsA for immunosuppressive treatment in newborn pigs which can serve as an excellent large-animal model for neonate and pediatric transplantation. In practical use, CsA is often dosed by target trough levels, correlated to a range of CsA dosage according to weight. However in previous studies, there was no definitive knowledge on the appropriate trough level, dose and toxicity of CsA for transplantation in newborn pig model. Concerning the immunosuppressive treatment, we only have data in (1) adult or juvenile swine models [272, 342, 363] and (2) in pediatric transplantation patients [364-366]. The oral CsA doses we choose for newborn pigs was in the range of 4~12 mg/kg/d. This choice was based on the CsA doses used in adult pigs and in human pediatric patients. We discovered that newborn pigs required much lower oral dose of CsA achieved comparable trough level or AUC levels than adult pigs. In newborn pig, CsA blood level required by immunosuppressive treatment can be achieved with an oral dose of only 4 mg/kg/d. Although during 14 d of treatment, higher doses of CsA at 8 and 12 mg/kg/d did not result in more serious acute renal effects, considering long-termed side effects on kidney and other organs such as testicle and mammary glands, we recommend a dose of CsA not superior to 4mg/kg/d for immunosuppressive treatment in newborn piglets. For CsA monitoring strategy in newborn pigs, because of the flat C-T curves, trough levels were highly correlated with AUC. Thus, CsA trough level wonderfully indicates the drug exposure during 24h.

III. Characterization of Rabbit anti-Pig Thymocyte Globulin in Neonatal Swines

III.1. Background

Creation of hematopoietic chimerism in solid organ transplant recipients by donor-derived hematopoietic stem cells infusion has been proved to be a promising solution for donor-specific tolerance induction [167, 367].

However, the underlying mechanisms of tolerance induction through multilineage chimerism are not completely understood. The qualitative and quantitative profiles or thresholds of multilineage chimerism needed for tolerance induction still lack of clear illustration. Besides, peripheral blood chimerism, no matter macro- or micro-chimerism, serves as a “window” for chimerism state in recipients; however, it has also proved that detectable (by flow cytometry or PCR protocol) permanent or long-term peripheral blood chimerism is not indispensable for maintenance of tolerance [167, 368]. In other words, transient peripheral blood chimerism may be adequate for induction of tolerance; thereafter, the maintenance of tolerance might largely depend on the engraftment of donor-derived immunocompetent cells in other immunologic tissues or organs, such as lymph nodes, spleen or thymus. Moreover, establishment of permanent peripheral blood chimerism in large-animals and human patients will encounter larger barrier than in rodents, thus will require a more intensive conditioning therapy which is not applicable in clinical solid organ transplantation. Therefore, the present study aims at creating a transient peripheral blood chimerism by donor-derived bone marrow cells transplantation under a clinically applicable, relatively non-toxic conditioning regimen.

Compared to total body irradiation, total lymphoid irradiation or thymic irradiation, T-cell depleting agents such as anti-thymocyte globulin (ATG), anti-CD52 antibody or CD3-immunotoxin [334, 369] have less toxic effects and have been shown to be able

to improve donor-derived cells engraftment and the outcome of tolerance induction to solid organ transplant in large-animals [368, 370]. In human hand transplantation, bone marrow cells infusion under T-cell depletion based induction therapy by alemtuzumab (anti-CD52 antibody) has also resulted in steadily tapering of immunosuppressive therapy [278]. In the world first face transplantation, T-cell depletion based induction therapy by ATG was also administered [3, 277].

In such background, we decided to develop an tolerance induction protocol based on T-cell depleting agents and other traditional immunosuppressants (such as CsA), combined with bone marrow cells transplantation. We hypothesized that in neonatal pigs which have a premature immune system, this less intensive induction protocol would be adequate to create a chimerism state for tolerance induction. The rabbit anti-pig thymocyte globulin (pATG) used in the present study is a special product fabricated by Genzyme with the same procedure as Thymoglobulin (ATG).

ATG is polyclonal antibody widely used in induction therapy and treatment of acute rejection for solid organ transplantation, in conditioning regimen and treatment of acute graft-versus-host disease (GVHD) for hematopoietic stem cell transplantation (HSCT), and the treatment of autoimmune diseases such as severe aplastic anemia (AA) [371, 372].

ATG consists of a mixture of different antibodies that interact with immune response components, adhesion molecules and cell trafficking molecules thus is able to induce a variety of depleting and non-depleting effects such as modulation of cell surface molecules [373].

T-cell depletion is the primary immunosuppressive effect of ATG. ATG induced T-cell depletion can be observed in peripheral blood, secondary lymphoid organs and bone marrow. The mechanisms of T-cell depletion effect had been well characterized not only by intensive clinical in vitro and in vivo studies, but also by thorough experimental studies on non-human primate model and murine model [371, 374, 375]. Now it's clear that ATG can eliminate T cells via different mechanisms including complement dependent cytotoxicity (CDC), antibody dependent cell-mediated cytotoxicity (ADCC) and Fas-dependent and -independent apoptosis, according to the

variety of ATG concentration range, different lymphocyte subsets and resident compartments [373].

Therefore, T-cell depletion effects of ATG may be dependent not only on the ATG dosage but also on the immunological status such as compliment level and function, phagocytic cells function of the patients in whom ATG is administrated, etc. We also hypothesize that the defect of neonatal immune system, especially of the innate immune, may impair the ATG depleting effect and result in a low response to ATG treatment, thus compared to older pediatric and adult patients, neonatal patients may require higher dose of ATG, as well as proper adjuvant therapies in order to achieve and maintain complete T cell depletion.

III.2. Publication

Characterization of Rabbit anti-Pig Thymocyte Globulin in Neonatal Swines

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Abstract

Background: Antithymocyte globulins (ATG) have been used for decades in different clinical applications (conditioning for allogeneic stem cell transplantation, prevention or treatment of acute rejection in organ allografts). The immunosuppressive activity of ATG has been widely characterized in human, non-human primate and murine models, demonstrating that ATG can deplete and interfere with the function of several immune effectors. However, study of ATG in swine, an excellent model for allotransplantation, has never been performed.

Methods: Experiments were conducted in neonatal pigs using rabbit anti-pig thymocyte globulins (pATG, Genzyme product) at different doses (2.5, 5, 20, 40 and 80 mg/kg). Mechanisms of action of pATG were monitored *in vitro* on peripheral blood mononuclear cells (PBMCs).

Results: pATG treatment induced a dose-dependent lymphopenia in the blood and lymph nodes, spleen, as well as thymus. No severe neutropenia was observed. Interestingly, reconstitution of T lymphocytes was initiated immediately in after pATG-induced lymphopenia, and could be completed within 3 days in blood. Pharmacokinetic studies showed dose-related pATG binding to remaining lymphocytes and neutrophils, but levels of free antibodies in circulating blood decreased rapidly. *In vitro*, pATG was shown to bind to lymphocytes and neutrophils in a dose-dependent manner; and to kill PBMCs by apoptosis and complement-dependent mechanisms.

Conclusion: The results demonstrated that T-cell depletion can be achieved in pig blood and lymphoid tissues at high doses of pATG. Short pATG treatment could therefore be used as novel T-cell depleting regimen in swines. Moreover, this study also provided clues for ATG use in newborns.

Keywords:

Anti-thymocyte globulin; T-cell depletion; transplantation; neonates; swines

1. Instruction

Rabbit or horse anti-human thymocyte globulin (ATG) consists of a mixture of different antibodies that interact with immune response components, adhesion molecules and cell trafficking molecules thus is able to induce a variety of depleting and non-depleting effects such as modulation of cell surface molecules [376]. ATG induced T-cell depletion, which can be observed in peripheral blood, secondary lymphoid organs and bone marrow, is the primary immunosuppressive effect of ATG [371].

ATG induced T-cell depletion is widely used in induction therapy and acute rejection treatment of solid organ transplantation (SOT) [377], hematopoietic stem cell transplantation (HSCT) [378] and vascularized composite allotransplantation (VCA) [379], as well as in the treatment of autoimmune diseases such as severe aplastic anemia (AA) [380]. In recently performed clinical trials, T-cell depletion also serves as an important strategy for donor-specific tolerance induction through development of chimerism in SOT [381].

In experimental studies of transplant tolerance induction, in order to mimic the clinical protocol, Thymoglobulin or murine ATG have been administered in non-human primates [371] and mice [375]. Non-human primate model can best reproduce human immune system, but it's often limited by animal cost and availability. Mouse model permits a thorough study of mechanism of ATG because of variability of murine model; however, knowledge from murine model often has limit utility in clinical practice because of the disparate immunological characters compared to human [318].

Swine is an excellent model for transplantation immunology research program because of its similarities to human on immunogenetics profiles and body size [323, 346]. Especially, several strains of inbred miniature swines have been developed and used in preclinical tolerance induction studies [322, 382]. In previous studies, T-cell depletion in swines was often induced by total body irradiation [383] and/or thymic irradiation [384] which are not routinely used in clinical SOT studies. From this viewpoint, a specific surrogate of Thymoglobulin for swine model will help to better mimic clinical protocol.

In this present study, we evaluate the effects of a rabbit anti-pig thymocyte globulin (pATG) in neonatal swines. This pATG is produced by Genzyme (Marcy l'Etoile, France) under the same procedure as Thymoglobulin. The choice of neonatal swines is related to our ongoing study of tolerance induction in neonatal swine model, which aims at tolerance induction for solid organ or vascularized composite allografts in human neonates and very young infants. Thus, this study also devoted to evaluate whether ATG may function via different mechanisms in neonates which have a relatively immature immune system.

2. Materials and Methods

2. 1. Animals

Newborn (5~6 days of age) Youna domestic piglets were housed together with sows under the standard circumstance for neonatal pigs in the animal research center of Institute Claude Bourgelat (VetAgro Sup Campus-Vétérinaire, Marcy l'Etoile, France). The sows were fed with a standard pig diet. Newborn piglets were breastfed exclusively and unrestrictedly. For *in vitro* studies, blood samples were collected from newborn or 3~6 months' old juvenile pigs. All animal experimentations were approved by the Ethical Committee of the Veterinary Campus of Lyon (Number – 2012/1205, 1257).

2. 2. pATG Production, Administration and Treatment Schedule

pATG was produced by Genzyme, following the same process used for Thymoglobulin®. Briefly, New Zealand white rabbits were immunized twice with thymocytes from 3~6 months' old Youna domestic pigs, and terminally bled 2 weeks following second immunization. Total rabbit IgG from the resulting serum was purified, and a ready to use liquid pATG solution containing 5mg/ml of purified IgG was prepared. Purified polyclonal rabbit IgG (AbD Serotec, France) was used as isotype control.

The pATG was administrated under general anesthesia. Briefly, intramuscular premedication (ketamine 10 mg/kg, xylazine 2 mg/kg) was administered 10 minutes before anesthesia induced with 5% isoflurane by facial mask and maintained with 2%

isoflurane by orotracheal intubation. A proper dosage of pATG according to animal body weight was diluted with isotonic sodium chloride and glucose solution to a total volume of 25ml, and then was slowly infused intravenously via epigastric vein or axillary vein during 2 hours (h) by means of a catheter and an automatic syringe. Continuous pulse oximetry, capnography and rectal temperature were monitored during the administration.

For *in vivo* study, pATG was dosed by body weight; otherwise, newborn swine body surface area was also taken into account according to the formula from DeRoth et al. [385]. Piglets were randomized into 5 groups according to doses of pATG, and administered with different amounts of pATG: Very Low Dose (VLD, 5 piglets), administered with 0.25/1/1.25 mg/kg/day on days 0/1/2 respectively; Low Dose (LD, 5 piglets), administered with 0.5/2/2.5 mg/kg/day on days 0/1/2 respectively; Median Dose (MD, 4 piglets), administered with 10 mg/kg/day on days 0/1; High Dose (HD, 4 piglets), administered with 20 mg/kg/day on days 0/1; Very High Dose (VHD, 4 piglets), administered with 40 mg/kg/day on days 0/1. The control group (Con, 8 piglets) received 25 ml of NaCl and glucose solution per day on days 0/1/2. Besides, 1mg/kg/day of 6-methylprednisolone was injected intravenously during the infusion of pATG or placebo.

Peripheral blood samplings were performed on days 0/1/2 before infusion of pATG and on days 4/7/10/14/17/20. Blood cell count was performed using a Sysmex 2000iV automation following a specific veterinary protocol (Laboratoire Biomédicale Biovelys, VetAgro Sup). Hepatic and renal functions were evaluated by measuring serum levels of aspartate aminotransferase (AST)/alanine aminotransferase (ALT)/creatinine (Cr) and blood urea nitrogen (BUN) using a KONELAB KL20 ISEND automatic analyzer. Four piglets (1Con, 1MD, 1HD and 1VHD) were killed on day 6 and 11, respectively, to collect lymphoid organs (lymph nodes, spleen and thymus). All animals were euthanized on day 21.

2. 3. Analysis on Blood and Lymphoid Tissues by Flow Cytometry

Peripheral blood samples were collected at the indicated times and proceeded to analysis of the alteration of lymphocyte subsets including total T and B cells, cytotoxic

T cells (T_c), helper T cells (T_h) as well as regulatory T cells (T_{reg}). Antibodies used for this analysis were listed in Table 1. To analyze lymphocyte subsets, 25 μ l of blood was centrifuged and incubated with anti-CD3 ϵ , CD21, CD4a and CD8a, then lysed red blood cells, washed twice with FACS buffer and proceeded to flow cytometer. Gate of Lymphocytes was set according to their light scatter signals in FSC-SSC dot plot. Different lymphocyte subpopulations were chosen for analysis by phenotypes according to Gerner et al. (Dev Comp Immunol 2009). T cells were identified as CD3 ϵ^+ CD21 $^-$, B cells were identified as CD3 ϵ^- CD21 $^+$. CD3 ϵ^+ CD21 $^-$ cells were discriminated by their expression of CD4a and CD8a either as CD4a $^+$ CD8 $^-$ rest T helper cells (rT_h), CD4a $^-$ CD8 high cytotoxic T cells and CD4a $^+$ CD8 $^+$ double positive T cells (dpT) of which the exact function has not been identified. To analyze regulatory T cells, 25 μ l of blood was incubated with anti-CD25 (Alexa Fluor® 647 labeled), CD3 ϵ and CD4a, then performed the intracellular staining of Forkhead Box P3 (Foxp3) staining and proceeded to flow cytometer. Regulatory T cells were identified as CD3 ϵ^+ CD4a $^+$ CD25 high Foxp3 $^+$ cells and expressed by their percentage in CD3 ϵ^+ T cells or their absolute count. Percentages for lymphocyte subsets were converted to absolute cell numbers per μ l of blood according to lymphocyte count in blood cell count.

Proper sizes of lymphoid organs (mandibular lymph nodes, spleen and thymus) were collected and weigh on the balance. Tissues were minced with sharp scissors and pound to release the cells. Cell suspensions were filtered through 100 μ m nylon cell strainers (BD Falcon™, France), centrifuged and red blood cells were lysed. Cellular concentrations were evaluated using BD Accuri C6 flow cytometer, and analysis of pATG binding and lymphocyte subsets were performed.

To evaluate the *in vivo* cell-bound pATG to various cells, 25 μ l of heparinized blood (or cells from lymphoid tissues) were stained with goat anti-rabbit IgG (GARIG) coupled to FITC (AbD Serotec) at 4°C for 30min. Following red blood cell lysis, pATG binding was analyzed by FACS analysis (BD Accuri C6) after electronic gating of lymphocytes and neutrophils windows. The binding of pATG and IgG was expressed by value of Median Fluorescence Intensity (MedFI). Circulating lymphocyte- and

neutrophil-specific pATG in serum were also determined for MD, HD and VHD groups. Briefly, peripheral blood cells were incubated with serum at 4°C for 30min before GARIG staining and FACS analysis (described above). Cell-bound pATG on lymphocytes and neutrophils was expressed by MedFI value. Subsequently, MedFI was qualified to be a relative concentration value with a standard curve defined by pATG binding test. The ranged of pATG concentrations used to define the standard curve was among 0.001 and 25 µg/ml.

Table 1 Antibody combinations used for flow cytometry staining

Specificity	Clone Name	Ig Isotype	Labelling	Provider	Cat. No.
<i>Conjugated Abs</i>					
Pig CD3ε	PPT 3	Mouse IgG1,κ	FITC	Clinisciences	4510-02
<i>Isotype</i>	N/A	Mouse IgG			0107-02
Pig CD4a	74-12-4	Mouse IgG2b, κ	PE-Cy7	BD Biosciences	561473
<i>Isotype</i>	27-35		PE-Cy5.5		558304
Pig CD8a	76-2-11	Mouse IgG2a, κ	Alexa 647	BD Biosciences	561475
<i>Isotype</i>	G155-178				557715
Human CD21	B-ly4	Mouse IgG1,κ	PE	BD Biosciences	555422
<i>Isotype</i>	MOPC-21				555749
Mouse/Rat Foxp3	FJK-16s	Rat IgG2a, κ	PE	eBioscience	72-5775-40
<i>Isotype</i>	eBR2a				12-4321
<i>Non-conjugated Abs</i>					
Pig CD25	K231.3B2	Mouse IgG1		AbD Serotec	MCA1736
<i>Isotype</i>	N/A				MCA928
Mouse IgG1 label kit			Alexa 647	Life Technologies	Z25008

pATG-Table 1: Antibody combinations used for flow cytometry staining.

2. 4. Rabbit IgG (ELISA) and Anti-rabbit IgG Antibody Response

Free circulating pATG was detected by immunoenzymatic assay (ELISA). Ninety six-well microtiter plates were coated with goat anti-rabbit IgG (Jackson ImmunoResearch, Britain). After incubation of pig sera (1/20~1/100 dilution), immune complexes were revealed with alkaline phosphatase-conjugate goat anti-rabbit IgG (Jackson ImmunoResearch) followed by development with p-nitrophenyl phosphate (CliniSciences, France). Absorbance was measured at 405 nm using an ELISA plate reader. Similar procedure was applied to detect anti-rabbit antibodies, except that plates were coated with diluted ATG and complexes were detected with alkaline

phosphatase goat anti-swine IgG (Jackson ImmunoResearch) and followed by p-nitrophenyl phosphate substrate (VWR).

2. 5. Immunohistological Analysis on Lymphoid Tissue Samples

Samples of inguinal lymph nodes, spleen, and thymus were frozen embedded in OCT compound sectioning medium (VWR) and stored at -80°C . Six micrometer sections were performed using a cryomicrotome (Leica), followed by overnight drying at room temperature. The analysis was performed according to standard Immunohistochemistry (IHC) protocol. Briefly, tissue sections were fixed in cold acetone for 10 min, immersed in 3% H_2O_2 for 10 min (to block endogenous peroxidase activity), saturated with PBS-1% BSA for 30 min, incubated for 30 min with biotin goat anti-rabbit IgG, washed and further incubated with peroxidase labeled streptavidin and finally revealed with DAB substrate kit (all from BD Pharmingen). Slides were counterstained with haematoxylin for 3~5 seconds, followed with bleuing under running tap water for 30 min. Sections were at last mounted in glycerol gelatin mounting medium.

2. 6. *In vitro* pATG Binding and Blocking Assays

In vitro pATG binding assay on blood lymphocytes and neutrophils was performed by similar protocol to that used for *in vivo* pATG binding assay, with an initial incubation of 25 μl heparinized blood in the presence of different concentrations of pATG or control rIgG before GARIG staining and FACS analysis.

For pATG blocking assay, PBMCs were isolated by standard density (1.077 g/ml) gradient centrifugation with PANCOLL Animal (Dominique Dutscher, France), and cultured in RPMI 1640 medium (VWR, France) supplemented with 10% heat-inactivated fetal bovine serum (VWR), 2mM L-glutamine (Dominique Dutscher), 20mM HEPES buffer (PAN-Biotech, Germany) and 1% penicillin/streptomycin (Life Technologies, France) in a humid atmosphere containing 5% CO_2 . To evaluate the blocking capacities of pATG on lymphocyte surface molecules CD3, CD4, and CD21, PBMCs from neonatal or juvenile pigs were incubated with indicated concentrations of pATG or control rIgG at 4°C for 30min. Cell surface molecule staining was then performed using anti-pig CD3 ϵ FITC, CD4a PE-Cy7, CD8a AF647 or anti-human

CD21 PE at 4°C for 30min. Blocking capacities of pATG (or control rIgG) were expressed by values of median fluorescence intensity (MedFI) analyzed by Accuri C6.

2. 7. *In vitro* Complement Mediated Cytotoxicity (CDC) Assay and pATG-induced Apoptosis Assay

To evaluate complement mediated cytotoxicity induced by pATG, PBMCs from neonatal or juvenile pigs were incubated with pATG or control rIgG at 4°C for 30min. Corresponding serum (the same origin with PBMCs) was used as source of complement components and added to the culture in 37°C, 95% humidity, and 5% CO₂ incubator for 30min. Cells were finally stained with Propidium Iodide (PI, BD Biosciences, France) and analyzed on BD Accuri C6 flow cytometer within 10~30 min.

To evaluate pATG-induced apoptosis, PBMCs of 5×10^5 cells/ml from neonatal or juvenile pigs were incubated with pATG or control rIgG at 37°C. pATG-induced cell apoptosis was detected by Annexin V (BD Biosciences) staining after 4h of culture, and cell lysis by PI staining after 6h of culture. Cells were analyzed on Accuri C6. To count the lysed PBMCs which had lost cell structure, PI⁻ cells in the gate of PBMCs on FCS-SSC plot after 6h of incubation. ATG-induced cell lysis was determined with the equation: % ATG-induced cell lysis = $(1 - \text{PI}^- \text{ cells concentration} / 5 \times 10^5 \text{ cells/ml}) \times 100\%$.

2. 8. Statistical Analysis

Data are expressed as arithmetical mean or mean \pm SD. To analyze the pATG depletive effects and pharmacokinetic profiles of *in vivo* study, counts of peripheral blood cells and lymphocyte subsets, or results of pATG concentrations, were tested by one-way analysis of variance (ANOVA) and multiple comparisons were subjected and least-significant difference (LSD) test [371, 375]. Correlation between depletion proportions and pATG doses was assessed by Spearman's rank coefficient (ρ). Variables were considered to be highly correlated when Spearman's ρ was >0.800 . For *in vitro* study, differences between means of two data sets (pATG-treated cells and non-treated cells, or differences between juvenile and neonatal pig cells) were analyzed by Student's t-test. A p value of <0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS Statistics Version 17.0.

3. Results

The pATG was tolerated by neonatal pigs in all treated groups without severe adverse events. However, in MD, HD, and VHD groups, mild fever and trembling were observed at D 0 and 1 after pATG infusion. No further abnormal clinical manifestation was observed among different groups thereafter.

3. 1. Alteration of Peripheral Blood Cell Counts (Figure 1)

In neonatal pigs of control group, lymphocyte count showed a natural increase during D 0 to 20 (age of 1 week to 4 weeks), in contrast to a natural decrease of neutrophil count and total WBC count. Meanwhile, natural increase of RBC count and HGB level were also observed. Besides, the monocyte and platelet counts in non-treated newborn pigs remained relatively stable. These natural dynamics of blood cell counts should be taken into consideration in the evaluation of pATG depletion effect.

Compared to lymphocyte count of Group Con, administration of pATG in neonatal pigs caused significant depletion in peripheral blood lymphocyte and monocyte in Groups MD, HD and VHD but not in Groups VLD and LD. In Groups MD, HD and VHD, maximum depletion of lymphocyte and monocyte was achieved at D 1. Compared to lymphocyte count at D 0 of the same group, proportions of lymphocyte depletion at D 1 was $29.52\pm 9.17\%$ in MD, $46.93\pm 6.25\%$ in HD and $56.09\pm 8.55\%$ in VHD. The lymphocyte depletion effect of pATG was shown to be dose-dependent among Groups MD, HD and VHD (Spearman's $\rho=0.828$). Compared to D 0, proportions of monocyte depletion on D 1 were $63.60\pm 19.04\%$ in MD, $82.34\pm 7.38\%$ in HD and $79.35\pm 15.38\%$ in VHD; while monocyte count kept stable in Group Con. At D 2, although only 24 h after pATG infusion in D 1, no continual reduction of lymphocyte and monocyte counts was observed in Groups MD, HD and VHD. In contrast, lymphocyte counts of these 3 groups increased to higher level than day 1 and returned to normal level at D 4; monocyte count returned to normal level at D 2.

Significant thrombocytopenia caused by pATG was observed in Groups HD and VHD at D 1 and D 2, with the decrease proportions of $48.65\pm 13.71\%$ and

-57.25±11.66%, respectively. Besides, compared to Group Con, none of the pATG-treated groups had significant decrease of neutrophil count, red blood cell (RBC) count and hemoglobin (HGB) concentration. Generally, from day 4 to day 20, peripheral blood cell counts of pATG-treated groups remained at comparable levels to those of control group.

pATG-Figure 1: Impacts of pATG on peripheral blood cell counts.

*Data are expressed by arithmetical mean. Blood cell counts of each treated group were compared with Group Con within the same day and significant difference was marked when a p value was <0.05. Significant difference was never observed in Groups VLD and LD. Triple asterisks (***) indicate a significant difference observed in all of MD, HD and VHD; double asterisks (**) refer to a significant difference in both HD and VHD; while single asterisk (*) signifies a significant difference only in Group VHD. Transient depletion of lymphocytes and monocytes was observed in MD, HD and VHD; depletion of platelets was observed in HD and VHD. After depletion, these cell counts increased rapidly and normal levels of cell counts were observed at day 4. No significant decrease was observed in RBC.*

3.2. Evolution of Peripheral Blood Lymphocyte Subsets (Figure 2)

In control group, B-lymphocyte count had increased spontaneously from $(0.34\pm 0.15)\times 10^3$ cells/ μ L to $(0.75\pm 0.22)\times 10^3$ cells/ μ L during observation ($p=0.002$),

while T-lymphocyte count showed a slight increase from $(2.97 \pm 0.97) \times 10^3$ cells/ μ L to $(3.55 \pm 0.86) \times 10^3$ cells/ μ L. In T subsets, proportion of $CD4^+CD8^- T_h$ cells decreased from $54.39 \pm 7.42\%$ to $43.62 \pm 5.88\%$ ($p=0.019$), belonged with steady increases of $CD4^+CD8^{high} T_c$ cells (from $13.30 \pm 3.23\%$ to $19.42 \pm 5.21\%$, $p=0.019$) and $CD4^+CD8^+$ DPT cells (from $6.75 \pm 1.88\%$ to $12.03 \pm 3.60\%$, $p=0.004$). Correspondingly, the ratio of T_h/T_c diminished from 4.38 ± 1.5 to 2.62 ± 1.15 (regardless of DPT cells). Notably, the percentage of $CD3^+CD4^+CD25^{high}Foxp3^+ T_{reg}$ cells gradually decreased from $3.56 \pm 0.44\%$ to $2.38 \pm 0.36\%$ ($p=0.001$).

Under pATG therapy, significant decrease of T-cell count was observed only in groups MD, HD and VHD. Maximum depletion of T cell was achieved at day 1. Compared to T-cell count of the same group at day 0, T-cell count reduction on day 1 was $29.36 \pm 17.63\%$ in MD, $53.81 \pm 10.43\%$ in HD and $69.45 \pm 10.78\%$ in VHD, indicating a dose-dependent T-cell depletion effect (Spearman's $\rho=887$). Such dose-dependent depletion effect was observed in all T cell subsets including $CD4^+CD8^- T_h$ cell, $CD4^+CD8^{high} T_c$ cell, $CD4^+CD8^+$ DPT cell and $CD3^+CD4^+CD25^{high}Foxp3^+ T_{reg}$ cells. However, T-cell depletion caused by pATG was transient; as compared to day 1, a rebound of T-cell count was shown in Groups MD, HD and VHD at day 2; normal level of T-cell count was regained at day 4 in these groups. Besides, after pATG infusion, no significant decrease in B-cell count was observed in all treated groups.

In reconstitution phase of T-cell subsets, compared to non-depleted Groups (Con, VLD and LD), Groups MD, HD and VHD showed lower proportion of $CD4^+CD8^- T_h$ cell and higher percentage of $CD4^+CD8^{high} T_c$ cell. Thus, after T-cell depletion, a decreased Th/Tc ratio of was observed in Group MD (1.69 ± 0.14), HD (1.75 ± 0.26) and VHD (1.51 ± 0.01) than Group Con (2.62 ± 1.15), VLD (2.93 ± 1.61) and LD (2.74 ± 0.88). Otherwise, after T-cell reconstitution, proportion of DPT cells in peripheral blood had no significant difference in Groups MD, HD and VHD compared to other groups. Compared to Group Con, absolute count and percentage of T_{reg} grew to normal level since D 4 in T-cell depleted groups. Thereby, no significant alteration of T_{reg} was observed in all pATG treated groups.

pATG-Figure 2: Analysis of lymphocyte subsets in pATG therapy.

(A) Analysis strategy of different lymphocyte subsets and T-cell subsets. (B) Analysis strategy of $CD4^+CD25^{high}Foxp3^+$ regulatory T cells. (C) Kinetics of lymphocyte depletion, repopulation and alteration of T_H/T_C ratio. Data are expressed by arithmetical mean. Cell counts and T_H/T_C ratio of each treated group were compared with Group Con of the same day and significant difference was marked when a p value was <0.05 . Significations of the asterisk markers (**, **, *) were similar to those used in Figure 1. In Groups MD, HD and VHD, pATG significantly depleted T-cell, but had no significant impact on B-cell count. Significant depletion can be observed in all T-cell subsets except $CD4^+CD25^{high}Foxp3^+$ T_{reg} . T-cell depletion was transient (only observed on day 1 and 2), thereafter T-cell count was rapidly restored to normal level. T_H and T_C showed different repopulation dynamics. T_H

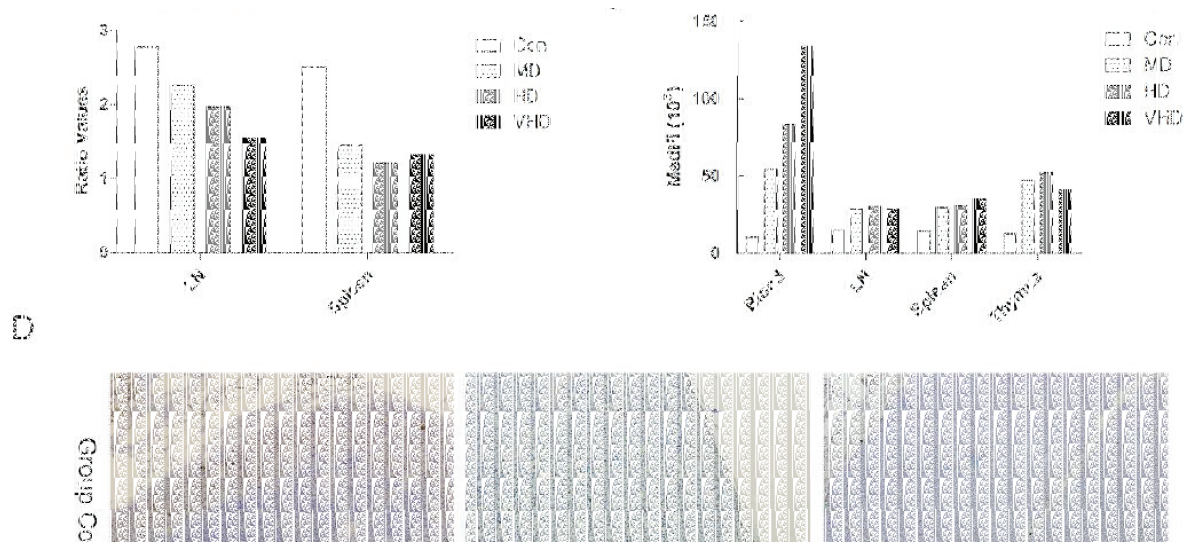
increased slowly after depletion, hardly recovered to normal level. In contrast, after regained normal count on day 4, T_c continued to increase to higher level in Group HD and VHD. Different repopulation dynamics of T_h and T_c resulted in a decrease of T_h/T_c ratio after T-cell depletion.

3.3. Lymphopenia and pATG Binding in Lymphoid Tissues (Figure 3)

In Groups MD, HD and VHD, lymphocyte depletion in LN, spleen and thymus was examined on D 6, 11 and 21. On day 6, lymphopenia was observed in LN, spleen and thymus. Lymphopenia persisted for longer term in LN, for which obvious lymphopenia was still exist at D 21. Otherwise, in spleen and thymus, the lymphocyte counts showed a gradually increase and were comparable to those in Group Con. In the thymus of Group Con, a noticeable result was that the lymphocyte count of day 6 was dramatically lower than day 11 and day 21. Such situation was not observed in LN or spleen.

The alteration of T-cell homeostasis after depletion was also evaluated in LN and spleen. Corresponding to peripheral blood, alterations of T_h/T_c ratio were also observed in LN and spleen. At D 21, T_h/T_c ratio in LN of Groups MD (2.26 ± 0.27), HD (1.97 ± 0.45) and VHD (1.54 ± 0.26) were lower than Group Con (2.78). Similar decrease of T_h/T_c ratio was also observed in spleen of Groups MD (1.45 ± 0.28), HD (1.21 ± 0.34) and VHD (1.32 ± 0.75) compared to Group Con (2.51).

The pATG binding effect in lymphoid tissues was measured by flow cytometer and further demonstrated by immunohistochemistry examination. The result of pATG binding was generally corresponding to pATG depletion effect. On day 6, significant bond pATG was observed in all 3 types of lymphoid tissues; dramatically, bond pATG on thymocytes was higher than those on lymphocytes in lymph node and spleen. On day 21, cell-bond pATG was still observed in all lymphoid tissues examined in Group MD, HD and VHD.



pATG-Figure 3: Lymphocyte depletion in lymphoid organs caused by pATG.

(A) Alteration of lymphocyte counts in lymphoid organs. By pATG therapy, reduced lymphocyte concentrations were observed on day 6 in all lymphoid organs including thymus. Lymphopenia was observed in mandibular lymph nodes (LN) until day 21; besides, in spleen and in thymus, lymphocyte concentration showed gradually recovery and approached to Group Con at day 21. (B) On day 21, LN and spleen of Groups MD, HD and VHD showed decreased T_H/T_C ratio after T-cell depletion. (C) Level of lymphocyte-bound pATG on day 6 in different tissues. Cell-bound pATG was detected with flow cytometer and expressed by MedFl. Lymphocyte-bound pATG was lower in lymphoid tissues than in

peripheral blood. Among lymphoid tissues, pATG binding on thymocytes was shown to be comparable with that on lymphocytes from lymph nodes and spleen. (D) Immunohistochemistry examination of Cell-bond pATG in lymphoid tissues of day 6. Cell-bond pATG was demonstrated as yellow staining in LN, spleen and thymus. In thymus, cell-bond pATG in medullary zone was more significant than that in cortical zone.

3.4. Kinetics of Cell-bond or Circulating pATG and Pig anti-Rabbit IgG Response (Figure 4)

In Groups MD, HD and VHD, lymphocyte- and neutrophil-bond pATG concentrations at day 1 were higher than those detected at day 2, although both of them were measured 24 hr after the same dose of pATG. These results were corresponding to the rebound of lymphocyte counts at day 2 in these groups, indicating the peripheral blood lymphocyte compartment was undergoing a restoration. The cell-bond pATG continued to decrease since day 2 and was still detectable at day 21. During day 4 to day 21, no significant lymphopenia was observed in peripheral blood despite the presence of cell-bond pATG.

Circulating pATG in serum was detected by specific and non-specific methods: cell-specific circulating pATG was detected by flow cytometer, while total circulating pATG (rlgG) was detected by ELISA. Different to cell-bond pATG, blood circulating cell-specific and total pATG had higher levels on d 2 than on d 1, indicating an accumulation of pATG in blood after the second dose of administration. Thereafter, both serum cell-specific and total pATG concentrations decreased rapidly to significantly lower level.

Notably, the serum total pATG was much lower than those observed in cynomolgus monkeys [371] or in mice [375] which have received comparable dose of ATG. For example, 24 hr after the second dose of pATG in Group HD (20 mg/kg/day), the serum total pATG concentration was 2.62 $\mu\text{g/ml}$, in contrast to more than 125 $\mu\text{g/ml}$ of serum total pATG concentration in mice at 24 hr after a single dose of mATG of 25 mg/kg [375]. Similar low concentration of circulating pATG was also confirmed by serum cell-specific pATG detection with flow cytometry.

ELISA of pig anti-rabbit IgG response was designed to examine whether administration of pATG had caused alteration in newborn pig serum IgG. Quantitation of absorb value was not performed because antibody used to coat plate was pATG (aimed at pig anti-pATG IgG, can also bind to other ordinary pig IgG) but not standard anti-swine IgG antibody. Results showed that in Group Con, pig serum IgG steadily decreased from D0 to D21. During the same period, pig serum IgG in Groups MD, HD and VHD demonstrated similar decrease kinetics and no significant alteration was observed. These kinetics were similar to serum total IgG kinetics in normal newborn pigs reported by previous study [386]. We thus concluded that pig anti-rabbit IgG response was not observed in newborn pigs after pATG administration.

pATG-Figure 4: pATG pharmacokinetics and pig anti-rabbit response in Groups MD, HD and VHD.

Data are expressed by arithmetical mean. (A) Peripheral blood lymphocyte- and neutrophil-bond pATG kinetics was examined by flow cytometer. Within these time-points, highest level of cell-bond pATG was observed at day 1, thereafter cell-bond pATG decreased rapidly. (B) Serum cell-specific pATG detection examined the kinetics of lymphocyte- and neutrophil-specific circulating pATG by flow cytometer. (C) Total serum circulating pATG was detected by ELISA. Low level of total serum

circulating pATG was observed even in Group VHD. (D) Pig anti-rabbit response in Groups MD, HD and VHD was measured by ELISA. Quantitation of absorb value was not performed. Compared to Group Con, absorb value in Groups MD, HD and VHD demonstrated similar decrease kinetics and no significant alteration was observed.

3.5. *In vitro* Characterization of pATG Effects (Figure 5)

In binding test, pATG showed high affinity to blood lymphocytes and neutrophils; quantity of pATG to these cells was dose-dependent. Generally, with the pATG concentration ranged from 5 to 250 $\mu\text{g/ml}$, MedFI values of pATG binding on lymphocytes had no significant difference between juvenile and neonatal pigs, except 25 and 250 $\mu\text{g/ml}$ of which MedFI values were higher on juvenile pig cells; MedFI values of pATG binding on neutrophils had no significant difference between juvenile and neonatal pigs at all concentrations, indicating that affinity of pATG to juvenile and neonatal pig cells were comparable.

In pATG blocking test, after incubated with pATG in the range of 5 to 50 $\mu\text{g/ml}$, lymphocyte surface molecules (CD3, CD4 and CD21) of both neonatal and juvenile pig PBMCs showed significantly lower values of MedFI compared non-treated PBMCs, indicating that pATG can effectively block these cell-surface molecules.

CDC assay showed significant difference between juvenile and neonatal pigs. Incubated with the same concentrations of pATG, equal volume of complement-containing plasma from juvenile pig lysed significantly higher percentage of PBMCs than plasma from neonatal pigs. Briefly, at low dose pATG of 5 $\mu\text{g/ml}$, juvenile pig plasma lysed $44.45 \pm 12.80\%$ of PBMCs and the rate of lysed cells reached the plateau (83.50~88.70%) since 25 $\mu\text{g/ml}$. In contrast, with newborn pig plasma only $8.50 \pm 5.71\%$ of PBMCs were lysed at 5 $\mu\text{g/ml}$ of pATG; rate of lysed PBMCs increased along with pATG doses and attained $64.33 \pm 15.45\%$ at 250 $\mu\text{g/ml}$ pATG.

Apoptosis assay showed that pATG can induce significant cell apoptosis even at 5 $\mu\text{g/ml}$ of pATG concentration. By increase of pATG concentration, corresponding increase of ongoing cell apoptosis was not observed; as in high pATG concentration, the apoptotic cells subjected to rapid, complete lysis of cell structure thus could no

longer be observed directly. The measurement of totally lysed PBMCs via ATG-induced apoptosis was performed at 6 hr of incubation. Results demonstrated that the pATG-induced apoptosis effect was highly dose-dependent ($p=0.967$ in juvenile pigs and $p=0.939$ in newborn pigs) and had not significant difference between juvenile and newborn pig PBMCs.



pATG-Figure 5: In vitro characterization of pATG.

Data are expressed by mean \pm SD. Single asterisks (*) indicates a significant difference ($p<0.05$) between neonatal pigs (NP) and juvenile pigs (JP). Double asterisks (**) signifies significant difference ($p<0.05$) between pATG treated peripheral blood mononuclear cells (PBMCs) and non-treated PBMCs. (A) In pATG binding test, lymphocyte- and neutrophil-bound pATG was comparable

between neonatal pigs and juvenile pigs (except significant difference of lymphocyte-bound pATG observed at the concentrations of 25 µg/mL and 250µg/mL). (B) In pATG CDC test, equal quantity of plasma from juvenile pig lysed higher percentage of PBMCs than plasma from neonatal pigs at all pATG doses. (C) In pATG cytotoxicity test, examination of the ongoing apoptotic cells showed that pATG had induced apoptosis in both NB and JP PBMCs even at low pATG doses; higher concentration of pATG had lysed more PBMCs, indicating that pATG-induced apoptosis effect was dose-dependent ($p=0.967$ in juvenile pigs and $p=0.939$ in newborn pigs). At the same dose, pATG induced comparable cell-lysis in JP-PBMCs and NB-PBMCs (except at 25µg/mL for which concentration pATG lysed more JP-PBMCs). (D) In pATG blocking test, significant pATG blocking effect on CD3e, CD4a and CD21 was observed in both neonatal pigs and juvenile pigs.

Discussion

This present study characterized the effects of rabbit anti-pig thymocyte globulin (pATG) which is a novel surrogate of Thymoglobulin[®] (Genzyme) for preclinical large-animal model; moreover, this study also described the impact of immature host immune system on the ATG-induced T-cell depletion effects.

As administration of pATG has never been reported in previous study, in present study pATG doses were arranged according to (i) Thymoglobulin doses administered in clinical studies [378, 387-389] and (ii) difference in body surface area versus body weight between neonatal normal pigs and adult human [385]. Besides, ATG dose choosing in experimental studies of Thymoglobulin (administered in cynomolgus monkeys) [371], as well as surrogate rabbit anti-murine thymocyte globulin (mATG) were also taken into account [375].

Great variation exists in the administration of Thymoglobulin for various therapeutic objectives according to numerous protocols from different medical units. Typically, described by Kho et al. , Thymoglobulin is administered for 3 days in induction therapy of kidney transplantation and total dose of Thymoglobulin was classed as ultra-lose dose (1.5 mg/kg), lose dose (3.0~6.0 mg/kg) and high dose (6.0~13.5 mg/kg) [389].

Analyzed by equation from DeRoth and Bisailon, relation of body surface area

versus body weight in neonatal swine was found to be comparable to that in New Zealand rabbit determined by Meeh-Rubner formula. Thus, similar to rabbit, in neonatal swine the ratio of body surface area versus body weight is 3-fold as that in human. Total dose of pATG infused in each treated group was 2.5 (VLD), 5 (LD), 20 (MD), 40 (HD) and 80 (VHD) mg/kg; if expressed by mg/m², should equals to 0.8 (VLD), 1.7 (LD), 6.7 (MD), 13.3 (HD) and 26.7 (VHD) mg/kg of ATG administered in human.

Administration of ATG at 0.8 mg/kg (0.2~0.3 mg/kg/day) is not currently used in clinics, but is sufficient to induced borderline immunosuppressive effect, as shown in cynomolgus monkeys [371]; infusion of ATG at 1.5 mg/kg (0.5 mg/kg/day) could result in depletion of peripheral T and NK cells for at least one week in kidney transplant patients [389]; ATG dose at 6.0~13.5 mg/kg is routinely used in induction therapy for solid organ transplantation and conditioning regimen of HSCT[389]; very-high ATG dose at 26.7 mg/kg is rarely used in clinical protocol, considering of the side-effects such as severe neutropenia and thrombocytopenia caused by ATG [371].

In present study, administration of pATG in neonatal pigs resulted in definite T-cell depletion in peripheral blood, however, only in Groups MD, HD and VHD, while T-cell count was never diminished in Groups VLD and LD. Thus, it indicated that neonatal pigs required higher dose of ATG than human patients or cynomolgus monkeys, if batch to batch variation of ATG effects is regardless. Besides, pATG administered in neonatal pigs was able to cause only a transient T-cell depletion even high-dose was given; furthermore, increase of pATG from high-dose to very-high dose did not receive dose-dependent longer term of depletion. After maximum T-cell depletion observed on D1, reconstitution of peripheral blood T-cell in neonatal pigs must have initiated rapidly within 24 hr and accomplished within 72 hr, as peripheral T-cell count showed an increase on D2 and regained normal level on D4. In contrast, by experimental studies of cynomolgus monkeys [371] and mice [375], full repletion of peripheral T cells did not achieved in these animal until 15~18 days after two dosed of Thymoglobulin or mATG.

This transient T-cell depletion effect caused by pATG should not be explained by

sequestration and release of T-lymphocyte between peripheral blood and lymphoid organs which is the typical mechanism of FTY720 [390, 391], because in present study, cell-bond pATG on peripheral lymphocytes was routinely examined, if sequestration and release of T cells did happened, on D4 and thereafter T cells bond with high pATG as on D1 should be detectable, by in fact, this phenomenon was never observed. Moreover, besides lymphocytes, monocytes and thrombocytes were also depleted in present study, which can only be explained by depletion effect of ATG and sequestration has never been reported as an effect of ATG. Notably, such transient T-cell depletion in pigs had also been documented by previous experiment: similar to our pATG study, intravenous infusion of porcine CD3-immunotoxin in miniature pigs of 12-week age transiently depleted T-cell, with normal T-cell level being regained within 2 to 3 days [334]. Thus, attempts of inducing T-cell depletion by pATG and porcine CD3-immunotoxin both resulted in transient outcome, suggesting that swines species, at least in neonatal and juvenile stages, have more powerful capacity in maintaining peripheral blood T-cell homeostasis than other species such as human, non-human primate or rodent.

As observed in studies of human patients and murine model, peripheral blood T-cell reconstitution after depletion can involve two pathways: thymic emigration of naive T lymphocytes into peripheral blood and homeostasis-driven proliferation of peripheral T cells [376, 392].

Porcine thymic reactivity may be firstly responsible for rapid reconstitution of peripheral T-lymphocyte within 2~3 days, because homeostasis-driven proliferation pathway may not be able to restore peripheral T-cell pool in such a short term. As shown by in vitro mixed lymphocyte culture, mitosis of porcine lymphocytes requires at least 3~5 days [393]. Besides, shortly after pATG administration in Groups MD, HD and VHD, that was from D2 to D4 in present study, homeostasis-driven proliferation related manifestation such as significant decrease or even reversed Th/Tc ratio was not observed; instead, an mild increase of Th/Tc ratio was noticed in Groups MD and HD.

Pig thymus is a long organ located both in the chest over the pericardium and

along the jugular groove associated with the carotid artery, as far as the pharyngeal region [322, 394]. Similar to human newborn infant, thymus is a relative large organ which can account for 0.5% of body weight in 4-week age newborn pig as examined in present study; then 0.13~0.35% (34.8~86.7 g) in young pigs with about 25 kg of body weight and 10 to 12 weeks of age [395]. It's also reported that in a young pigs of 26 kg body weight, the average number of lymphocytes was 3.21×10^{11} and 44% of them distribute in thymus (1.41×10^{11} thymocytes and 20-25% of them are produced by cell division each day), while only 3% in blood (9.63×10^9) [395]. In these young pigs, each day 1% of thymocytes (up to 15% of blood lymphocytes) can emigrate into peripheral blood under steady stage [394]. This routine thymic exportation can explain the rapid restoration of peripheral blood lymphocytes in young pigs experienced T-cell depletion by CD3 immunotoxin. Moreover, peripheral T-cell depletion may have stimulated and increased thymic exportation.

The total number and distribution of lymphocytes in 1-week age neonatal pigs is unavailable. Although they may have less thymocyte number in per gram of thymic tissue because of perinatal natural high level of corticosteroids which will suppress the development of thymocytes; but in contrast, higher ratio (up to 0.5%) of thymus weight versus body weight, as well as decreased lymphocyte count in per milliliter of blood (1/4 of that in young pigs). Thus, in present study, taking the parameters in young pigs as a reference, thymic emigration can also be the first explanation of rapid repletion of peripheral blood lymphocytes.

Another pathway of blood T-cell reconstitution after depletion by pATG is homeostasis-driven proliferation, which may have played important role since D4 or D7. It has been demonstrated that homeostasis-driven proliferation after T-cell depletion is associated with changes in the ratios of (1) $CD4^+$ to $CD8^+$ T cells and (2) naive to memory T cells [376]. In present study, rapid decrease of T_H/T_C from D0 to D2 can be explained by the finding that $CD8^+$ T cells are more resistant to depletion by ATG [396], as from D0 to D2 in T-cell depleted groups, proportion of $CD4^+CD8^-$ T_H in total T cells decrease rapidly, while proportion of $CD4^-CD8^+$ T_C remained relatively stable. Stable or mild increase of T_H/T_C observed from D2 to D4 or D7 should be due to

higher proportion of CD4⁺ cells in thymic emigration as mentioned in paragraphs above. However, since D4 (VHD) or D7(MD and HD), T_h/T_c showed a sharp decrease, which became stable since D14 to end to observation. At the meantime, in blood as well as in lymph nodes and spleen, the percentage of CD4⁺CD8⁺ DPT cells, which represent the memory and effector T cells proportion [397-399], increased significantly in Groups MD, HD and VHD. Alterations in both of T_h/T_c ratio and DPT cell proportion indicated that homeostasis-driven proliferation has participated in the restoration of peripheral T-cell pool.

Under pATG therapy, lymphopenia state in lymphoid organs persisted for longer term than in blood, especially in lymph nodes. These results may be explained by different T-cell depletion mechanism in different tissues. In blood, ATG induces lymphocyte depletion primarily by complement-dependent cytotoxicity (CDC) mechanism which largely depends on the dose of ATG administered and the density of antibody coating the surfaces of cells. High concentrations of Thymoglobulin (in the range of 100 to 1000 µg/ml), can activate the complement pathway and induce lysis of T cells in peripheral blood [376].

Lymphocyte depletion in the peripheral lymphoid tissues, such as the spleen and lymph nodes, occurs mainly by T-cell apoptosis. ATG concentrations in the range of 10 to 100 µg/ml have been shown to be able to trigger Fas/FasL dependent T-cell apoptosis [376, 400].

Lower concentration of ATG, in the range of 0.1 to 1 µg/mL, is able to induce lysis of T cells through antibody dependent cell-mediated cytotoxicity (ADCC) mechanism in lymphoid organ. As proved by mATG study, ATG induced ADCC is primarily mediated by macrophages [374, 376].

An important finding in present study was that pATG has induced significant thymocytopenia in the thymus of neonatal swines, as observed on D6; meanwhile, pATG binding on thymocytes was shown to be higher than those on lymphocytes in spleen and lymph nodes. These results were first observed by flow cytometry and subsequently confirmed by immunohistochemical examination. Thymocytopenia caused by ATG, as well as in vivo ATG binding on thymocytes had never been

reported in previous study. In thorough experimental studies of Thymoglobulin and murine ATG which were conducted in mature animals, ATG was proved to be unable to bind to thymocytes and cause T-cell depletion in thymus at any dose level, indicating that ATG has limited access to thymus, at least in mature cynomolgus monkeys and mice [371, 375, 376]. This limited access of ATG is due to blood-thymus barrier between blood and thymic tissue. The adult blood-thymus barrier consist continuous endothelial cell and complete basement membrane of endothelium inside of capillary, macrophages in peri-vessel space, basal lamina of epithelial reticular cells and processes epithelial reticular cells with desmosome [401].

Because of this barrier, it is difficult that large molecular substances, such as antigen and some medicine in blood pass into the parenchyma of thymus, especially in cortex, thus can provide a stable environment for lymphocytes development.

However, in neonatal stage, it has been proved that natural high level of corticosteroid, as well as prednisolone administered with pATG, can improve the permeability of blood-thymus barrier. Current cognition also proposed that increased permeability of the blood-thymus barrier is one of the anatomical basis of neonatal central tolerance [401].

In this regard, because of reversed permeability of the blood-thymus barrier, ATG can have increased access to thymocytes in neonates. Nevertheless, in present study, by immunochemical examination it's also confirmed that pATG was still restricted around the medulla zone and could not spread freely in the cortex of thymus where the blood-thymus barrier is more strengthened.

Increased accessibility of pATG to thymus in neonatal pigs may explain the elevated dose of pATG to cause T-cell depletion and low concentration and rapid decrease of serum circulating pATG, considering the huge number and active reproductivity of thymocytes in neonates.

Another explanation for the need of high dose pATG was the immature immune system in neonatal pigs. As mentioned in previous paragraphs, pATG can cause lymphocyte depletion mainly via 3 mechanisms: CDC, ADCC and apoptosis.

In vitro study of pATG shown that lymphocytes from neonatal pigs and juvenile

pigs have no significant difference in pATG binding, blocking and apoptosis tests. However, in CDC test, equal quantity of complement-containing serum from neonatal pigs showed significant lower capacity to lyse pATG-bound lymphocytes than the serum from juvenile pigs, indicating qualitative and/or quantitative defects of neonatal complement system. These findings were corresponding to those from human neonates of which T lymphocytes are proved to be largely mature and immunocompetent, however, compared to the adults, neonatal complement components have significant lower concentrations and reduced activity [158-160].

Theoretically, pATG-induced ADCC should be similarly impaired in neonatal pigs, as macrophages, which predominantly mediated ADCC by pATG, do not show mature morphological parameters and phagocytic function until 7 days after birth [402]. Thus, due to increased pATG targets as well as reduced CDC and ADCC functions, it's reasonable that higher dose of pATG is necessary for lymphocyte depletion in neonatal pigs.

Immature immune system in neonatal pigs may partially explain the absent of pig-anti-rIgG immune response in this study. Newborn pigs have lower proportion of B lymphocytes and impaired function to produce specific IgG when encounter foreign antigens (the porcine IgG circulating in serum of pig neonates are primarily from the sow via breast-feeding) [386, 403]. Besides, binding of pATG on B-cell surface molecules may result in modulation and impairment of B-cell function to amount effective IgG response [376, 404]. The absent of pig-anti-rIgG response, subsequently resulted in the difficulty of complete clearance of serum rIgG which may be either non-reactive pATG or non-specific rIgG. Long term effects of the residual circulating rIgG are unknown.

Many protocols which were shown to be effective for tolerance induction in rodents have failed when applied to large-animal or patients [13], suggesting the necessity of preclinical study in large-animals. So far, swine is the only large-animal model which can provide the selective and reproductive MHC matching/mis-matching as parameters of transplantation [322, 382]. Thus we choose swine for the production of a large-animal-specific surrogate of Thymoglobulin in order to make future

preclinical study more applicable and reproducible. T-cell depletion is proved to be able to prevent acute rejection post-allotransplantation and improve donor cells engraftment in donor-specific tolerance induction protocol which involved in hematopoietic stem cells transplantation and chimerism creation [405]. Lymphocyte depletion by pATG in neonatal pigs is transient but may be important to create a “window phase” for the homing of donor cells [334, 369]. Routine maintenance therapy with calcineurin inhibitor (tacrolimus, cyclosporine A) and anti-metabolic medication (mycophenolate mofetil) may be able to prolong the T-cell depletion effect of pATG in neonatal pigs. On the other hand, in transplant patients received Thymoglobulin, subsequent homeostasis-driven proliferation of T lymphocytes inevitably results in reversed ratio of Th/Tc and excessive expansion of memory T cells, which are considered to important obstacle for tapering or weaning of immunosuppression [406, 407]. Decrease Th/Tc ratio and expansion of memory T cells are also observed in present study, however, much less significant in neonatal swines. Future studies of pATG on juvenile or adult, normal or miniature swines, with or without maintaining therapy, will help to fully characterize the properties of this pATG.

At last, this present study may also provide implication for the administrations of anti-thymocyte/lymphocyte globulin or serum (ATG/AST) in neonatal or very young infants, as immature, defective immune system in these patients may probably impaired the lymphocyte depletion effects cause by ATG/AST, thus may call for higher dose of ATG/AST. Besides, the involvement of thymus in these young pediatric patients should be taken into account, as it may cause great difference in the ratio of total lymphocyte number versus body weight between neonates and adults.

IV. A Short-Term Pharmacokinetics Study of Mycophenolate Mofetil in Neonatal Swines

IV.1. Background

In this project, Mycophenolate mofetil (MMF) is used as adjuvant immunosuppressant with pATG in order to maintain longer term of peripheral blood lymphocyte-depletion. In the study of pATG, it was shown that administration of high dose pATG in neonatal swines can cause T -cell depletion in peripheral blood. However, T-cell depletion in neonatal swines was transient and reconstitution of peripheral blood T-cell can initiate and accomplish rapidly; peripheral blood T-cell count can increase to normal level within 72 hours after the last infusion of pATG. The reconstitution of peripheral T cell should be because of exportation of thymocytes and homeostasis-driven proliferation of peripheral T cells.

Maintenance of longer term lymphocyte-depletion state may reduce allorejection to composite allograft and should be favorable for the engraftment of donor-derived HSCs as well as tolerance induction. For this purpose, MMF was introduced into the present immunosuppressive protocol.

MMF is an ester prodrug of the active immunosuppressant mycophenolic acid (MPA) which is a more recent immunosuppressive drug which does not interfere with the actions of calcineurin and will not cause nephrotoxicity [408-410]. By suppressing the *de novo* biosynthesis of guanosine nucleotides which is critical for the proliferation of T- and B- lymphocytes, MPA acts as a selective inhibitor on proliferation of lymphocytes. Moreover, different to CsA, MMF does not interfere with the actions of calcineurin and will not cause nephrotoxicity.

MMF has been used in porcine experimental transplant studies of VCA [411, 412]. In these studies, MMF was administered in juvenile pigs with a dose of 500 mg/day, with concomitant CsA or tacrolimus. These combinations of immunosuppressants

successfully prevented rejection to VCA. However, pharmacokinetic profiles of MMF in swines have never been defined in these studies. Significantly, MPA is primarily metabolized by glucuronidation via uridine diphosphate-glucuronosyltransferases (UGTs) in the gastrointestinal tract, liver, and kidney. Whether the same UGTs are expressed in newborn swines and have similar metabolic characteristics as in human, remain to be unknown.

Pig neonates are born with low peripheral blood lymphocyte count which is termed as neonatal lymphopenia [386]. This neonatal lymphopenia subsequently results in homeostasis-driven proliferation of lymphocytes in peripheral blood [413]. Thus, natural increase of blood lymphocyte count can be observed in newborn pigs. Theoretically, administration of MMF in neonatal pigs will interfere with the homeostasis-driven proliferation of lymphocyte, thus resulting in stable or even decrease of blood lymphocyte count. Therefore, blood lymphocyte count can be used as parameter for evaluation of MMF immunosuppressive effect on homeostasis-driven proliferation in newborn pigs.

A short-term pharmacokinetics study of MMF in neonatal swines was thus designed in order to answer these questions below. (1) Whether MMF can be tolerated, absorbed, metabolized by newborn pigs? (2) What are the pharmacokinetic profiles of MMF in newborn pigs? (3) Whether MMF can suppress homeostasis-driven proliferation in newborn pigs?

IV.2. Publication

Short-term Pharmacokinetic Study of Mycophenolate Mofetil in Neonatal Swines

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Abstract

Background: Mycophenolate mofetil (MMF) is an effective immunosuppressive agent which has been frequently used in laboratory animals including swines; however, pharmacokinetic characters of MMF in swines have never been studied. This short-term study was designed to evaluate the feasibility and the pharmacokinetic profiles of MMF therapy in neonatal swines.

Materials and Methods: Respecting the balance of body weight among groups, twelve neonatal pigs were randomized into 4 groups: 1 control and 3 treated groups with oral MMF administered at 0.5, 1, and 2 g/m²/day for 4 days, divided by 2 half-doses at 9:00 and 17:00 (except day 4 on which MMF was not given at 17:00). Blood samples were collected at 9:00 on day (D) 0, 2, 3 and 4 for complete blood count (CBC) and hepatic/renal function examination; the trough concentration of plasma mycophenolic acid (MPA) was also determined. On D2 and D4, blood was collected to determine the area under the curve (AUC) of plasma MPA concentration. Animal body-weight growth and manifestations of MMF side-effects such as anorexia, vomit and diarrhea were also observed.

Results: MMF has no acute hepatic/renal toxicity in newborn pigs, however, less body-weight growth was observed in treated-groups.

In control group, lymphopenia-driven, spontaneous increase of lymphocyte count was observed; in contrast, MMF therapy with doses of 1 and 2 g/m²/day reduced both lymphocyte and monocyte count of piglets. Oral MMF had high bioavailability in neonatal swines. MPA-AUC_{0-12h} of doses 0.5, 1 and 2 g/m²/day was 22.00 ± 3.32, 57.57 ± 34.30 and 140.00 ± 19.70 ug×h/mL, respectively. Neither MPA trough concentration (MPA-C₀), nor MPA maximum concentration (MPA-C_{max}) or MPA-AUC_{0-6h} had high correlation with MMF-dose. For surveillance of MPA exposure, MPA-C₀ had significant correlation with MPA-AUC_{0-12h} (Spearman's $\rho=0.933$, $AUC_{0-12h}=17.882\times C_0+14.479$, $r^2=0.966$).

Conclusion: In order to reach adequate drug exposure and to reduce dose-dependent side effects, an MMF dose of 1 g/m²/day is recommended to be used

as initial dose for immunosuppressive therapy in piglets and MPA-C₀ monitoring is the most practical strategy for experimental transplant study.

Keywords: mycophenolate mofetil, pharmacokinetics, transplantation, neonates, swines

1. Introduction

Mycophenolate mofetil (MMF) is a more recent immunosuppressive drug which does not interfere with the actions of calcineurin and will not cause nephrotoxicity [408-410]. As an ester prodrug of the active immunosuppressant mycophenolic acid (MPA), MMF is metabolized mainly by liver and intestinal esterases [414].

MPA is a noncompetitive, selective and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) which is an important enzyme in guanosine nucleotides *de novo* biosynthesis. As proliferation of T- and B- lymphocytes is critically dependent on *de novo* synthesis of purines, whereas other cell types can recover purines by utilizing salvage pathways, thus MPA has specific cytostatic effects on lymphocytes. MPA can inhibit proliferation responses of T- and B- lymphocytes to both mitogenic and allogenic stimulation [415]. MPA has no effect on the production or release of the cytokines (IL-1 and IL-2) associated with early T-cell signal transduction, so it is not effective in the treatment of ongoing acute rejection [416].

MMF is now frequently used in transplant recipients as adjuvant immunosuppressant which effectively helps to reduce the administration of CNIs and relevant nephrotoxicity [417]. A decreased rate of acute rejection was reported for adult and pediatric renal transplant patients who achieved a MPA exposure value, expressed by area under the plasma MPA concentration-time curve from 0 to 12 hours (MPA-AUC₀₋₁₂) after MMF administration, greater than 30 mg*h/L [418, 419]. Clinical studies also showed that the pharmacokinetic parameters of MPA exhibit wide inter- and intra-patient variability, and that MMF doses are not correlated with MPA plasma concentrations in each patient [420].

Swine is an excellent model for transplantation immunology research program because of its similarities to human on genetics and body size. MMF has been used in porcine experimental transplant studies [411, 412, 421-423]. Representatively, in the studies of vascularized composite allotransplantation (VCA) in juvenile pigs [411, 412], MMF was administered with a dose of 500 mg/day, with concomitant cyclosporine (CsA, 40 mg/kg/day) or tacrolimus (FK506, 1.5 mg/kg/day). These combinations of immunosuppressants successfully prevented rejection to VCA. However,

pharmacokinetic profiles of MMF in swines have never been defined in these studies.

In order to develop a preclinical model for study of tolerance induction in neonates and very young infants, we have previously established a vascularized composite tissue allograft model in newborn pigs [347]. We subsequently studied the pharmacokinetics and acute nephrotoxicity of oral cyclosporine in newborn pigs (article in revising). This present study was designed to characterize MMF pharmacokinetic profiles, side-effects, as well as immunosuppressive effects on homeostasis-driven proliferation of lymphocytes in newborn pigs, in order to choose proper initial dose and practical monitoring strategy of MMF in neonatal swine model.

2. Materials and Methods

2.1. Animals

Twelve neonatal domestic piglets with a patrilineal pedigree of Large White and a matrilineal pedigree of Youli, the same age of 5~6 days, weights ranged from 1.22 to 2.16 kg, were housed together with the sow in one cage under the standard procedure for neonatal pigs in the Institute Claude Bourgelat, animal research center of the national veterinary school of Lyon (VetAgro Sup-Campus Vétérinaire de Lyon). The sow was fed with a standard pig diet. Newborn piglets were breastfed exclusively and unrestrictedly. All animal experimentations were approved by the Ethical Committee of the Veterinary Campus of Lyon (Number – 2013/1322) and conformed to the European Guideline 2010/63/EU, for the care and use of animals for scientific purposes.

2.2. MMF Administration and Clinical Observation

MMF (CellCept[®], 1g/5mL, oral suspension) was purchased from Hoffmann-La Roche Ltd., Switzerland. Piglets were divided randomly in 4 groups respecting to the weight balance among groups, 3 piglets/group. In control group (Group 0), piglets were administered with saline solution. In treated groups (Group 1/2/3), different doses of MMF (0.5/1/2 g/m²/day) were given. During day (d) 0~4, piglets were weighed every morning at 8:30, and then body surface area was calculated according

to body weight by a specific formula from DeRoth et al. [385]. MMF was administered orally by 2 equal doses per day at 9:00 and 17:00 from day 0 to day 3. To evaluate AUC_{0-12h} , only 1 dose of MMF was given at 9:00 on Day 4. Animal weight was recorded each day before drug administration and weight growth during 4-day MMF therapy was calculated as $Weight\ Growth = (Weight_{d4} - Weight_{d0}) / Weight_{d0} \times 100\%$ and then compared between treated groups and control groups. During therapy period, manifestations of anorexia (motivation in feed competition), vomit and diarrhea were recorded.

2.3. Blood Sampling

The blood samples were drawn from the jugular vein under general anesthesia. Anesthesia was induced by 5% isoflurane (Laboratoires Belamont, Paris, France) in O_2 , and then maintained with 1-2% isoflurane in O_2 . For measurement of MPA predose concentration (C_0), as well as examination of blood cell count and renal/hepatic functions, blood was collected on day 0, 2, 3, 4 at 9:00 before MMF administration (thus MPA- C_0 was detected at 16 hr after last dose of MMF).

On day 2, blood samples were also collected at 1h, 2h, 3h, 4h and 6h after MMF administration in order to measure MPA- AUC_{0-6h} . On day 4, for evaluation of MPA- AUC_{0-12h} , blood samplings were performed at 1h, 2h, 3h, 4h, 6h, 8h, 10h, 12h after drug administration.

2.4. Complete Blood Count and Examination of Renal/Hepatic Functions

On day 0, 2, 3 and 4, complete blood count was performed using a Sysmex 2000iV automation following a specific veterinary protocol (Laboratoire Biomédicale Biovelys, VetAgro Sup), while hepatic and renal functions were evaluated by measuring serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Cr) and blood urea nitrogen (BUN) using a KONELAB KL20 ISEND automatic analyzer.

2.5. Plasma MPA Concentrations

For MPA plasma concentration measurement, blood samples were collected and stored at 4°C until centrifugation (< 60 minutes after collection). Following centrifugation, the plasma samples were stored at -80°C until MPA assay.

MPA plasma concentrations were determined using high-performance liquid chromatography (HPLC) equipped with diode array UV and mass spectrometer detectors. Briefly, after protein precipitation by acetonitrile and centrifugation, samples were evaporated under nitrogen stream. Dry residues were dissolved in the mobile phase and injected. Calibration curves were established with commercial calibration standards (0 to 5.28mg/L). MPA concentrations were calculated from the signals obtained with the two detectors to ensure the purity of chromatographic peaks. Accuracy and precision were evaluated at three levels of quality control concentrations (0.463 – 2.35 – 4.72 mg/L). The inter-day precision was less than 6% with a bias lower than 3%.

2.6. Measurement of MPA Variability

The measurement of variability of MPA concentration and MPA-AUC was according to previous study [415]: firstly the interquartile range (IQR) (difference between the upper quartile and the lower quartile) was determined, subsequently a nonparametric standard deviation was calculated by the Tukey method: $\sigma = \text{IQR}/1.35$, then a nonparametric coefficient of variation (CV_{np}) was calculated: $\text{CV}_{np} = \sigma / \text{median} (\%)$.

2.7. Statistical Analysis

Data of animal weight growth, hepatic/renal function parameters and blood cell counts are expressed as mean \pm SD. For these results, difference between means of two data sets were analyzed by Student's *t* test, whereas multiple comparisons were subjected to one way analysis of variance and least-significant difference (LSD) test. A *p* value of <0.05 was considered to be statistically significant.

MPA concentration and AUC were presented mainly by mean \pm SD, description of median and range was also introduced. Correlations between variables were assessed by Spearman's rank coefficient (ρ). Variables were considered to be highly correlated when Spearman's ρ was >0.8. Regression of MPA concentrations (C_0 or C_{max}) and MPA-AUC_{0-12h} was performed in linear model.

All statistical analyses were performed using SPSS Statistics Version 17.0.

3. Results

3.1. MMF has no severe, acute effects in newborn pigs

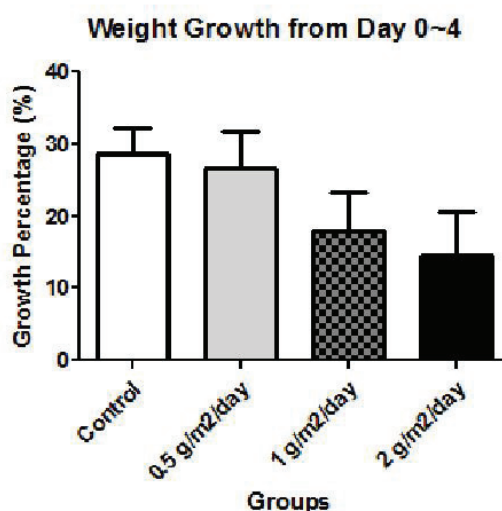
MMF didn't cause any obvious acute manifestation in all treated groups. MMF treated piglets didn't show noticeable anorexia. No vomit or diarrhea was observed and no piglets died during therapy.

Compared to control group, a dose-dependent decrease in weight growth can be observed in treated groups (Figure 1). However, this difference did not reach statistical significance ($p < 0.05$).

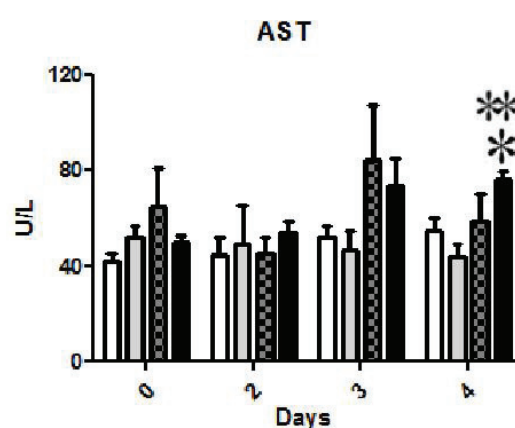
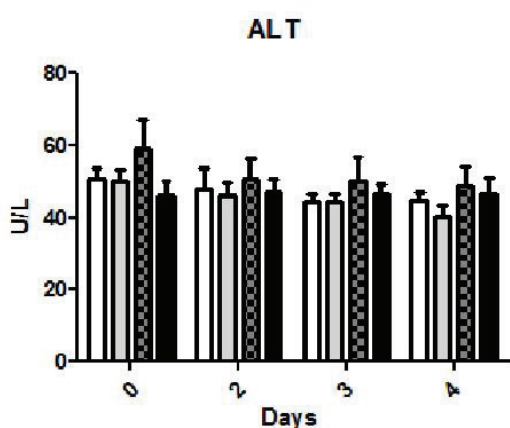
In parameters of hepatic and renal function (Figure 1), no dose-related, significant change of AST, ALT, Cr and Urea was observed in all treated groups.

A. Weight Growth

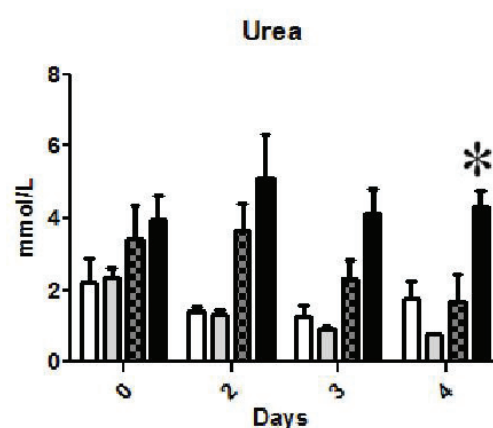
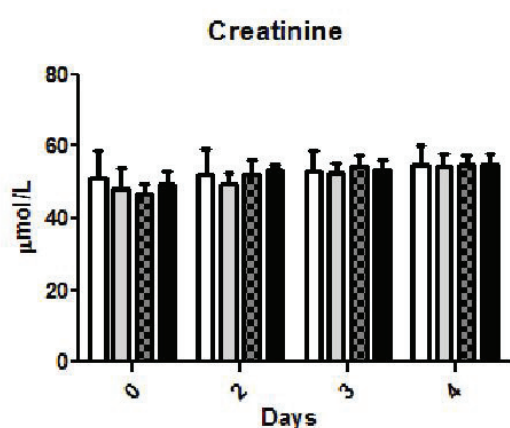
- Group 0: Control Group
- ▒ Group 1: 0.5 g/m²/day
- ▣ Group 2: 1 g/m²/day
- Group 3: 2 g/m²/day



B. Hepatic Function



C. Renal Function



MMF-Figure 1: MMF has no severe, acute effect in neonatal pigs.

Single asterisk signifies a significant difference ($p < 0.05$) in comparison of blood cell count on day 4 versus day 0 within the same group, while double asterisks refer to a significant difference ($p < 0.05$) in

comparison of blood cell count on day 4 between each treated group versus control group. **(A) Weight growth.** Animals in control group grew $28.7 \pm 6.1\%$ from day 0 to day 4, while animals grew $26.7 \pm 8.8\%$ in Group 1, $17.9 \pm 9.3\%$ in Group 2 and $14.7 \pm 10.2\%$ in Group 3. **(B) Hepatic function.** In Group 2g/m²/day, AST on d4 showed a significant increase compared to d0; but it was also significantly higher than AST of control group on d4. **(C) Renal function.** In Group 2g/m²/day the Urea on d4 was also significantly higher than all other groups; however, it didn't show an increase compared to day 0.

3.2. MMF reduces lymphocyte and monocyte counts in newborn pigs

In neonatal pigs, short-term administration of MMF may cause alteration in several peripheral blood cells. Two types of comparisons were performed to confirm the alteration caused by MMF: comparison of blood cell count on day 4 versus day 0 within the same group, as well as comparison of blood cell count on day 4 between each treated group versus control group (Figure 2).

MMF treated groups showed a dose-related decrease of lymphocyte count after 4 days of treatment, statistically significant difference ($p < 0.05$) in Group 3 (2 g/m²/day). Significant decrease of monocyte count caused by MMF administration was observed in both Groups 2 and 3 (1g/m²/day and 2g/m²/day respectively) but not in Group 1 (0.5 g/m²/day). MMF treatment didn't cause dose-dependent, significant alteration in neutrophil count and total leukocyte count.

Significant reduced red blood cell (RBC) count and hemoglobin (HGB) concentration was observed in Group 1g/m²/day during MMF treatment. However, such effect was not observed in Group 2g/m²/day. No significant change was shown in platelet count.

MMF-Figure 2: Alteration of blood cell counts by short-term treatment of MMF in neonatal pigs.

Unit was 1000/ μ L except special mark. Single asterisk signifies a significant difference ($p < 0.05$) in comparison of blood cell count on day 4 versus day 0 within the same group, while double asterisks refer to a significant difference ($p < 0.05$) in comparison of blood cell count on day 4 between each treated group versus control group. **(A) Leukocyte sub-populations and total leukocyte count.** **(a) Lymphocytes:** in contrast to control group in which lymphocyte count increased spontaneously from $(3.48 \pm 1.34) \times 10^3 / \mu\text{L}$ to $(4.84 \pm 1.36) \times 10^3 / \mu\text{L}$, Group 0.5 $\text{g}/\text{m}^2/\text{day}$ showed a stable lymphocyte count. In Group 2 $\text{g}/\text{m}^2/\text{day}$, statistically significant decrease ($p < 0.05$) of lymphocyte count was observed, from

(4.69 ± 0.75) $\times 10^3/\mu\text{L}$ to (3.34 ± 0.43) $\times 10^3/\mu\text{L}$. Steady decrease of lymphocyte count was observed in Group 1 $\text{g}/\text{m}^2/\text{day}$ from (5.35 ± 1.22) $\times 10^3/\mu\text{L}$ to (3.26 ± 1.73) $\times 10^3/\mu\text{L}$, but statistical significance was not obtained. **(b) Monocytes:** significant decrease of monocyte count caused by MMF administration was observed in both Groups $1\text{g}/\text{m}^2/\text{day}$ and $2\text{g}/\text{m}^2/\text{day}$. **(c) Neutrophils:** Although neutrophil count of day 4 in Group $0.5\text{g}/\text{m}^2/\text{day}$ showed a decrease compared to day 0, it did not show significant difference to control group. Moreover, no dose-dependent effect was observed by results from two other treated groups. **(B) Red blood cell (RBC), hemoglobin and thrombocyte count.** Compared to control group, Group $1\text{g}/\text{m}^2/\text{day}$ showed significantly lower RBC count on day 3 and lower HGB concentration on day 3 and 4; however, such effect was not observed in Group $2\text{g}/\text{m}^2/\text{day}$. No significant change was observed in platelet count.

3.3. MPA trough concentrations

During day 2, 3 and 4, MPA- C_0 of Group 1, 2 and 3 were 0.96 ± 0.32 , 2.27 ± 1.22 , 5.47 ± 3.07 $\mu\text{g}/\text{mL}$, respectively (Table 1 and Figure 3). Values of CV_{np} of MPA- C_0 were 37.40% (Group 1), 92.30% (Group 2) and 85.47% (Group 3), indicating a high variability of MPA- C_0 , especially in Group 2 and Group 3. MPA- C_0 was not highly correlated to MMF dose ($p=0.786$); moreover, because of high MPA- C_0 variability, estimate of MPA- C_0 by MMF dose was not reliable by linear regression model ($r^2=0.521$).

Table 1 MMF Doses and MPA Exposure

		Group 0.5 $\text{g}/\text{m}^2/\text{day}$	Group 1 $\text{g}/\text{m}^2/\text{day}$	Group 2 $\text{g}/\text{m}^2/\text{day}$
C_0 (n=27, $\mu\text{g}/\text{mL}$)	Mean \pm SD	0.96 ± 0.32	2.27 ± 1.22	5.47 ± 3.07
	Median(Minimum, Maximum)	1.01 (0.47, 1.46)	1.89 (0.77, 4.04)	5.20 (1.44, 9.83)
	CV _{np}	37.40%	92.30%	85.47%
C_{max} (n=18, $\mu\text{g}/\text{mL}$)	Mean \pm SD	5.58 ± 1.39	11.54 ± 4.56	19.48 ± 9.36
	Median(Minimum, Maximum)	5.325 (4.34, 8.19)	11.76 (5.60, 17.25)	19.67 (4.13, 33.42)
	CV _{np}	24.76%	51.08%	38.39%
AUC_{0-6h} (n=18, $\mu\text{g}^*\text{h}/\text{mL}$)	Mean \pm SD	17.85 ± 4.60	39.69 ± 17.65	74.53 ± 29.83
	Median(Minimum, Maximum)	16.29 (13.62, 26.15)	36.54 (19.65, 60.68)	80.21 (19.05, 105.78)
	CV _{np}	20.01%	45.58%	20.99%
	Correlation with C_0 or C_{max}	with C_0 : $p=0.932$ ($p<0.001$); with C_{max} : $p=0.888$ ($p<0.001$)		
AUC_{0-12h} (n=9, $\mu\text{g}^*\text{h}/\text{mL}$)	Mean \pm SD	22.00 ± 3.32	57.57 ± 34.30	140.00 ± 19.70
	Correlation, Linear regression with C_0	$p=0.933$ ($p<0.001$), $AUC_{0-12h}=17.882 \times C_0 + 14.479$, $r^2=0.966$		
	Correlation, Linear regression with C_{max}	$p=0.900$ ($p<0.001$), $AUC_{0-12h}=8.689 \times C_{\text{max}} - 29.141$, $r^2=0.940$		

Abbreviations: C_0 , trough concentration; C_{max} , maximum concentration; AUC, area under the concentration-time curve; SD, standard deviation; CV_{np}, nonparametric coefficient of variation.

MMF-Table 1: MMF doses and MPA exposure.

3.4. MPA maximum concentrations

MPA maximum concentrations (MPA- C_{\max}) of day 2 and 4 were 5.58 ± 1.39 , 11.54 ± 4.56 , 19.48 ± 9.36 $\mu\text{g/ml}$, respectively in Group 1, 2 and 3. Among the MPA- C_{\max} data, 11/18 of them were achieved at 1 hr after drug administration, 5/18 at 2 hr and 2/18 at other time-points (Table 1 and Figure 3). Thus, the time to MPA- C_{\max} (time-to-peak) can be regarded as 1 to 2 hr, similar to the results observed in human patients of whom the MPA- C_{\max} usually occurred within 0.5 to 2 hr of drug intake (Tett, Saint-Marcoux et al. 2011). Values of CV_{np} of MPA- C_{\max} were 24.76% in Group 1, 51.08% in Group 2 and 38.39% in Group 3, less than the CV_{np} of MPA- C_0 during day 2, 3 and 4. Similar to MPA- C_0 , highly correlation of MPA- C_{\max} with MMF dose was not observed ($\rho=0.656$) and estimate of MPA- C_{\max} by MMF dose was not reliable by linear regression model ($r^2=0.508$).

3.5. C-T curves and MPA-AUC

In Group 1, 2 and 3, MPA-AUC_{0-6h} of day 2 and 4 were 17.85 ± 4.60 , 39.69 ± 17.65 and 74.53 ± 29.83 $\mu\text{g}\cdot\text{h/ml}$. MPA-AUC_{0-12h} on day 4 were 22.00 ± 3.32 , 57.57 ± 34.30 and 140.00 ± 19.70 $\mu\text{g}\cdot\text{h/ml}$ (Table 1 and Figure 3).

Values of CV_{np} of MPA- AUC_{0-6h} were 20.01% in Group 1, 45.58% in Group 2 and 20.99% in Group 3. MPA-AUC_{0-6h} and MMF dose was not highly correlated ($\rho=0.774$). Estimate of MPA-AUC_{0-6h} by MMF dose was not reliable by linear regression model ($r^2=0.614$). MPA-AUC_{0-6h} was highly correlated with MPA- C_0 ($\rho=0.932$, $p<0.01$) and MPA- C_{\max} ($\rho=0.888$, $p<0.01$).

Similar to MPA-AUC_{0-6h}, MPA-AUC_{0-12h} of day 4 was also highly correlated with MPA- C_0 ($\rho=0.933$, $p<0.01$) and MPA- C_{\max} ($\rho=0.900$, $p<0.01$). Thus the linear regression of MPA- C_0 or MPA- C_{\max} to MPA-AUC_{0-12h} was performed. Results showed that $\text{AUC}_{0-12h}=17.882\times C_0+14.479$, $r^2=0.966$ and $\text{AUC}_{0-12h}=8.689\times C_{\max}-29.141$, $r^2=0.940$.

MMF-Figure 3: Pharmacokinetic profiles of MMF in neonatal swines.

(A) Variability in dose-concentration relationship: MPA- C_0 was significant but not highly correlated to MMF dose (Spearman's $\rho=0.786$, $p<0.001$), indicating that increase of MMF dose will correspondingly result in elevated MPA- C_0 ; however, because of high MPA- C_0 variability, estimate of MPA- C_0 by MMF dose was not reliable by linear regression model ($r^2=0.521$). Similar dose-concentration relationship was also observed in MPA- C_0 (Spearman's $\rho=0.656$, $r^2=0.508$ in linear regression model) and MPA-AUC $_{0-6h}$ (Spearman's $\rho=0.774$, $r^2=0.614$ in linear regression model). **(B) Correlations between MPA-AUC $_{0-12h}$ and MPA- C_0 or MPA- C_{max} :** MPA-AUC $_{0-12h}$ was highly correlated with MPA- C_0 ($\rho=0.933$, $p<0.01$) and with MPA- C_{max} ($\rho=0.900$, $p<0.001$). The linear regression of MPA- C_0 or MPA- C_{max} to MPA-AUC $_{0-12h}$ was also performed. **(C) C-T curves of plasma MPA concentration:** MPA C-T curves showed high bioavailability of MMF in neonatal swines. The time to MPA- C_{max} (time-to-peak) was ≈ 1 hr, similar to the results observed in human patients.

4. Discussion

This study described the MMF pharmacokinetics profiles, immunosuppressive effect on lymphopenia-driven lymphocyte proliferation, as well as side-effects with neonatal swine model, in order to define a proper, safe dose of MMF and an applicable monitoring strategy for immunosuppressive therapy in experimental transplant studies with newborn pigs.

MMF is widely used in maintenance immunosuppressive therapy in pediatric solid organ transplantation. Generally, the overall efficacy and tolerability of MMF in pediatric patients are comparable to those in adult patients, except for a higher prevalence of gastrointestinal adverse effects in children younger than 6 years [410]. The currently recommended dose in pediatric patients with concomitant cyclosporine is 1200 mg/m²/day in 2 divided doses; the recommended MMF dose with concomitant tacrolimus or without a concurrent calcineurin inhibitor is 900 mg/m²/day in 2 divided doses [410, 424]. As in adults, there is an approximately 10-fold variability in dose-normalized MPA–AUC_{0–12} values between pediatric patients after renal transplantation, strengthening the argument for concentration-controlled dosing of the drug [420].

The currently recommended therapeutic window for MPA exposure in conjunction with full-dose CNI therapy in the initial period after pediatric renal transplantation to minimize the risk of acute rejection is 30~60 mg · h/L (high-performance liquid chromatography, HPLC) or 37~70 mg·h/L (enzyme multiplied immunoassay technique, EMIT). Predose plasma concentrations (C₀) should be between 1.0 and 3.5 mg/L (HPLC) or 1.3 and 4.5 mg/L (EMIT) [425, 426]. Maximum plasma concentrations (C_{max}) determined by a HPLC method should be at the level of 14.5±4.21mg/L [427].

MMF is also administered in experimental transplant studies conducted with swine model [411, 412, 421-423]; however, the pharmacokinetics of MMF in pigs has never been reported. Moreover, concerning to the therapeutic effects, MPA is primarily metabolized by glucuronidation via uridine diphosphate-glucuronosyltransferases

(UGTs) in the gastrointestinal tract, liver, and kidney. Whether the same UGTs are expressed in swine species and have similar metabolic characteristics as in human, remain to be unknown.

Related to the previous studies conducted in our lab which focus on transplant tolerance induction in neonates and infants [347], this present study chose neonatal pigs as a preclinical model. Similar to mouse and human neonates, pig neonates are born with low peripheral blood lymphocyte count which is termed as neonatal lymphopenia [386]. This neonatal lymphopenia subsequently results in homeostasis-driven proliferation of lymphocytes in peripheral blood [413]. Thus, natural increase of blood lymphocyte count can be observed in newborn pigs. Theoretically, administration of MMF in neonatal pigs will interfere with the homeostasis-driven proliferation of lymphocyte, thus resulting in stable or even decrease of blood lymphocyte count. Therefore, blood lymphocyte count can be used as parameter for evaluation of MMF immunosuppressive effect in newborn pigs.

In present study, 4 days of MMF therapy did not cause severe, acute hepatic or renal toxic effects. Although manifestations of vomit and diarrhea were not observed, MMF-treated piglets showed dose-related, lower body weight growth, indicating a potential gastrointestinal side-effect of MMF.

Short-term administration of MMF effectively interfered with the lymphopenia-driven proliferation of lymphocytes in neonatal pigs. The lymphocyte count in control group showed steady increase from $(3.48 \pm 1.34) \times 10^3/\mu\text{L}$ to $(4.84 \pm 1.36) \times 10^3/\mu\text{L}$ (although not statistically significant). Corresponding to the increase of MMF dose, Group 1 showed a stable of lymphocyte count, Group 3 showed a significantly reduced lymphocyte count. Although statistical significance was not obtained, a lymphocyte count decrease from $(5.35 \pm 1.22) \times 10^3/\mu\text{L}$ to $(3.26 \pm 1.73) \times 10^3/\mu\text{L}$ was observed in Group 2. We thus considered that in neonatal pigs the immunosuppressive effect of MMF is efficient and dose-related. Besides, by short-term MMF therapy with doses from 0.5~2 g/m²/day in present study, although dose-related neutropenia was not evident, reduced monocyte count was significant in both Groups 2 and 3 but not Group 1.

The present study observed that MMF pharmacokinetics profiles in neonatal pigs are of similar characteristics to those in human patients. In day 2 and 4, 16/18 of the C_{\max} was reached at 1 or 2 hr after MMF administration, indicating a high bioavailability of MMF in newborn pigs. High interindividual variability of each MPA pharmacokinetic parameter (C_0 , C_{\max} and AUC_{0-6h}) was also confirmed. As in human patients, reasons for MPA pharmacokinetic variability may include differences in albumin, bilirubin, hemoglobin concentrations, renal and hepatic functions, as well as genetic polymorphisms in uridine diphosphate (UDP)-glucuronosyltransferase (UGT) expression. Co-administration of CsA inhibits transport of the metabolite mycophenolate glucuronide (MPAG) into the bile, results in less or no enterohepatic recycling of MPA, hence leads to lower drug exposure; while tacrolimus does not interact with MPA.

MMF administration in piglets with the dose of 1 g/m²/day lead to a drug exposure more corresponding to the approved MPA level in clinical transplantation, characterized by MPA- AUC_{0-12} of 30~60 $\mu\text{g}\cdot\text{h}/\text{ml}$, MPA- C_0 of 1.5-3.5 $\mu\text{g}/\text{ml}$ and MPA- C_{\max} of 10-20 $\mu\text{g}/\text{ml}$ (all detected by HPLC as present study). Besides, MMF dose of 1 g/m²/day also keep better balance between desired therapeutic effects and adverse side-effects. We thus concluded that a MMF dose at 1 g/m²/day may be used as an initial dose in immunosuppressive therapy.

Similar to pediatric patients, AUC_{0-6h} , C_0 and C_{\max} of MPA have no high correlation with MMF doses in piglets. In order to achieve optimal immunosuppressive outcome, MPA exposure should be monitored and doses should be adjusted [420, 424].

AUC_{0-12h} can be considered as the most informative parameter of drug exposure; however, the blood sampling schedules for this monitoring strategy are not practicable in transplantation studies. Especially in experimental research with swine model, frequent blood collections may result in porcine stress syndrome, manifested by perturbation of immune system which involves in sudden, severe increase or decrease of lymphocyte or neutrophil counts [428, 429]. Therefore, MMF monitoring strategies in pigs should be balanced to their own harmful effect. From this aspect,

MMF monitoring by one single time-point is undoubtedly a more practical strategy than limited sampling strategies (LSS) which required several blood samplings.

Both C_0 and C_{\max} ($\approx C_1$) were shown to be significantly correlated to AUC_{0-12h} , thus are justified to be used for monitoring of MPA exposure. In transplant studies, MMF is often administered with CsA or tacrolimus which are routinely monitored with C_0 . By this consideration, C_0 monitoring which does not require once more blood sampling and anesthesia, will cause less harmful intervention than C_1 monitoring thus may be a more practical strategy for MMF surveillance in pigs. From standpoint of clinical pharmacokinetic study, MPA- C_0 is not a very informative parameter and not the best time point to choose for MPA monitoring; however, in experimental study C_0 monitoring may be the most practicable one.

In this study, in order to make the experiment schedule more convenient, MPA- C_0 was measured at 16 hr after the last dose of MMF (but not 12 hr as a standard interval in clinical study). What should be notice was that, on day 4 the MPA- C_0 at 12-hr interval can be counted as 0.89 ± 0.48 , 3.08 ± 1.50 and 10.14 ± 7.06 $\mu\text{g/ml}$ in 3 treated groups, relatively higher than the MPA- C_0 at 16-hr interval.

The other limitation is that in present study MMF was administered without concomitant therapies of calcineurin inhibitors and corticosteroids. The recommended MPA- AUC_{0-12h} of 30-60 $\mu\text{g} \cdot \text{h/ml}$ was derived from the clinical trial in adult renal transplant patients receiving CsA, MMF, and corticosteroids [408]. In human patients, co-administration of cyclosporine interferes with the MPA pharmacodynamics and leads to lower MPA exposure, as mentioned above. Thus the recommended initial dose of 1 $\text{g/m}_2/\text{day}$ has to be confirmed in future transplant study if co-administered with CsA. Besides, longer term of MMF therapy is required to study the evolution of MMF pharmacokinetics, immunosuppressive effects and side effects, in order to adjust the MMF dosage in long-term study.

5. Conclusion

As in pediatric patients, oral MMF has high bioavailability in neonatal swines; however high interindividual variability in the dose-concentration relationship was also

confirmed in present study. MMF administered at proper dose can cause decrease of lymphocyte and monocyte count in pig neonates, at the mean time less body weight growth was also observed. By balancing the therapeutic- and side-effects, an MMF dose of 1 g/m²/day is recommended to be used as initial dose for immunosuppressive therapy in piglets, which leads to the MPA-AUC_{0-12h} of 30~60 ug×h/ml. With current doses of MMF used in present study, predose MPA concentration (C₀) was proved to be significantly correlated to MPA-AUC_{0-12h}; thus C₀ monitoring can serve as a practical strategy for surveillance of MPA exposure.

Part III: Conclusions and Perspectives

I. Conclusions of Previous Studies

This present project is devoted to explore an applicable tolerance induction protocol for vascularized composite allograft (VCA) in neonates or infants with severe congenital hand or face defects.

For this objective, a series of preclinical experiments in neonatal swines has already been performed step by step, including: (a) establishment of VCA model, (b) pharmacokinetics and nephrotoxicity of cyclosporine A (CsA), (c) characterization of rabbit anti-pig thymocyte globulin (pATG), (d) pharmacokinetics and immunosuppressive effects of mycophenolate mofetil (MMF). This chapter is written to draw collective conclusions of these previous studies.

1. In the study of building VCA model in newborn swines, heterotopic transplantation of osteo-chondro-myocutaneous free flap of the right knee joint was performed in 2 series: Series 1 of 20 autografts and Series 2 of 10 allografts without immunosuppression. We concluded that: (1) heterotopic hind-limb VCA model can be successfully established in piglets weighing over 2 kg and aged more than 6 days with low rates of both vascular failure (0/10) and postoperative death (0/10), permitting the use of this model for research in newborn VCA; (2) newborn piglets with age of 6 days or more are competent to mount effective alloimmune response and to reject allograft (at 4~8 post-transplantation day) if immunosuppressive therapy is absent.

2. In the research of oral CsA pharmacokinetics and nephrotoxicity, CsA was administered for 15 days, twice daily in 4 groups of piglets with different doses (0, 4, 8 and 12 mg/kg/day), 3-4 pigs/group. Conclusions of this study were as below. (1) To achieve comparable CsA trough concentration and AUC_{0-12h} adequate to induce an immunosuppression status, neonatal piglets need much lower dose of CsA, about 1/7 of the dose administered in adult pigs; in other words, CsA administration at the dose of 4 mg/kg/day in neonatal piglets can result in comparable drug exposure to 30 mg/kg/day of CsA in adult pigs. (2) Oral CsA treatment with the doses range from 4 to 12 mg/kg/day for a 15-day course is safe for newborn piglets in spite of very high blood concentrations of CsA were reached. CsA caused significantly increase in

serum BUN but without any change in serum Cr and renal histological examination. Increase of serum BUN concentration should be due to increased urea reabsorption induced by CsA and may indicate a potential decrease of renal blood flow (RBF) and glomerular filtration rate (GFR), which should be examined in future study and iohexol clearance assay may be considered. (3) We recommend a dose of CsA not superior to 4 mg/kg/day for immunosuppressive treatment in newborn piglets to achieve a CsA level of 400-800 ng/ml for immunosuppressive treatment in newborn piglets (immunosuppressive effects equal to 100-200 ng/ml in human). (4) For CsA monitoring strategy in newborn piglets, because of flat C-T curves of CsA, trough concentrations were highly correlated with AUC_{0-12h} . Thus, CsA trough concentration wonderfully indicates the drug exposure during 24h.

3. The study of pATG included *in vivo* experiment and *in vitro* experiment. pATG *in vivo* experiment was conducted in 6 groups of neonatal pigs with different total doses (0, 2.5, 5, 20, 40 and 80 mg/kg, administered in 2-3 days), 4~8 pigs/groups. pATG *in vitro* experiment including pATG binding effect, blocking test, cytotoxicity test, pATG-induced CDC (complement dependent cytotoxicity) test. By this study, several interesting findings were obtained. (1) Administration of pATG in neonatal swines can cause T cell depletion in peripheral blood and lymphoid tissues including thymus. However, T cell depletion in neonatal swines requires high dose of pATG. (2) Reconstitution of peripheral blood T lymphocyte can initiate and accomplish rapidly in neonatal swines. The lymphopenia state in lymphoid tissues persists relatively longer term than that in peripheral blood. (3) In neonatal pigs, requirement of high dose of pATG to cause T cell depletion in peripheral blood is partially because of deficient CDC mechanism in neonatal swines; deficient ADCC mechanism should be also involved. However, the apoptosis mechanism is shown to be effective in neonatal pigs. (4) In neonatal pigs, pATG can bind to thymocytes, thus resulting in thymocytopenia in thymus. This phenomenon is different to adult mice and non-human primates in which pATG had limited access into thymus. The access of pATG to thymocytes also explains the rapid consuming of pATG in neonatal pigs. (5) No pig anti-rapid humoral immune response was observed after pATG administration

in newborn pig, which may be due to B cell function is modulated by pATG or B cell is functional deficient in neonatal pigs. (6) The reconstitution of peripheral T cell should be because of exportation of thymocytes and proliferation of peripheral T cells.

4. In the study of pharmacokinetics and immunosuppressive effects of MMF, oral MMF was administered in 4 groups with different dosages (0, 0.5, 1 and 2 g/m²/day), divided in 2 doses/day, 3 pigs/groups. We proved that: (1) as in pediatric patients, oral MMF has high bioavailability in neonatal swines, however high interindividual variability in the dose-concentration relationship was also confirmed in present study; (2) MMF administered at proper dose can cause decrease of lymphocyte and monocyte count in pig neonates, at the mean time less body weight growth was also observed; (3) by balancing the therapeutic- and side-effects, an MMF dose of 1 g/m²/day is recommended to be used as initial dose for immunosuppressive therapy in piglets, which leads to the MPA-AUC_{0-12h} of 30~60 ug×h/ml; (4) with current doses of MMF used in present study, predose MPA concentration (C₀) was proved to be significantly correlated to MPA-AUC_{0-12h}, thus C₀ monitoring can serve as a practical strategy for surveillance of MPA exposure.

As our plan, the global study of donor-specific tolerance induction in neonatal swine through hematopoietic stem cells transplantation (HSCT) under less intensive conditioning therapy, which consist of pATG, CsA and MMF, will be performed in years 2014~2015. The tolerance induction protocol of this study is presented in the following chapter.

II. Protocol: Tolerance Induction to Vascularized Composite Allografts through Mixed Hematopoietic Chimerism in Neonatal Swines

II.1. Introduction

Since the world first hand transplantation was successfully performed in 1998 in Lyon (France) [2], nowadays Composite tissue allotransplantation (CTA) has achieved great development in limb or face reconstruction in adults. CTAs can also serve as a potential alternative to prosthetics and complex reconstructive surgery for severe congenital limb and face deformities which can't be reconstructed by traditional surgical intervention, as proposed by A. Gazarian [7] and K.M. Washington [8]. Moreover, the surgical feasibility and functional recovery outcome have been confirmed by V. Pathmanathan who has performed the first hand transplantation in newborn identical twins in year 2000 in Malaysia [9], and then by R.M. Zurker [430] who has performed the first successful lower limb transplantation between newborn ischiopagus identical twins and the 6-year follow-up showed excellent functional recovery [431]. Additionally, the feasibility of immunosuppressive therapy in neonates has been documented by heart or lung transplantation in neonates. Today, as listed by L.L. Bailey, about 400 transplants are reported per year to the International Society for Heart and Lung Transplantation, 25% of which are in infants [432].

However, until today CTAs have never been performed on children. As a non-life-saving therapy, performing hand or face allotransplantation remains to be unacceptable because life-long immunosuppressive therapy may be required and will result in the nephrotoxicity and hepatotoxicity of calcineurin inhibitors such as cyclosporine A and tacrolimus, the side-effects of glucocorticoides drugs on childhood growth and development, as well as the risk of tumor and opportunistic-infections [6]. The chronic rejection of allografts is also the main concern because undoubtedly children have greater demand for prolonged graft survival [13]. Induction of

donor-specific tolerance may be the only solution for these obstacles.

Neonatal transplant recipients might be able to benefit from their relatively immature immune system which is still antigen-inexperienced and consists of high percentage of naïve or even functionally defective immunologic components [406]. Theoretically, neonate stage is an immune “window phase” susceptible for induction of central transplantation tolerance [13]. We hypothesize that donor-specific central tolerance may be induced in neonates through hematopoietic stem cells transplantation under less intensive conditioning therapy and we consequently propose that CTAs may be performed on newborn infants for treatment of severe congenital hand or face defects. The objective of the present study is to induce donor-specific tolerance to vascularized composite allografts through hematopoietic bone marrow cells transplantation in neonatal swines by a relatively non-toxic conditioning protocol.

II.2. Materials and Methods

II.2.1. Animals

- **Swines used for recipients:** Fifteen newborn domestic YOUNA_piglets with a patrilineal pedigree of Large White and a matrilineal pedigree of Youli, the same age of 5~10 days, weights ranged from 2 to 3 kg, regardless of sexes, will be housed together with the sow in one cage under the standard circumstance for neonatal pigs in the Institute Claude Bourgelat, VetAgro Sup, Lyon. The sow will be fed with a standard pig diet. Newborn piglets will be breastfed exclusively and unrestrictedly.

- **Swines used for donors:** Fifteen newborn, domestic, pure Large White piglets (or other race, have to be defined) with the same age of 5~10 days, weights ranged from 2 to 4 kg, regardless of sexes.

- **Swine used for third part animal:** One newborn domestic Youli piglet (or other race, have to be defined), age of 5~10 days, weights ranged from 2 to 4 kg, regardless of sexes.

II.2.2. Groups and Immunosuppressive Regimen

○ **Groups**

- Group 0: Control Group, VCA without Immunosuppressive (IS) therapy, 4 piglets;

- Group 1: IS Group, VCA with IS, without bone marrow transplantation (BMT), 5 piglets;

- Group 2: BMT+IS Group, VCA with BMT + IS, 6 piglets.

○ **Immunosuppressive regimen**

- ATG: 20mg/kg/day, Day -2, -1;

- Prednisone: 1mg/kg/day from Day -2~Day 3, then taper as 0.5, 0.25, 0mg/kg/day at Day 4, 5, 6.

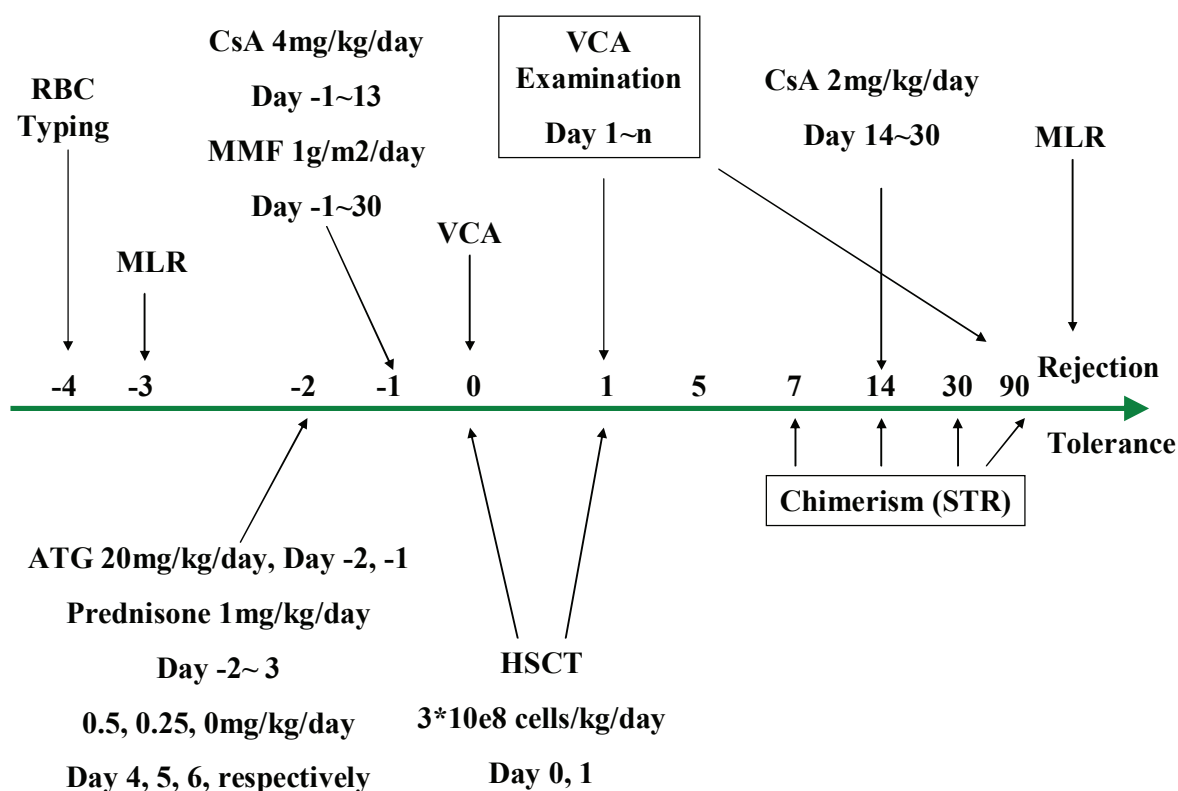
- CsA: 4mg/kg/day, Day -1~13;

 - 2mg/kg/day, Day 14~30;

- RAPA: 1mg/kg/day, Day 14~30;

- MMF: 1g/m²/day (or another dose depending on MMF study), Day -1~30.

○ **Schedule of protocol**



Tolerance Induction-Figure 1: Schedule of Tolerance Induction for VCA in Neonatal Swines.

II.2.3. Porcine Red Blood Cell Typing

Porcine RBC typing will be performed on Day -4 by ICB, Vetagro Sup according to established protocol [433].

II.2.4. Mixed Lymphocyte Reaction

MLR will be performed on day 14, 30, 60 and 90. Responder YOUNA pig PBMCs previously stain with CFSE. Responder YOUNA pig PBMCs (2×10^5) and irradiated (2500 cGy) Large White or Youli pig PBMCs (2×10^5) are combined in 200 μ l of complete tissue culture medium in flat-bottom wells in triplicate. Complete tissue culture medium for MLR assays consisted of RPMI 1640 (Life Technologies) supplement with 6% fetal porcine sera, 100 U/ml penicillin, 135 μ g/ml streptomycin, 50 μ g/ml gentamicin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, and 5×10^{-5} M β 2-ME. The cultures are incubated at 37°C

in humidified air containing 7% CO₂ for 5 days. MLR results are evaluated on Accuri C6.

II.2.5. Vascularized Composite Allotransplantation Procedure

VCA is performed as previous described [F. Solla, JRS, 2012]. On the donor, an osteo-chondro-myocutaneous free flap of right knee (containing distal femur, proximal tibia and fibula, knee articulation, periarticular muscles and tendons, and medial skin of the knee), vascularized by femoral vessels is harvested. The composite allograft is heterotopically transplanted in a subcutaneous inguinal pocket and latero-terminal anastomosis is performed on left femoral vessels.

II.2.6. ATG Administration

The pATG used in this study is a ready to use liquid solution prepared by Genzyme containing 5mg/ml of purified IgG. The osmotic pressure of the pATG solution is 272 mOsm/L. The pATG is stored at -80°C refrigerator until use.

The pATG is administrated under general anesthesia as previous study [347]. For each administration, proper dosage of pATG according to animal weight will be diluted with isotonic sodium chloride and glucose solution to a total volume of 25ml, and then will be slowly infused intravenously via epigastric vein or axillary vein during 2h with a catheter and an automatic syringe. Continuous pulse oximetry, capnography and rectal temperature will be monitored during the administration.

II.2.7. BMC Isolation, Infusion and Preservation

- **Isolation:** BMCs are harvested from the donor femurs, tibias, ilium and sternum. The bones are cut into small pieces; the marrow is minced, resuspended in RPMI 1640 with 1 mg/mL DNase, and filtered through sterile nylon mesh before centrifugation. Red blood cells are lysed with ACK buffer and the preparation washed with Hank' s buffered saline solution (HBSS) with Ca²⁺. Concentration of BMCs is adjusted to 5x10⁷ cells/ml.

- **Infusion:** On day 0, after Isolation, 6ml of BMCs (3*10⁸ cells) solution is infused immediately into the recipient through high flowed vein.

- **Preservation:** Residual BMCs solution is preserved in 4°C for the same dose of infusion on day 1.

V.2.8. Postoperative Care and General Observation

Postoperative Care will be performed as previous study [347]. Besides, the piglets are weighed every morning at 9:00AM and drug doses are calculated according to their weights. During ATG administration, continuous pulse oximetry, capnography and rectal temperature are monitored. Signs as trembling will be also observed and recorded on Day -2 and -1. Signs of Edema of soft tissues, skin lesions on anterior ankles should be examined at each time of blood sampling. The animals will be also monitored for signs of distress, sepsis, or wound complications.

II.2.9. Graft Observation

Graft Observation will be performed as previous study [433] [310, 337].

- **Gross observation:** the transplant will be monitored on a daily basis for signs of rejection occurring in a reproducible sequence of epidermilysis, desquamation, eschar formation, and necrosis. Criteria of gross rejection will also include absence of bleeding from the muscle and bone marrow.

- **Histological observation:** biopsy of donor skin, muscle will be obtained at specific predetermined times (day 7, 15, 30, 45, 60, 90, then once/month). Besides, samples of donor skin, muscle, inguinal gland lymphoid tissue, cartilage, bone and bone marrow will be harvested at the time of clinically evident rejection. Tissues will be fixed in 10% neutral buffered formalin, sectioned, and stained with hematoxylin and eosin. At the clinically defined end point, animals will be euthanized.

II.2.10. Blood Sampling

Peripheral blood samplings will be collected under anesthesia, from Day -3, then exactly before each administration of pATG, and then each morning of Day 0, 1, 4, 7, 10, 14, 17, 21, 25, 28, 32, 35...twice/week until animals are euthanized, in order to perform relevant detections.

II.2.11. Blood Cell Counts

For each blood sample, complete blood cells count assay will be performed using Sysmex 2000iV automation with specific veterinary protocol (Laboratoire Biomédicale Biovelys, VetAgro Sup campus Vétérinaire, Marcy l'Etoile, France).

II.2.12. Examination of Renal, Hepatic Function

Examination of hepatic and renal functions will be also performed by measuring the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Cr) and blood urea nitrogen (BUN) with a KONELAB KL20 ISEND automatic analyzer in Laboratoire Biomédicale Biovellys.

II.2.13. CsA Trough Level Monitoring

CsA whole blood concentration is measured just before drug administrations in the morning of Days -1, 1, 4, 7, 10, 14, 17, 21, then twice/week with each blood sampling to determine the changes of the trough concentration. CsA whole blood concentrations will be measured by BIOMNIS (Lyon, France) using the antibody-conjugated magnetic immunoassay (ACMIA) method on the Dimension® RxL Max® Integrated Chemistry System (Siemens Healthcare Diagnostics S.A.S., Saint Denis, France).

II.2.14. MMF Trough Level Monitoring

MMF will be monitored by MPA serum concentration on Days -1, 4, 7, 14, then once/week with each blood sampling to determine the changes of the trough concentration. MPA serum concentration will be measured in UF de Pharmacologie Spécialisée, Hôpital Edouard Herriot, Lyon.

II.2.15. ATG Pharmacokinetics

ATG pharmacokinetics will be monitored by pATG binding on lymphocytes and neutrophils on Days -2, -1, 0, 1, 4, 7, 10, 14, 17, 21, then twice/week with each blood sampling. Briefly, 25µl peripheral blood sample will be washed twice with FACS buffer, stained with Goat anti Rabbit IgG (H/L)-FITC at 4°C for 30min, then lysed red blood cells with BD FACS™ Lysing Solution at 4°C for 15min, washed twice with FACS buffer and proceeded to BD Accuri™ C6 flow cytometer.

II.2.16. Analysis of Lymphocyte Subsets in Peripheral Blood and Lymphoid Tissues by Flow Cytometer

Peripheral blood samples will be collected at the indicated times and proceeded to analysis of the alteration of lymphocyte subsets (T, B and NK cells) and regulatory T cells. To analyze lymphocyte subsets, 25 µl of heparinized blood will be centrifuged for one time with FACS buffer in a 1.7 ml micro centrifuge tube, then incubated with

FITC-Mouse anti-Porcine CD3 ϵ , PE Mouse Anti-Human CD21, PE-CyTM7 Mouse anti-Pig CD4a and Alexa Fluor® 647 Mouse Anti-Pig CD8a at 4°C for 30min, then lysed red blood cells with BD FACSTM lysing solution at 4°C for 15min, washed twice with FACS buffer and proceeded to BD AccuriTM C6 flow cytometer. Gate of Lymphocytes will be set according to their light scatter signals in FSC-SSC dot plot. Different lymphocyte subpopulations will be chosen for analysis by phenotypes according to [434]. T cell is identified as CD3 ϵ ⁺CD21⁻, B cell is identified as CD3 ϵ ⁻CD21⁺. CD3 ϵ ⁺CD21⁻ cell is discriminated by their expression of CD4a and CD8a either as CD4a⁺CD8a⁻ T helper cells, and CD4a⁻CD8a^{high} cytotoxic T cell. CD3 ϵ ⁻CD21⁻ cell is examined by their expression of CD8a to identify CD3 ϵ ⁻CD21⁻CD8a⁺ NK cells. Percentages for lymphocyte subsets will be converted to absolute cell numbers per μ l of blood according to lymphocyte count in blood cell count.

To analyze regulatory T cells, 25 μ l of heparinized blood will be centrifuged, then Mouse Anti-Pig CD25 labeled with Zenon Alexa Fluor® 647 Mouse IgG1 kit at 4°C for 30min, washed for one time with FACS buffer, stained with FITC-Mouse anti-Porcine CD3 ϵ and PE-CyTM7 Mouse anti-Pig CD4a, then performed the intracellular staining of Foxp3 with PE-anti-mouse/rat Foxp3 staining set (Ref. 72-5775-40, Clone: FJK-16s, eBioscience, France) according to indicated intracellular staining protocol, then proceeded to BD AccuriTM C6 flow cytometer. Regulatory T cells will be identified as CD3 ϵ ⁺CD4a⁺CD25^{high}Foxp3⁺ cells and expressed by their percentage in CD3 ϵ ⁺ T cells or their absolute count.

Surrounding tissues will be removed from mandibular lymph nodes, spleen, and thymus. Proper sizes of tissues will be collected and weighed on the balance, respectively. Tissues will be minced with sharp scissors and then pounded with rubber plugs from 5ml syringes to release the cells into a Petri dish containing RPMI 1640/10% FBS. Cell suspensions will be filtered through 100 μ m nylon cell strainers (Ref. 352360, BD FalconTM, France) into 50 ml FACS tubes. Centrifuged cell suspensions and lysed red blood cells with BD FACSTM lysing solution. Centrifuged for one time, and then counted cells with BD AccuriTM C6 flow cytometer. Adjusted

concentrations of cell suspensions to 1×10^6 cells/ml. Analysis of the lymphocyte subsets is performed on cells extracted from lymphoid tissues according the protocol for peripheral blood.

II.2.17. Analysis of Chimerism in Peripheral Blood and Lymphoid Tissues by STR Typing

Existence of chimerism in PBMCs at specific predetermined times (day 4, 7, 14, 30, 60, then once/month). Besides, chimerism in recipient inguinal/ mandibular gland lymphoid tissue, spleen, thymus and bone marrow will be detected at the time of euthanasia of animals.

STR typing will be performed according to previous studies [435, 436]. Primers will be designed as (more primers may be required):

SW24 Forward: 6FAM- CTTTGGGTGGAGTGTGTGC

SW24 Reverse: ATCCAAATGCTGCAAGCG

S0107 Forward: 6FAM- CAAGGATGCCTGTAAGTGGTGCAG

S0107 Reverse: TCCTTAAGGCCTCGTAGGATCTGT

Briefly, PCR reactions will be carried out in a GeneAmp™ PCR System 9700 cycler in a total volume of 12.5 μ l, containing 336 μ M of each dNTP, 1.2 \times PCR buffer (containing 15 mM MgCl₂, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 0.01% gelatin), 1.8 U Taq polymerase and 20~100 ng DNA. The primers of selected microsatellite sites markers will be used in concentrations from 100 nM to 600 nM. PCR conditions included an initial denaturation step of 10 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 58°C, 1 min at 72°C and a final extension of 60 min at 72°C.

Each 1 μ l of PCR product and 0.5 μ l of an internal lane size standard will be loaded in 11.5 μ l of deionized formamide. The samples will be then denatured at 95°C for 5 min and inserted into the ice. The polymorphism of microsatellite sequences will be examined by the PCR method and capillary electrophoresis, based on laser scanning of fluorescence-marked DNA fragments. Genotyping will be done on ABI PRISM 310™ Genetic Analyzer by fluorescent fragment analysis and evaluated by software GeneScan® 3.7 NT and Genotyper® 3.7 NT.

II.2.18. Assessment of GVHD

Assessment of GVHD in piglets will be performed as previous study [437]. Briefly, the intensity of recipient skin GVHD changes will be assessed by daily observation. The grading system used is: grade 0, no skin changes; grade 1, skin lesions localized to a few areas of the body; grade 2, widespread eruption and mild crusting, and grade 3, aggressive form of 2 with thick crust and ulceration. Skin biopsy will be performed on selected cases to confirm the observed changes. Histological features of GVHD in the skin will be graded normal, mild, moderate or severe. At autopsy, all major organs, skin and lymph nodes were examined and subjected to histological examination.

II.2.19. Assessment of Thymic Function

Thymic function will be evaluated by detection of T-cell receptor excision cycles (TREC) in PBMCs at specific predetermined times (Day 7, 14, 30, 60, then once/month) and at the time of euthanasia of animals, according to previous study [438]. Briefly, primers will be designed for QPCR reaction, with the expected PCR product containing the RSS of the δ -rec and ψ J α regions.

In a total volume of 25 μ L, each reaction included:

(1) 800 nM of ψ J α forward primer: 5'-TCT AAA GAG GAA GAA CAA GGT TGG CG-3',

(2) 800nM of δ -rec reverse primer: 5'-TGT GCA AAG CTG TGA AAT GCT CCC-3',

(3) 200 nM of labeled probe: 5'-/56-FAM/ATG CAG GAG GGC CAC GAG TGA AGA GCA GAC AGA/36-TAMSp/-3'.

Other reagents included:

(4) 0.25 μ L Hot start Taq polymerase (Qiagen),

(5) 2 μ L 10 mM dNTPs, (6) 150 ng of DNA template,

(7) 2.5 μ L of 10 \times Hot start Taq buffer.

The cycling conditions will be as follows: 95°C \times 15 min, followed by 50 cycles of the steps: 94 $^{\circ}$ \times 30 s, 55°C \times 60 s, 72°C \times 30 s. All QPCR reactions and quantitative analyses will be performed using a *Stratagene*.

II.2.20. Assessment of Humoral Reaction in Serum

Humoral reaction will be evaluated by measurement of the pig Ig in peripheral

blood serum at the mean time of peripheral B lymphocytes detection with ELISA. The 96-well plate will be coated with Goat Anti-Swine IgG (H+L) (Jackson ImmunoResearch, Ref. 114-005-003), pig serum Ig will be detected with Alkaline Phosphatase-AffiniPure Goat Anti-Swine IgG (H+L) (Jackson ImmunoResearch, Ref. 114-055-003).

II.2.21. Assessment of Humoral Reaction in the Graft

Humoral reaction by Immunohistochemistry of porcine IgG and C4d in graft skin and muscle will be assessed at the same time with H-E histological examination, as previous study [439]. Porcine IgG will be detected by Biotin-SP-AffiniPure Goat Anti-Swine IgG (H+L) (Jackson ImmunoResearch, Ref. 114-065-003). Porcine C4b will be detected by Complement C4B antibody (antibodies-online, Ref. ABIN1106789).

II.2.22. Assessment of T_{reg} in the Graft by Immunohistochemical Examination

Assessment of Treg in graft skin will be assessed by immunohistochemical examination at the same time-point which H-E histological examination, as previous study [303]. Briefly, the skin specimens will be formalin-fixed and paraffin-embedded. Five-micrometer thick sections will be stained immunohistochemically with antibody detecting nuclear FoxP3 (Anti-Mouse/Rat Foxp3 Biotin, ebioscience, Ref. 13-5773-82), after antigen retrieval. Negative controls were obtained by omitting the first layer antibody.

II.2.23. Skin Graft

In piglets which have prolonged VCA survival time until day 90, a non-vascularized graft of frozen skin (from the same donor with VCA) will be performed, in order to evaluate whether the tolerance to non-vascularized skin graft (which is more allogenic than vascularized skin component in VCA) has been induced in these animals. This skin graft can also be used to confirm the outcome of donor-specific tolerance in these animal. The skin graft will be performed with a procedure described in previous study on pig [273].

II.3. Perspective

In summary, we will evaluate tolerance induction and maintenance by measuring

chimerism after transplantation using short tandem repeats (STR) tests in molecular biology. If chimerism is missing or insufficient, cyclophosphamide can be added in the next experiments. If donor-specific tolerance for VCA could be induced with present protocol, in year 2016 we will subsequently elaborate an applicable tolerance induction protocol and hand allotransplantation program in human newborn infants. If favorable results are obtained, we will launch this program and search proper candidates of donors and recipients in year 2017.

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Appendix A: List of Abbreviations

APCs, antigen-presenting cells;

ALS, anti-lymphocyte serum;

ATG, rabbit anti-human globulin;

BM, bone marrow;

BMCs, bone marrow cells;

BMT, bone marrow transplantation;

CBMT, cellular bone marrow transplantation;

CD, cluster of differentiation;

CD40L, CD40 ligand;

CML, cell-mediated lymphocytotoxicity;

CM-PBMCs, cytokine-mobilized peripheral blood mononuclear cells;

CNI, calcineurin inhibitor;

CsA, cyclosporine A;

CTL, cytotoxic T lymphocyte;

CTLA-4, cytotoxic T-lymphocyte antigen 4;

DCs, dendritic cells;

FK506, tacrolimus;

FOXP3, forkhead box P3;

GVHD, graft-versus-host-disease;

HLA, human leukocyte antigen;

HSCs, hematopoietic stem cells;

HSCT, hematopoietic stem cells transplantation;

IFN, interferon;

IL, interleukin;

IUHSCT, in utero hematopoietic stem cells transplantation;

LPS, lipopolysaccharide;

LC, Langerhans cells;
mDCs, myeloid DCs;
MDDC, monocyte-derived dendritic cells;
MHC-I, major histocompatibility complex-class I;
MLR, mixed lymphocyte reaction;
MLV, murine leukemia virus;
MMF, mycophenolate mofetil;
mTECs, medullary thymic epithelial cells;
PAMPs, pathogen-associated molecular patterns;
pATG, rabbit anti-pig thymocyte globulin;
PBSCs, peripheral blood stem cells;
pDCs, plasmacytoid DCs;
PerC, peritoneal cavity;
PLTs, platelets;
PRRs, pattern-recognition receptors;
PTD, post-transplantation day
qPCR, quantitative polymerase chain reaction;
RBCs, red blood cells;
STR, short tandem repeats;
SLA, swine lymphocyte antigen;
TBI, total body irradiation;
TCR, T-cell receptor;
T_h, helper T cells;
TLI, total lymphoid irradiation;
TREC, T-cell receptor recombination excision circles;
T_{reg}, regulatory T cells;
VBMT, vascularized bone marrow transplantation;
VCA, vascularized composite allograft;
WBC, White Blood Cell.

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