Investigations relating to the Induction of Immunological Tolerance through Spleen Transplantation in Miniature Swine

Frank Johan Marinus Frederik Dor

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Aan allen die aan mijn persoonlijke en professionele ontwikkeling hebben bijgedragen

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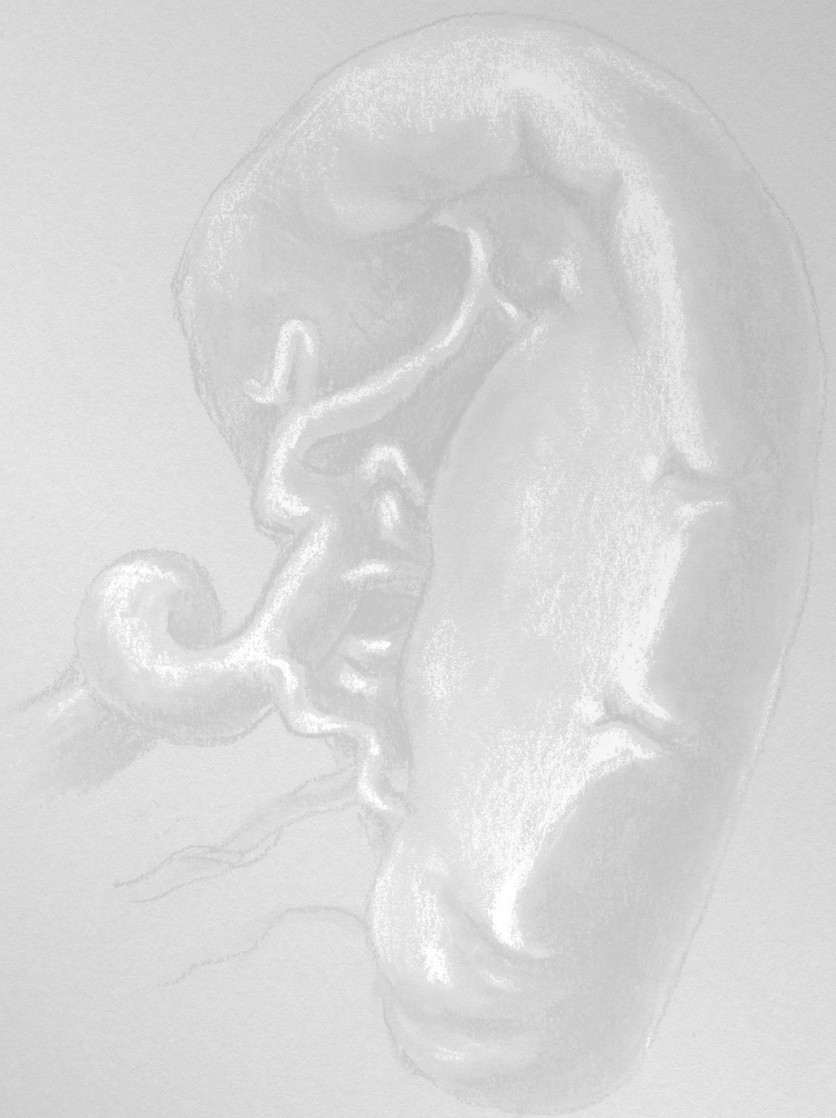
General Introduction



1

If you desire the spleen and will laugh yourself into stitches, follow me.
William Shakespeare (1564-1616) (Twelfth Night, Act 3;2:62-63)

This general introduction gives an extensive review on topics that are covered in this thesis, such as the spleen, transplantation and tolerance, as well as the Massachusetts General Hospital miniature swine model in relation to transplantation. It is the author's opinion, that this background information is beneficial for a better understanding of the studies and their aims as described in the chapters following the introduction.

HISTORICAL VIEW ON THE SPLEEN

The spleen is an enigmatic organ with a peculiar anatomy and physiology; its history is full of fables and myths, but it is also full of realities. The question "Este igitur splenatam necessarius?" ("Is the spleen necessary for survival?") has been the central question for physicians such as Galen, Vesalius, Malpighi, Billroth, Gray, Gross, and Mayo. It remained unanswered until recently.

The modern English term *"spleen"* descends from the Grecian "*σπλήν*" *(splén),* which may have been derived from *"σπλαγχνον" (splanchnon:* a viscus) or from *"σπάω" (spaw:* to draw*)*. The ancients believed that this viscous organ draws spoiled parts of the blood to itself, making both of these derivations equally plausible. Take away the *"sp"* in *splén* and the Latin term *lien* is born. The Teutonic term (and modern Dutch term) for spleen, *milt*, and its Germanic form, *milz*, offer additional suggestions as to possible splenic function; they are related to the milt (roe or spawn) of a male fish, meaning soft like a spleen. *Milt* is also related to the Icelandic *melta*, which means "to digest" (1,2).

In ancient times the spleen was thought to be related to the digestive system. Erasistratus believed that the spleen maintained the symmetry of the abdomen, but had no further function. Plato claimed that its function was to keep the liver "bright and shining". Hippocrates proposed a vital balance of four essential humours of the body: blood, phlegm, golden bile and black bile. In spite of the lack of anatomical and histologic knowledge in this period, he had described the anatomy of the spleen with remarkable accuracy. The description of function, however, was described differently from that of today; the liver was supposed to be the source of golden bile and the spleen of black bile. Galen believed that "humours unsuitable for its nutriment are discharged by the spleen through a canal into the stomach". He called the spleen: "Splenum mysterii organon" (3).

During the 17th and 18th century the main contributions to the study of the spleen consisted of careful anatomical dissections. In 1777 William Hewson recognized associations with the lymphatic system. Rudolph Virchow demonstrated in 1846 that the follicles in the spleen were related to the white blood cells, and in 1885 Ponfick recognized the ability of the spleen to remove particles from the blood (4).

About thirty years later, Morris and Bullock described the spleen as an important organ in the resistance to infections (5). O'Donnell reported a case of "acute septicemia" in a 6-year-old boy 2 years after splenectomy in 1926. The boy's father who had had a splenectomy in 1919 also died "of septic pneumonia, manifesting a similar lack of resistance to the disease" (3). Perla and Marmoston discussed the role of the spleen in resistance against infection in 1935 (6). It was only after the publication of King and Shumacker on postsplenectomy infections in 1952 (7), that there was a rise in concern over the decrease in resistance against infections as a consequence of splenectomy. After this publication, the immunological aspects of the spleen became increasingly the targets of scientific interest.

THE SPLEEN

Now we come to the spleen, of it we know nothing. So much for the spleen.
Emil du Bois-Reymond (1818-1896)

Embryology

During embryogenesis, the formation of the splanchnic mesodermal plate (SMP) at day 12 is the initial event in the development of the spleen, part of the formation of the asymmetrical left-right axis. When formation of the SMP is defective, then no spleen is formed (8,9). Progenitor cells of the erythroid and myeloid lineages are the first cells to colonize the spleen, and, at day 14.5 hematopoietic stem cells lodge in the spleen (10). From the 4th week of gestation, mesenchymal cells in the dorsal mesenterium of the foregut form lobules. Several of these lobules fuse, resulting in the definite form of the spleen. The dorsal part (spleno-aortic ligament, between spleen and dorsal body wall) of the dorsal mesenterium, containing the splenic artery and pancreatic tail, lengthens and is pulled to the left by the growing greater curvature of the stomach. Then, it fuses with the dorsal parietal peritoneum at the level of the left kidney, becoming splenorenal ligament. The ventral part of the dorsal mesenterium becomes the gastrosplenic ligament (together with the more caudal greater omentum) with, cranially, the short gastric vessels and, caudally, the left gastro-epiploic vessels (11).

Anatomy

The spleen is an oblong, aubergine-coloured organ, with the size of a clenched fist and the shape of a bean. In adult humans, the size varies from 12-15 cm (length), 4-8 cm (width), 3-4 cm (thickness). Its weight is approximately 150g in healthy adults (12). The spleen is located in the left upper quadrant of the abdominal cavity, sheltered by the 9th-11th ribs (13) (Figure 1). The spleen is soft in consistency, friable and is shaped by adjacent, firmer viscera. Together with the visceral peritoneum it forms strong suspensory attachments to the stomach (gastrosplenic ligament), diaphragm (phrenicosplenic ligament), kidney and pancreas (splenorenal or pancreaticosplenic ligament), colon (phrenicocolic ligament) and sometimes with a peritoneal fold to the abdominal wall on the left posterolateral aspect (14) (Figure 2). The medial side of the spleen has a long fissure; this forms the hilus, which is the site of the main entrance and exit for the blood vessels (13). There are no lymphatics leading into the spleen; in contrast to the lymph nodes, which are inserted in the lymph circulation, the spleen is inserted in the blood circulation.

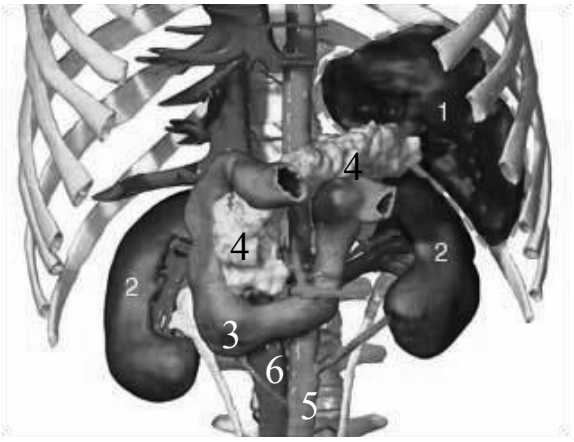


Figure 1. Anatomical position of the human spleen.
1, spleen; 2, kidney; 3, duodenum; 4, pancreas; 5, abdominal aorta; 6, inferior vena cava.
See addendum for colour version

Around the spleen is a strong fibrous capsule with collagenous trabeculae extending into inside the parenchyma. The splenic parenchyma can be divided into white and red pulp as can be seen on fresh surgical specimens.

The spleen is the largest lymphoid tissue of the body, accounting for about 25% of the body's lymphocytes. Blood flow through the spleen is immense relative to its size: this 150g organ in adult humans (less than 0.3% of the body weight) receives about 200-300 ml/min (~5%

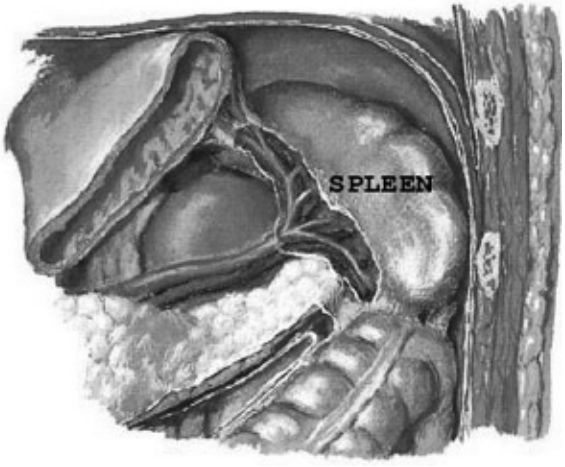


Figure 2. Anatomy of the upper abdomen with respect to the spleen.
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of cardiac output) (15). The bulk of this blood flow percolates through the splenic red pulp – the part of the spleen which functions as a general filter, removing particulate matter and damaged erythrocytes from the circulation (16-19). However, this blood flow also supplies the other, more immunologic compartment of the spleen with both antigen and lymphocytes. This compartment, the white pulp (accounting for approximately 5-20% of the splenic parenchyma in humans (20)), is the splenic version of secondary lymphoid tissue, and it constitutes the major initiator of immune responses against blood-borne antigens (21). The white pulp is organized as lymphoid sheaths, with B and T cell compartments, around the branching arterial vessels.

The splenic artery enters the spleen at its hilum and immediately branches into smaller "trabecular" arteries, which in turn branch into even smaller "central" arteries. These central arteries course through the splenic parenchyma, eventually branching into "penicilliary" arterioles that empty into the red pulp. Investing the central arteries and penicilliary arterioles is a concentric cuff of lymphocytes, the great majority of which are T cells. These cuffs, termed the periarteriolar lymphocytic sheaths (PALS), constitute the T cell zones of the spleen. Periodically, usually at arterial branch points, B-lineage lymphoid follicles, either primary or secondary, appear as outgrows of the PALS.

Surrounding the primary follicle or mantle zone of the secondary follicle (and the PALS in rodents) is the marginal zone (MZ), a uniquely splenic lymphoid compartment. The MZ contains both B and T cells (usually a majority of the former), and is thought to be the site in which splenic B and T cells initially encounter blood-borne antigens (21,22). Indeed, the MZ appears to be a primary site of entry of both B and T lymphocytes into the white pulp. MZ B cells in the spleen

are strategically positioned at the blood-lymphoid interface and are programmed to initiate a fast and intense antibody response to blood-borne viral and bacterial agents (23,24). Their ability to respond vigorously to antigen and polyclonal activators make MZ B cells key players in the early response to pathogens in the blood stream. The specialized functions of these innate-like lymphocytes bridge the gap between the early innate immune response and the slower adaptive antibody response, affected mainly by the more prolific follicular B cells (25).

The spleen lacks high endothelial venules (HEV; as in lymph nodes), but it is thought that specialized MZs subserve an analogous function to the HEVs in other secondary lymphoid sites (26). MZs are not found in secondary lymphoid tissues other than spleen.

Histology

The splenic parenchyma can be divided into two general components: the white pulp (± 5-20%) and the red pulp (± 85%) enclosed by a capsule and interspersed by trabeculae (27).

Capsule

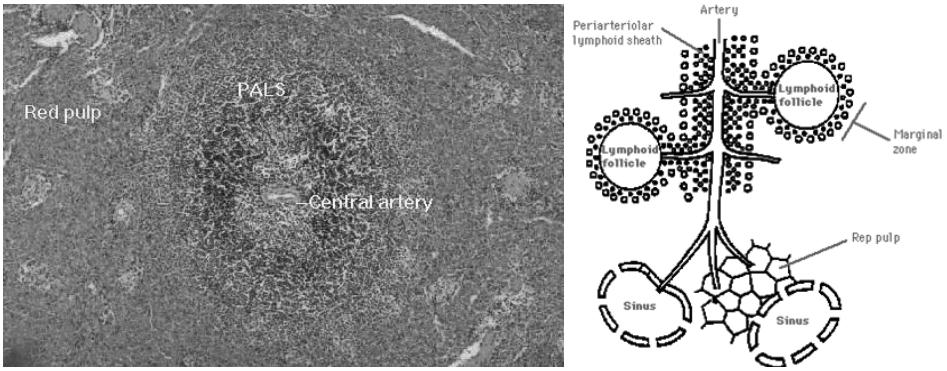
The capsule is composed of dense connective tissue with a few smooth muscle cells, reflecting the minimal contractile capacity of this capsule in man (in dogs it is highly contractile) (28). Serosa covers the capsule except at the hilus where vessels enter the spleen. From the inner surface of the capsule a branching network of trabeculae subdivides the spleen into communicating compartments. These trabeculae carry the blood and lymph vessels into the splenic pulp (21,29,30).

White pulp

The white pulp is composed of lymphatic tissue, primarily lymphocytes. In the fresh state, it appears as small, round pale white or gray areas surrounded by red pulp (hence the name "white pulp"). However, by H&E with light microscopy, it appears deeply basophilic due to the concentration of lymphocytes.

The white pulp is composed of 3 major compartments that are easily recognized in routinely stained histological sections (Figure 3A). These compartments are the PALS, the lymphoid follicle (LF) and the MZ (31).

The PALS will parallel the course of the central arteries. The lymphocytes that make up the PALS are primarily T-lymphocytes. The presence of the artery helps distinguish the PALS from LFs that may also form along the course of the central artery. The PALS is the T-lymphocyte compartment of the white pulp in which T-lymphocytes are interspersed in concentric layers of stromal cells around a central artery (Figure 3A). The lymphocytes of the PALS are mostly recirculating cells. The PALS is a site of T-cell clonal expansion. A small percentage of the T-cells



A B

Figure 3.
(A) Cross section of the spleen with white and red pulp zones (Giemsa-stained).
(B) Schematic representation.
See addendum for colour version

within the PALS are in an activated state, demonstrated by IL2-receptor (CD25) expression. The other T-cells are in a resting state (31).

The LFs of the spleen are globular structures attached to the PALS (Figure 3B) with similar structure as LFs in other lymphoid organs (32). Like LFs in the lymph node, these are composed chiefly of B-lymphocytes. Germinal centers (GC) with mantle zones (B-cells) and MZs (B- and T-cells) may be present and result in the formation of large nodules known as Malpighian corpuscles. They can be differentiated into primary and secondary LFs. The primary LF consists of a homogeneous aggregate of small B-cells in an inactivated state. Upon activation the primary LF will become a secondary LF with a GC surrounded by a small rim of remaining small B-cells, the lymphocyte corona (LC) (Figure 4). This GC of the secondary LF consists of differentiated B-cells (centroblasts and centrocytes), a few T-cells, and follicular dendritic reticulum cells. In the LFs, a special type of dendritic cells (follicular dendritic cells) is found (33), which are able to bind immune complexes. They can maintain immune complexes on their cell surface for a long period without phagocytosis. This seems to play a role in the down-regulation of the production of plasma cells (34).

GCs provide a site for rapid proliferation of B-cells with isotype switching and affinity maturation. Upon maturation of the GC, cell division stops and cells differentiate into memory cells or plasma cells, which acquire the ability to migrate out of the GC (35). A specific type of mononuclear macrophages is present that phagocytose defective lymphoid cells and debris in the GC (36).

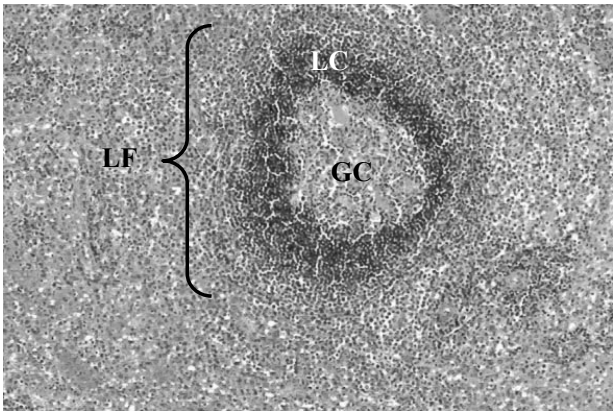


Figure 4. Lymphoid follicle (LF) in the spleen. LF, lymphoid follicle; GC, germinal center; LC, lymphocyte corona.

The GC is surrounded by a small border of small lymphocytes (in fact the pre-existent cells of the former primary follicles); these form called the LC or mantle zone. The LC in its turn is enclosed by medium-sized lymphocytes, mostly B-cells, the MZ. The MZ is an anatomical demarcation between the white pulp and the red pulp. The real border is formed by the perifollicular zone (27). A great part of the arterial circulation within the MZ terminates intercellularly, and at the outer side of the MZ sinuses are present, which are smaller than the sinusoids of the red pulp. The MZ provides an environment, which by its low blood flow allows a prolonged and intimate contact between antigens in the bloodstream and the lymphocytic system (31,36,37).

Lymphoid cells in the MZ have been demonstrated to possess surface immunoglobulin (Ig) (mainly IgM, in absence of IgD), as well as receptors for Fc-fragments and complement factors (C3b and C3d) (31,36,37). It is because of this low flow in combination with a specific type of B-cells that the MZ is supposed to have an important role in the primary immune response to T-cell independent antigens type 2 (TI-2 antigens) like the polysaccharide encapsulated bacteria, e.g., *pneumococci, meningococci, and Haemophilus influenzae*. The MZ is a distinct anatomical lymphocyte compartment in the spleen with unique immunohistological features (38).

Red pulp

The red pulp consists of a loose reticular tissue, rich in capillaries and venous sinusoids. These sinusoids comprise approximately 30% of the volume of the red pulp. They form a meshwork with many interconnections, but also bulb-like extensions with blind ends projected into the cord tissue (27,39). The sinusoids have an unique endothelium of longitudinally arranged cells. These run parallel to the long axis of the sinusoids like the staves of a barrel, and possess close junctional complexes at regular intervals along their lateral surfaces to the white pulp veins.

Slit-like spaces, which can be penetrated by cells flowing from the pulp cords, separate the endothelial cells. The basal membranes have been shown to contain actin and myosin, which can probably contract to vary the tension in the endothelial cell and the dimensions of the interendothelial slits. The interendothelial slits are a critical point in the pathway of particulates through the spleen and in the filtration function (18,40-42).

Part of the red pulp tissue has a reticuloendothelial nature with small aggregates of B- and T-lymphocytes and many mononuclear phagocytes. Morphometrically, the size of the lymphoid, non-filtering red pulp compartment seems to equal that of the white pulp (43). The macrophages are not simply phagocytic cells, but also have secretory capacities and in this way enhance the immunogenicity of antigens. They have the ability to produce components of complement factors, interferon, hematopoietic colony-stimulating factors and fibroblast-stimulating factors. This whole system is part of the so-called mononuclear phagocytic system (MPS) (44).

Vasculature, lymphatics and innervation

Arterial supply

As 5-10% of the cardiac output at rest passes through the spleen, the spleen has to be richly vascularized. It is supplied by the tortuous (in later life often calcified) splenic artery, which is the largest of the three branches of the celiac trunk, which originates from the abdominal aorta. After passing the upper body of the pancreas horizontally, giving a few branches to the stomach (left gastro-epiploic artery and short gastric artery) and pancreas (large pancreatic artery), the splenic artery divides into several branches about 1-3 cm from the hilum. These branches will divide further, into superior and inferior branches (enabling partial splenectomy), subdividing into several smaller branches and finally enter the spleen in the hilus. Ramifications of the splenic arterial branches develop internally into trabecular arteries, which pass through the white pulp as central arteries, branches of which supply the lymphatic nodules in the white pulp. From the centre of the lymphatic node the artery can pass through to the red pulp or split into branches in the marginal zone. Via marginal zone sinuses the blood can also reach the red pulp. The red pulp is assumed to have two systems for the blood circulation, which will be described under *Microcirculation*, below.

Venous drainage

The venous drainage commences in the venous sinusoids, located in the red pulp, subsequently draining into trabecular veins. The trabecular veins terminate in branches that unite to form the splenic veins and follow the arteries at the hilus of the spleen. Here, it receives the short gastric and left gastro-epiploic veins. The large retropancreatic splenic vein represents 40% of the total portal flow. It passes along the dorsal and superior part of the pancreas and,

with the superior mesenteric vein, becomes the portal vein. At a short distance before the superior mesenteric vein, the inferior mesenteric vein empties into the splenic vein.

Lymphatic drainage

In contrast to the extensively-distributed blood vessels, the lymphatic vessels in the spleen are few in number. Lymphatic capillaries originating in the splenic capsule and trabeculae converge in lymph nodes of the hilus outside the spleen and subsequently drain along the arteries into parapancreatic, celiac and para-aortal nodes.

Innervation

The spleen merely has a vasomotor innervation from the celiac ganglion and plexus.
The nerve supply follows the splenic artery in the hilus and innervates the musculature of the branching vessels. Sympathetic nerve fibres densely innervate the spleen. Their endings contact immune cells within the spleen, particularly in areas of T cells and macrophages (building the neuroimmune junction). Neurotransmitters are released into the vicinity of nerve terminals and bind to specific postsynaptic receptors on the surface of these cells. Local bi-directionality exists through cytokines and neurotransmitters from immune cells that modulate the release of sympathetic neurotransmitters from nerve terminals. This complex 'dialog' depends on microenvironmental factors such as infectious agents, and this 'conversation' is needed to balance the function of both the sympathetic nerve terminal and the immune system. Activation of the sympathetic nervous system and also the resting sympathetic nervous tone are important for controlling innate and adaptive immune responses (45). Also, preganglionic parasympathetic fibres of the right vagal nerve follow the splenic artery into the spleen, although the role of parasympathetic innervation of the spleen remains obscure (46).

Microcirculation and lymphocyte circulation

Microcirculation

The microcirculation of the blood in the spleen is perhaps the most complex of any organ in the body. It contains blood with a packed cell volume twice that of arterial blood. Most studies of the microcirculation in the spleen have been performed in animals and the results were often extrapolated to the human spleen. It is not clear whether these results are sufficiently representative for the human situation, because the histology and subsequently the microanatomy of the human spleen seem to differ between species (21, 27, 31, 47). However, because of difficulties in investigation of the spleen in humans, we have to rely on well-designed animal experiments to provide useful hints in the elucidation of the complex mechanisms in the human spleen.
The spleen is the only organ specialised in the filtration of blood. It has been suggested that there is a fast and a slow pathway of the bloodstream in the red pulp of the spleen for which

two compartments are assumed to exist for this bloodstream within the spleen. The first system is the closed circulation with direct connection via the sinusoids and collecting veins to the trabecular veins (Figure 5). The second (more important) system is the open circulation with arterial vessels ending blindly in the red pulp cord spaces. From the cords, the blood runs intercellularly and subsequently is collected in sinusoids, from which it will be transported by pulp veins to trabecular veins. The fast compartment is intra-vascular, whereas the slow compartment is in the reticular meshwork (19,48).

Some arterial capillaries of the red pulp show cyclic changes in luminal calibre, with sometimes a very low to absent flow. Erythrocytes pass through interendothelial slits in venous sinus walls always from the reticular meshwork into the sinuses (18).

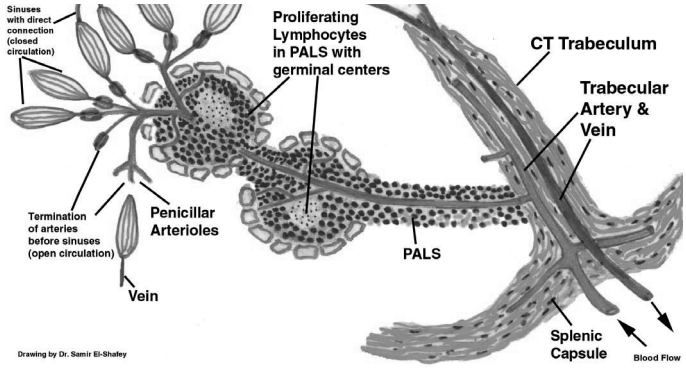


Figure 5. Schematic representation of the blood stream in the spleen.

Lymphocyte circulation

A unique feature of lymphocytes, in contrast to all other cells of the blood, is their continuous migration between lymphoid and non-lymphoid organs through the lymphatic and blood vessels. Granulocytes and monocytes mostly remain in organs once they have left the blood stream, but lymphocytes may temporarily leave the blood stream and return to it at a later stage (lymphocyte recirculation). This recirculation of lymphocytes is important for their ability to recognise antigens throughout the body and for the interaction between accessory and lymphoid cells in initiating immune reactions (27). The extent of lymphocyte recirculation in the spleen by the blood far outweighs the total number of lymphocytes using the classical route via lymph vessels and thoracic duct. In a young adult human about $2{,}5x10^{14}$ lymphocytes recirculate through the spleen per day, which is approximately 8 times more than through all lymph nodes (49). The lymphocytes enter the spleen through the arterial blood stream and migrate to several splenic compartments.

T-lymphocytes rapidly enter the central part of the PALS, while B-lymphocytes persist in more peripheral parts of the PALS, and by 24 hours are evenly distributed throughout the LC. A few migrating B-cells are found in the GCs, but no T-cells. It is unknown as yet whether the venous route or the lymphatic route is the most important outflow for lymphocytes of the spleen; probably the venous route is more important than that via lymphatic vessels. The exact migratory mechanisms and routes of the lymphocyte subsets through all the splenic compartments are very complex and have not yet been fully clarified (50,51).

Functions of the spleen

The spleen is a unique organ in the immune system; first, it is the only organ that can clear low opsonized antigens from the blood stream, secondly, it is the sole organ that is specialised in producing antibodies in a short time after contact with antigens. Besides this, the spleen is a true lymphoid organ with several organized lymphoid compartments. Because of its central position in the blood stream and its large blood supply, the spleen represents an important meeting point between antigenic information transported by the blood and the immune system. It possesses a wide range of the immune cell repertoire and its specific architecture allows unique functions. The functions of the spleen can be divided into non-immunological and immunological.

Non-immunological

Filtration of the blood is the best known and a(n) (quantitatively) important function of the spleen. The reticular meshwork in the red pulp, with the terminal arterial vessels and the venous sinusoids, is specialised in filtration of the blood. When blood passes the endothelial wall of the sinusoids, blood cells have to pass through the interendothelial slits. These slits are only small in diameter; hence, during this passage the blood cells have to deform, subsequently to regain their normal form (Figure 6). If the cells loose their deformation capacity or if the cell walls are

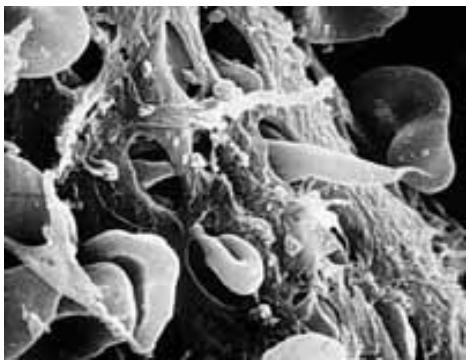


Figure 6. Passage of an erythrocyte through a sinusoid.

too fragile, they cannot pass through this filtration system (52). Erythrocytes with intracellular inclusions (pittings, Howell-Jolly bodies, intra-cellular organisms such as malaria, etc.) can be cleared of these inclusions during the passage without destroying the entire cell. The membrane of the cell reseals and subsequently passes into the sinuses and the general circulation. The perisinusoidal phagocytic cells will clear the inclusions and the aged blood cells (53, 54).

The spleen has a reservoir function for a large number of all kinds of blood cells by means of a process that is not yet understood. This storage function is mainly for platelets, but also for erythrocytes and lymphocytes. Of all platelets in the body, approximately 30% may be stored in the spleen. The spleen is the largest lymphoid organ with 25% of all white blood cells of the body, mainly lymphocytes. Only 5% of the red cells are supposed to be stored in the spleen. The number of blood cells in the spleen at a given time depends on the presence or absence of pathology in the spleen and/or of the blood cells. In the reticular meshwork, the hematocrit of the blood is twice that of arterial blood. The spleen appears to function as a "nursery" for reticulocytes after their release from the bone marrow (BM) (55), and is supposed to play a role in final maturation. Reticulocytes have a reduced negative surface charge, are less flexible, contain unnecessary organelles and are bigger than mature red cells. The reticulocytes will be sequestered in the red pulp for two days because of these properties, thus allowing them to mature. After maturation, the erythrocytes will be remodelled, loose certain adhesion molecules, and then emerge into the circulation (56-58).

Additional known functions of the spleen are hematopoiesis during fetal life and, when hematopoiesis in the BM is failing, e.g., from dysplasia or myelofibrosis, the spleen can regain its fetal role in extramedullary hematopoiesis (59). It also has a positive effect on factor VIII serum levels, inhibits serum angiotensin-converting enzyme activity, and participates in reutilization of iron from destroyed erythrocytes (56,60,61). It is even suggested that the spleen has a role in lipid metabolism, demonstrated by lower HDL-cholesterol and higher triglyceride serum levels after splenectomy (62).

Immunological

Phagocytosis of foreign particles can be promoted by interaction with opsonins, serum factors that enhance their uptake by specific phagocytosing cells. The spleen has a prominent role in the generation of opsonins (4,63). Phagocytes in the spleen, together with macrophages in the liver, synthesise the majority of components of the classical pathway of complement (63). Whereas the MPS in the liver is the main site of phagocytosis of opsonized particles, the spleen is the major organ for the phagocytosis of non-opsonized foreign particles.
In experiments in rabbits, the phagocytosing capacity for non-opsonized particles appeared to be sixty times as effective in spleen as in liver when corrected for weight (reviewed by Lockwood) (63). The unique microvasculature of the spleen supposedly contributes to the

specialised function of the spleen in the phagocytosis of insufficiently-opsonized particles (34,42,64). The greatly retarded blood flow in the red pulp cords allows a very intimate and prolonged contact between antigens and phagocytes. Thus, particles can be ingested without specific ligand-receptor interactions.

An important practical implication of this specialised phagocytosing capacity is that the spleen is the most important site of clearance in the early phase of bacterial invasion before sufficient amounts of specific antibody have been produced. This is particularly important for blood-borne antigens, like polysaccharide-encapsulated micro-organisms (e.g., pneumococci) (56,65).

The spleen also plays a part in the alternative complement pathway. Complement factors work synergistically with antibodies in promoting phagocytosis of bacteria. In the presence of the complement factor C3b, the Ig opsonization degree required for phagocytosis is decreased 100-fold. Moreover, lysis of bacteria can take place by complement factors only. In the primary immune response to TI-2 antigens, C3d is also an important factor, and a high density of C3d-receptors is found in the MZ of the spleen (66).

Generation of specific antibody is primarily dependent upon the spleen. The spleen is the site of IgM-specific antibody generation very early after exposure to blood-borne antigen. The first contact of antigens entering the spleen via the blood and immunocompetent cells occurs in the MZ, a structure exclusively present in the spleen (67, 68). The MZ has a unique microvasculature, enabling a very low blood flow. Also, specialised macrophages, antigen presenting cells, and a subset of intermediate-sized B-cells with a specific phenotype (IgM+, IgD-, and strongly CD21+) are predominantly found (67-70). Although B-cells with this phenotype can also be found in other lymphoid tissues, the splenic MZ contains the largest accumulation of this type of B-cells in the body. When the blood enters the MZ sinusoids, there is a considerable increase in flow area diameter, with a subsequent decrease in blood flow. Similar as in the red pulp, a sluggish flow results; this enables close contact between antigens and phagocytes or lymphoid cells, and between different cell subtypes involved in the immune response (34,64).

Because the spleen is a major lymphoid organ, it also plays an important role in the primary humoral and secondary immune response. After encountering antigens in the MZ-sinuses, antigen-specific B-cells migrate to the LFs (36). From here they can either differentiate to produce antibodies (plasma cells) or to B-memory cells. The primary immune response is very important and can provide antibody production within 6 hours after the first contact with an antigen. The MZ B-cells are particularly well-equipped for rapid and easy activation in a primary immune response (68).

The spleen is also of importance in the secondary immune response, since it especially promotes the formation of memory cells of B- and T-lymphocytes (56,63). It is specifically involved in the immune response to TI-2 antigens. These antigens, generally polysaccharides, are the antigenic component of the capsule of encapsulated bacteria such as *Streptococcus pneumoniae, Haemophilus influenzae and Neisseria meningitides*. The immune response to TI-2 antigens is characterized by the need of T-cell-produced factors, although it is independent of the actual presence of T-cells (71-73). After splenectomy, this response is significantly decreased or even absent. The initiation of the response to polysaccharide antigens (TI-2 antigens) takes place in the MZ (74). In rodents, TI-2 antigens were found to localize specifically on antigen-presenting cells in the MZ, and the elimination of MZ cells abrogated the immune response to such antigens (75). As described above, MZ B-cells have a distinct immunophenotype. By their high expression of CD21, the receptor for complement fragment C3d, MZ B-cells play a specific role in the immune response to TI-2 antigens, as these antigens are able to bind C3d (64,66). In this way, TI-2 antigen-C3d complexes can bind to, and activate, MZ B-cells (64,66).

Another special feature of the spleen is the generation of tuftsin, originating from the Fc-fragment of IgG. This is a tetrapeptide reported to exert stimulatory effects on activity and migration responses of phagocytic cells (76,77).

Recently, Feleder et al. demonstrated a new function of the spleen, namely an antipyretic response to lipopolysaccharides (LPS) (78,79). They postulate that the spleen naturally limits the fever response by inhibiting the avidity of Kupffer cells to LPS. The latest data indicate that this inhibitory factor is a lipid produced by the spleen (79). A summary of the functions of the spleen is given in Table 1.

Table 1. Functions of the spleen.

White Pulp	Red Pulp
Antibody production	Filter function
Initiation of humoral response	Phagocytosis (in particular to TI-2 antigens & badly-opsonized particles)
Lymphocyte reservoir	Reservoir of thrombocytes and immature erythrocytes
Hematopoiesis (fetal life, extramedullary hematopoiesis)	Hematopoiesis (fetal life, extramedullary hematopoiesis)
Role in alternative complement pathway	Inhibition of angiotensin converting enzyme
	Iron reutilization
	Tuftsin production
	Positive effect on factor VIII
	Lipid metabolism
	Inhibition of LPS-induced fever

Consequences of splenectomy

Although incidental reports mentioned a relationship between splenectomy and infection, it was not until 1952 that a causative association was reported between splenectomy (for congenital haemolytic anaemia) and the occurrence of meningitis with sepsis (7). Since then the increased risk of infection and septicemia directly related to splenectomy has been well defined in the literature. Such infections are now generally termed "Overwhelming Post-Splenectomy Infections" (OPSI). In most cases, the OPSI syndrome is caused by one of the following micro-organisms: *Streptococcus pneumoniae* (50%), *Neisseria meningitides* (12%), *Escherichia coli* (11%), *Haemophilus influenzae* (8%) and *Staphylococcus aureus* (8%), but also by mycobacteria, viruses and parasites (80-84).

The frequency of OPSI is dependent on age and the cause of splenectomy. The highest frequencies were found after thalassemia and Hodgkin's disease and the lowest frequencies after trauma (85). Singer reported an overall frequency of 4.25% with a mortality of 2.52% (86). In patients who had had a splenectomy for traumatic splenic rupture the mortality due to sepsis was 0.58%; after thalassaemia, however, it was 11.0%. In the total population, the incidence of mortality due to sepsis was 0.01%. In a more recent review about OPSI in 12514 post-splenectomy patients, under 16 years of age an OPSI frequency of 4.4% was found with a mortality of 2.2%, but for adults these Figureures were 0.9% and 0.8% respectively. Overall there was 3.6% morbidity and 1.8% mortality. The highest incidence of OPSI is generally found in infancy and childhood. Patients who have undergone splenectomy for hematologic diseases, reticuloendothelial diseases or portal hypertension have a higher incidence than those undergoing splenectomy for trauma (80, 86-92). Most frustrating is the high overall mortality rate of OPSI of about 50% (56,80,81,85,93,94).

As indicated above, there is a significant decrease in the primary immune response to bacterial capsular polysaccharide antigens after splenectomy (80-82). These antigens belong to the group of TI-2 antigens, and other antigens of this type also give a similar decreased immune response after splenectomy (24,95). Another cause for the increased risk of OPSI is a decrease in phagocytic activity, in particular with respect to phagocytosis of poorly- or non-opsonized antigens. After a splenectomy, the phagocytic function will be partly taken over by the liver. However, the liver needs a higher level of antigen opsonization. This may present an important problem, especially with respect to TI-2 antigens, like encapsulated bacteria, which are badly opsonized (24), in particular because the spleen-dependent specific TI-2 antibody response is hampered. A lower phagocytic activity also results from decreased tuftsin concentrations after splenectomy (93,96).

The general ability to generate a specific antibody response after the first contact with blood-borne antigen, the primary immune response is also reduced. This is consistent with a low production of IgM after splenectomy (97-99). The alternative complement pathway also seems to be impaired after splenectomy, with normal functioning of the classical pathway.

After splenectomy, the ability of the body to filter the blood will be reduced, which results in an increase of erythrocytes with inclusions, like vacuoles and Howell-Jolly bodies and with surface pits, in the circulation (100,101). The ability to remove intracellular organisms, such as malaria and bartonella, is also reduced. The loss of splenic maturation for reticulocytes causes a high percentage of immature erythrocytes and reticulocytes in the blood stream (56).

Another less important impairment that can have consequences is a decreased reservoir function for blood cells. There will be an increase of platelets and a prolonged residence time of lymphocytes in the blood shortly after splenectomy. However, after a few months the platelet counts seem to be normalised (56). The effects of splenectomy in humans and animals are summarized in Table 2.

Table 2. Effects of splenectomy.

Immunological	Non-immunological
Reduced phagocytic activity of badly opsonized antigens	Reduced filter function
Decreased tuftsin formation	Increase of reticulocytes
Lower IgM serum level	Thrombocytosis
Prolonged survival of lymphocytes in blood	Increase in serum ACE
Reduced alternative complement pathway activity	lower HDL-cholesterol, higher triglyceride
Increased auto-antibody activity	
Diminished numbers T-suppressor cells	
Diminished antipyretic response to LPS	

The pig spleen

Aliter catuli longe olent, aliter sues.
(Puppies and pigs have a very different smell.)
Plautus (254 BC-184 BC)

Since the experiments described in this dissertation are related to pig spleen transplantation, a short section on the pig spleen in relation to spleens from other mammals seems to be justified.

In comparison with the human spleen, the porcine spleen is a much longer (up to 1 meter in length), more fish-shaped organ, with an average weight of 8g/kg body weight, which is approximately 4 times more than a human spleen. The bright-red colored organ has a surface marbling produced by the prominence of the splenic copuscles. It has a more or less dorsoventral orientation (Figure 7A); its dorsal end fits between the fundus of the stomach, the cranial pole of the left kidney, and the distal pancreas; these relationships are not immediate as fat invariably intervenes. The spleen is more tightly attached to the stomach by the short

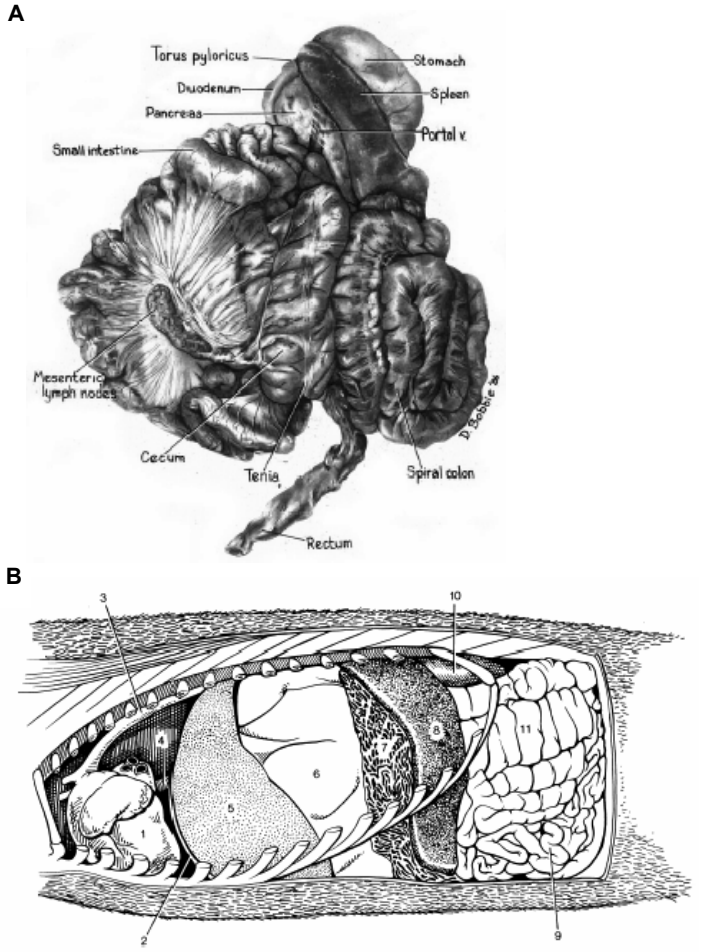


Figure 7.

A. Abdominal viscera of the pig, ventral view. *From: Swindle MM. Comparative anatomy of the pig. Technical Bulletin SRC 2002.*

B. The Abdomen of the pig. Left lateral view. *From: Textbook of Veterinary Anatomy,3rd Ed. Dyce, Sack, Wensing (eds.) 2002, Elsevier, pp776-785.*

1, heart; 2, diaphragm, cut; 3, aorta; 4, caudal vena cava; 5, left lateral lobe of the liver; 6, greatly distended stomach; 7, greater omentum (gastrosplenic ligament); 8, spleen; 9, jejunum; 10, left kidney; 11, mass of ascending colon.

gastric blood vessels and therefore is not as pedunculate an organ as it is in the dog (102). Its middle portion is in contact with the stomach and the intestine. When the stomach is moderately full, the spleen lies opposite the last three or four ribs, and its ventral end crosses the costal arch or even the median plane. When the stomach is greatly dilated, the spleen is pushed to the vicinity of the last rib (Figure 7B).

The accent of the different functions of the spleen differs quite a bit in different animal species. For example, the human spleen's main function, besides its filter function, is the immunological defense of the body, whereas in the dog and horse, the spleen plays a major role in storage of cells and is less important as a lymphoid organ. The pig spleen plays an intermediate role between defense and storage, but resembles the human spleen greatly. Histologically, there are no major differences between the pig spleen and the human spleen (Houser SL, personal communication).

TRANSPLANTATION TOLERANCE

Background

Fifty years ago, Dr Joseph Murray and colleagues performed the first kidney transplantation in Boston, USA (103). They could not have imagined that organ transplantation is now considered one of the two or three most important advances in medicine to have been made in the twentieth century (104). How transplantation came to be a clinical discipline can be pieced together perusing two volumes of reminiscences collected by Paul I. Terasaki during 1991-1992 from many of the persons who were directly involved. One volume was devoted to the discovery of the major histocompatibility complex (MHC), with particular reference to the human leukocyte antigens (HLA) that are widely used today for tissue matching (105). All of the contributions described in both volumes can be traced back in one way or the other to the demonstration more than a half century ago by Peter Brian Medawar that the rejection of allografts is an immunologic phenomenon (106, 107).

Ten years later (1953) Billingham, Brent and Medawar (108) showed that tolerance to skin allografts could be induced by inoculating fetal or prenatal mice with immunocompetent *spleen* cells from adult donors. Because of their immunologic immaturity, the recipients were incapable of rejecting the spleen cells whose progeny survived indefinitely. Specific unresponsiveness to donor strain tissues was retained as the recipient animals grew to adult life, whereas normal reactivity evolved to third party grafts and other kinds of antigens. They and others (109-111) observed in animal models that siblings became permanent hematopoietic chimeras if fusion of their placentas existed in utero, allowing fetal cross-circulation; such ani-

mals permanently accept each other's skin (112). It was predicted that this "natural chimerism" would induce tolerance to other donor-specific tissues and organs.

However, they soon learned (113-115) in rodents that the penalty for infusing immunocompetent hematopoietic cells could be graft-versus-host disease (GVHD) unless there was a close genetic relationship (i.e., histocompatibility) between the donor and recipient. This was the beginning of modern transplantation immunology, an extensive history of which has been written by Brent (116), one of its principal architects.

During the past three decades, enormous progress has been made in the field of transplantation, due in large part to the availability of effective immunosuppressive drugs. Although all of these agents suppress the immune response nonspecifically with respect to antigen, the most effective ones exhibit sufficient selectivity so that rejection can be avoided without undue compromise of the host's ability to respond to microbial pathogens. Nevertheless, patients on immunosuppressive medications are constantly walking a tightrope between the consequences of too little suppression (i.e., rejection) and too much suppression (infections or cancer) of their immune system (117). In addition, even in patients without complications due to their immunosuppression, there is an inexorable loss of transplanted organs due to chronic rejection at a rate of approximately 5% per year (118).

For these reasons, ever since the description of acquired tolerance to allografts in mice by Medawar and colleagues appeared in 1953 (108), a major goal of both clinicians and immunologists in the field of transplantation has been the induction of donor-specific tolerance in transplant recipients.
Tolerance is long-lasting non-reactivity of the immune system to a specific set of antigens, maintained without ongoing immunosuppression. The key feature of the immune system is tolerance to self-antigens. In transplantation, the ultimate goal is tolerance to donor-antigens. What has been most frustrating about this quest has been the fact that a very large number of successful approaches to the induction of tolerance have been reported in rodent models, but have failed when attempted in large animals, especially in nonhuman primates and in humans (Table 3). Indeed, as clinical results of organ transplants using standard immunosuppression are so good, at least in the short-term, most clinicians are no longer interested in testing new approaches to tolerance induction unless their effectiveness has already been demonstrated in large animal models.

But why should there be such a difference in the ability to induce tolerance in mice versus large animal species? Since it is much easier and far less expensive to carry out experiments in mice than in large animals, an answer to this question could have important practical as well as theoretical implications. Adams et al. (119) demonstrated that sequential exposure

Table 3. Methods that have induced transplantation tolerance.

Method	Mice	Large animals and humans
Enhancement	+	-
DST	+	-
Peptides	+	-
Anti-MHC mAb's	+	-
Calcineurin inhibitors	+	-
ALS	+	-
Anti-CD24	+	-
Anti-CD25	+	-
Total lymphoid irradiation	+	+/-
Anti-CD3 toxin	+	+/-
Costimulatory blockade	+	+/-
Chimerism	+	+

ALS=anti-lymphocyte serum; DST=donor-specific transfusion

From: Sachs DH. Tolerance: of mice and man. J Clin Invest 2003;111:1819-1821.

to sublethal infections by certain pathogenic viruses makes mice more resistant to tolerance induction by a protocol previously shown to result reproducibly in mixed chimerism and tolerance in mice (120). Assays for alloantigen-primed T cells in vitro following viral exposure confirmed that the priming led to cells with specificity cross-reactive between the pathogens and the allogeneic cells, a hypothesis, which, as they point out, has been proposed before as a possible reason for the frequent association of clinical rejection episodes with intercurrent viral infections (121).

On the other hand, the implications of these studies for the more general question of why it is more difficult to induce tolerance in large versus small animals, are not entirely clear. Indeed, the induction of tolerance through mixed chimerism is one of the few methodologies (Table 3) that has been shown to work not only in mice, but also in large animals (122,123) and most recently in humans (124,125).

Furthermore, the most obvious difference between small and large animal species with regard to tolerance induction is in the response to vascularized organ allografts (126). Skin graft survival is the hardest to prolong (127) unless the grafts are placed after a vascularized graft from the same donor strain, which suggests that vascularized grafts are themselves tolerogenic (128). Thus, studies utilizing a protocol for induction of tolerance to a vascularized organ allograft are needed for answering this general question.

Among the differences between rodents and large animals that have been suggested to account for this discrepant behavior in response to vascularized grafts are the markedly different tissue expression patterns of class II MHC antigens (129). These antigens, which are the most potent stimulators of the helper pathway in rejection reactions, are notably absent from

the vascular endothelial cells of rodents, but expressed constitutively in all large animals that have been studied, including humans. Indeed, in the Transplantation Biology Research Center at the Massachusetts General Hospital in Boston, USA, it has been shown, using intra-MHC recombinant lines of pigs, that matching for class II antigens permits uniform induction of tolerance to renal allografts by a short course of cyclosporin (130), one of the many methods that allows tolerance induction to vascularized organ allografts in mice across full MHC barriers (Table 3). The importance of an intact thymus to the induction of tolerance by this route has also been demonstrated (131), something that is markedly affected by age, stress, drugs, and infection - all of which may also be relevant to the difference between large and small animal models.

Rejection

Graft rejection occurs as a consequence of polymorphisms in histocompatibility genes, primarily those located within the MHC (132). T cells respond to foreign (allogeneic) MHC molecules in the same fashion as to any foreign protein: they secrete cytokines, divide, and differentiate (133). This generates a large population of activated effector cells, primarily T cells and macrophages, which are the primary mediators of graft destruction. Alloreactive T cells can recognize antigens present in transplanted tissues by 1 of 2 distinct pathways. In the direct pathway, the responding T cells recognize intact allogeneic MHC molecules on the surface of donor-derived antigen presenting cells (APCs), whereas in the indirect pathway, recipient APCs process donor-derived allo-MHC molecules into peptides and then present those peptides to T cells on self-MHC molecules. It is generally accepted that the direct pathway predominates in the immediate aftermath of transplantation, when graft-resident APCs (passenger leukocytes) migrate to the surrounding lymphoid tissue, where they stimulate alloresponsive T cells. As donor-derived APCs are relatively short lived, the indirect pathway of allorecognition is generally believed to predominate as the alloresponse progresses (134).

Central versus peripheral tolerance

Experimental methods to induce transplantation tolerance are typically divided into 2 categories. "Central" tolerance refers (in most instances) to the use of BM transplantation as a means to induce hematopoietic chimerism (135). This results in the coexistence of donor- and recipient-derived lymphoid and myeloid cells. As a result, developing T cells that are donor-reactive are deleted before they can exit the thymus, in the same manner as self-reactive T cells (136). "Peripheral" tolerance refers to the use of antibodies (or occasionally pharmacologic agents) that block or modulate T cell activation or growth factor receptor pathways in mature T cells. In most instances, this has the net result of promoting apoptosis among the T cells that are responding to alloantigens (137).

An important characteristic of alloimmune responses is the high frequency of T cells that are able to recognize and respond to alloantigens (primarily the products of genes encoded within the MHC) (138). Because of this, and based on data from studies on rodent models, many investigators believe that it is necessary to achieve large-scale deletion of alloreactive T cells in order to create transplantation tolerance (137). Both central and peripheral tolerance strategies achieve this during the early "induction" phase of therapy (i.e., the first 1–2 weeks after transplantation). In the case of central tolerance, this alone appears to be sufficient, as newly developing T cells with potential anti-donor reactivity will be eliminated within the thymus following encounter with donor-derived cells (135,136). However, in the case of peripheral tolerance strategies, a large body of data derived from experimental animals suggests that following depletion, the "maintenance" phase of tolerance requires regulatory T cells (Tregs) that can act on both any remaining alloresponsive T cells and on new thymic emigrants (Figure 8).

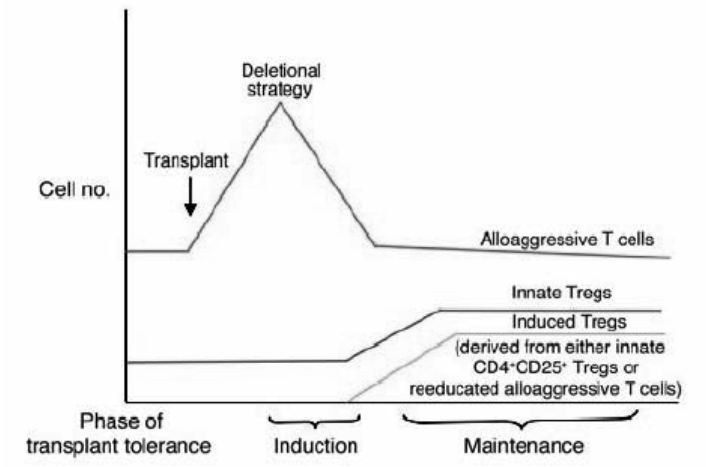


Figure 8. *From Walsh PT, Taylor DK, Turka LA. J Clin Invest. 2004; 114: 1398–1403.*
Deletional strategies employed at or around the time of transplant reduce the number of potentially alloreactive T cells (effector T cells) and facilitate the action of Treg subsets. During the maintenance phase of tolerance, these Tregs, either naturally-occurring or induced, can thus act more efficiently on a greatly reduced number of effector T cells. Cell number, as denoted on the *y* axis, represents an illustration as to how the relative ratio of effector versus Treg subsets alters during the establishment of transplant tolerance.

TRANSPLANTATION TOLERANCE IN THE CLINIC?

There are two major problems in the field of clinical transplantation. First, although short-term graft survival rates have been improved, these have not been accompanied by improvements in long-term outcomes (139). Accompanying the problem of late graft loss are the complications of continual immunosuppressive therapy, which include markedly increased

risks for cardiovascular disease, opportunistic infections, and malignancy. Secondly, there is the problem of the organ shortage. For those without living donors, waiting times for renal transplantation can exceed 5 years, although they can be maintained with dialysis. Since a chronic substitute for heart, lung or liver function is not available, in the year 2003 more than 2700 patients in the USA alone died, waiting for transplants.

Therefore, transplantation research has been focused on designing effective ways to induce transplantation tolerance. Tolerance would improve graft outcome, obviate the need for chronic immunosuppression, and expand the supply of donor organs (140). Given the recent experimental evidence for tolerance induction through a number of strategies, such as co-stimulation blockade, lymphocyte depletion and chimerism (141,142), the next problem is how to move these findings to the clinic. The dilemma arises of how to test new approaches in patients for which there is already exists a therapy that is successful in the short-term. One option is to test tolerance-inducing strategies by adding them to the existing immunosuppressive drug combinations. But then how could one distinguish tolerance from global immunosuppression? There is also concern that many tolerance-inducing strategies would be inhibited by the present regimen of immunosuppressive agents (143, 144).

There are several possible solutions. The first trials might aim to induce partial tolerance, and require that patients continue on at least one well-tested immunosuppressive agent (145). In a recent study, the CD52 antibody alemtuzumab (CAMPATH-1H) was given peri-transplant to partially deplete T cells and B cells in recipients of cadaveric kidney grafts (146). Patients were then maintained on monotherapy with half-dose CyA. The alemtuzumab induction allowed satisfactory long-term patient and graft survival equivalent to that seen with standard triple immunosuppression, while avoiding steroid therapy (146,147). From the patient's viewpoint, a steroid-free regimen with the security of a small dose of CyA, may be more acceptable than the immunologists' dream of tolerance.

It may also be possible to initially test tolerance induction therapies in patients for whom conventional immunosuppressive therapy is not effective, such as organ recipients that have been previously sensitized to donor antigens from blood transfusions or from previous failed transplants. This difficult setting offers one 'ethical' outlet for trials of new therapeutic strategies, although it would be difficult to determine, should graft rejection occur, whether failure is due to the experimental therapy, or the difficulty of imposing tolerance on a primed immune system.

At the Massachusetts General Hospital in Boston, USA, a study was initiated for the induction of renal allograft tolerance in patients with multiple myeloma and consequent kidney failure with the idea that concomitant BM transplantation may not only allow establishment of immuno-

logical tolerance, but might also produce a "graft-versus-myeloma" effect. The protocol used for tolerance induction was derived from rodent studies (148,149), and was translated into clinical studies for the treatment of patients with hematological malignancies without further treatment options, and the low toxicity and relatively low incidence of GVHD of this mixed chimerism approach was thereby confirmed in a clinical setting (150,151). The protocol was extended to myeloma patients with end-stage renal failure, a patient group that is usually not considered for BMTx due to poor general health. The tolerance protocol includes cytoreductive treatment with cyclophosphamide, T cell depletion with ATG and 7 Gy thymic irradiation followed by combined renal allograft and BM transplantation from the same HLA-identical related donor with a very short course of post-transplant CyA (approximately 60 days). In total, six patients have been treated so far, with a follow-up period between 2 months and 5 years. Four of these patients have been transplanted under a protocol sponsored by the Immune Tolerance Network (ITN). All six patients achieved initial macrochimerism. Results have been published on the two initial patients (not on the ITN protocol), in whom tolerance has been achieved at 3.5 and 5.5 years of follow-up (124,125). These initial results provide proof of principle that tolerance can be intentionally achieved in patients using combined simultaneous nonmyeloablative BMTx and kidney transplantation. Further details of the ITN study will be published soon.

This protocol was used as the basis for another ITN clinical tolerance protocol for combined kidney/BM transplantation in haploidentical transplant recipients without hematological malignancy. While only recently initiated, preliminary results of this trial are encouraging.
Diligent patient monitoring should also be included in tolerance clinical trials, to detect any signs of graft rejection at the earliest possible stage, and to allow rescue therapy with conventional drugs to prevent organ damage. The availability of surrogate biomarkers of rejection or tolerance, preferably blood-borne, would enable better monitoring and individual tailoring of pharmacological and biological therapies. Traditional functional responses such as mixed lymphocyte reaction or cytotoxic T-lymphocyte assays, or even their limiting dilution equivalents, are labor-intensive and are not sensitive enough to adequately predict whether or not a patient has become tolerant to the graft. It is thus imperative for immunologists to define sets of biomarkers as diagnostics for graft rejection or acceptance.

In the end, the ideal tolerance-inducing strategy may be one that involves multiple approaches. The present regulatory requirements make it unlikely that a tolerance-inducing strategy can be licensed for use before safety and efficacy are demonstrated. If that strategy is only effective in combination with another agent, then the chances for its development are diminished. Additional complications arise because a successful tolerance-inducing regimen may require administration over only a short time. As a result, the profit margin to a pharmaceutical may be smaller than it is for immunosuppressive therapies. If for this and the preceding commercial reasons, pharmaceutical companies are unable to progress with tolerance-induction

studies, it will be up to academic or health-service-based manufacturing facilities to develop these strategies. Establishing tolerance induction as a viable alternative for immunosuppression after organ transplant is, however, a reasonable goal that can be achieved through cooperation between basic and clinical researchers, regulatory authorities and industry.

THE MASSACHUSETTS GENERAL HOSPITAL MINIATURE SWINE MODEL

I like pigs. Dogs look up to us. Cats look down on us. Pigs treat us as equals.
Sir Winston Churchill (1874 - 1965)

Swine, *Sus scrofa domesticus,* are widely used in medical research. Most of the animals are small domestic farm breeds, but miniature swine are widely used for chronic studies where the significant growth of the domestic breeds would be an issue. They share anatomic and physiologic characteristics with humans that make them a unique and viable model for biomedical research. The cardiovascular anatomy, physiology and response to atherogenic diets have made them a universally standard model for the study of atherosclerosis, myocardial infarction and general cardiovascular studies. Their gastrointestinal anatomy has some significant differences from that of humans; however, the physiology of their digestive processes has made them a valuable model for digestive diseases. The urinary system of swine is similar to humans in many ways, especially in the anatomy and function of the kidneys. Swine are also a standard model for skin and plastic surgical procedures and have been developed as models of transdermal toxicity. The anatomy and physiology of organs such as the liver, pancreas, kidney and heart have also made this species the primary species of interest as potential organ donors for xenotransplantation. All swine commonly used in research are *Sus scrofa domesticus,* whether they are farm or miniature breeds. The main difference between breeds is size at sexual maturity. Domestic breeds typically reach 100 kg by 4 months of age and miniature breeds typically range from 25-50 kg at the same age (152) (Figure 9).

The MGH miniature swine herd was developed for studies of transplantation biology and has been selectively inbred at the MHC, termed Swine Leukocyte Antigens (SLA) in swine, and intentionally outbred for minor antigens, although one strain is available with near-compatibility for minor antigens also (153,154). Three independent MHC alleles derived from the founding breeding pair are maintained, as well as four additional intra-MHC recombinants, which have been recognized and subsequently bred to homozygosity (Figure 10). These animals exhibit many physiologic and immunologic similarities to humans, including MHC class II expression on renal endothelium, and they provide a large animal model that allows transplant combinations approximating most relevant human transplants.

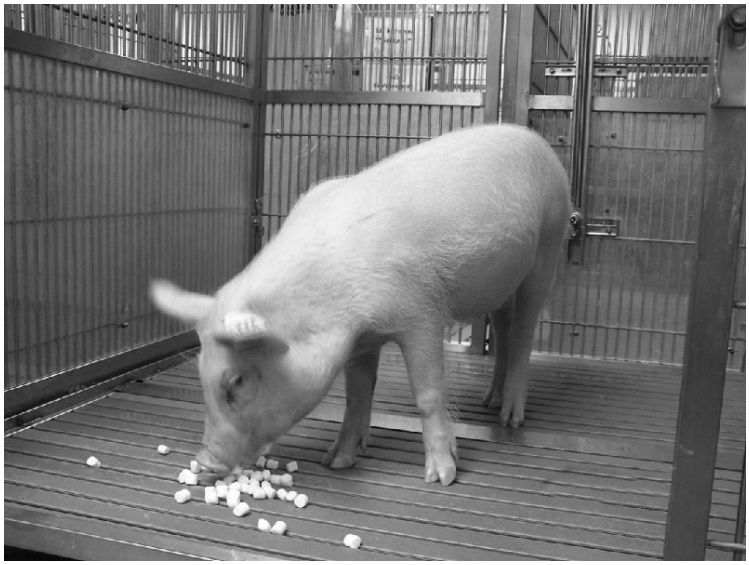


Figure 9. MGH miniature swine, 4 months old, weighing 25 kg.

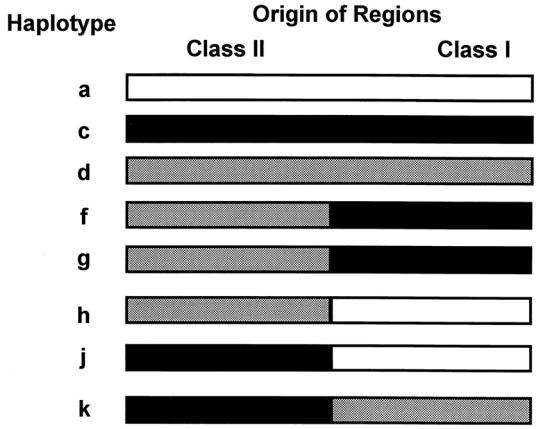


Figure 10. Schematic diagram of the origin of available homozygous porcine MHC (SLA) haplotypes.
Partially inbred SLA^{aa}, SLA^{cc}, and SLA^{dd} haplotypes were derived from the original founder miniature swine. Recombination events between the MHC class I and class II haplotypes have been identified and maintained as homozygous, recombinant haplotypes SLA^{ff}, SLA^{gg}, SLA^{hh}, SLA^{ij}, and SLA^{kk}.

Of relevance to the present studies is that kidney allografts (but *not* heart allografts) between MHC two-haplotype class I-mismatched pigs that receive a 12-day course of cyclosporine A (CyA) are uniformly accepted (155). A subsequent kidney allograft, matched to the original donor, will be accepted long-term without the need for further immunosuppressive therapy (156). Furthermore, a successfully transplanted kidney induces tolerance to a donor-specific or donor-matched (i.e., class I-mismatched to the recipient) heart allograft transplanted at the same time, whereas the heart, if transplanted in the absence of a kidney (but with a 12-day

course of CyA), is rejected within 55 days (33-55 days) and begins to develop chronic allograft vasculopathy (CAV) after 4 weeks (155). The mechanism whereby the kidney induces tolerance to the heart remains uncertain, though both passenger leukocytes and parenchymal cells appear to play a role (157). The tolerogenic effect of the kidney is not due to antigen load alone since the transplantation of two (matched) hearts, although resulting in long-term graft survival (>190 days), does not prevent CAV from developing (158). There is evidence that peripheral blood lymphocytes (PBL) from tolerant animals, when primed *in vitro* with donor antigen, suppress antidonor cytotoxic T cell reactivity by naïve recipient-matched PBL (159). The suppression is cell dose-dependent, radiation-sensitive, and requires cell-to-cell contact, suggesting that the presence of peripheral regulatory cells plays a role in the induction of tolerance. Antigen-specific regulation of the IL-2α expression on CD8 single-positive PBL is thought to be an important event associated with the development of this tolerance (160).

However, kidney Tx across a class I disparity is not successful in older swine (> 55 months), presumably because the thymus is involuted (161). The potential clinical approach of transplanting a kidney in order to induce tolerance to a heart (even if it is considered justified to transplant a kidney solely for this purpose) is therefore unlikely to be applicable in adult patients. The importance of the thymus in this model has been confirmed by Yamada and his colleagues (162-164). Prior thymectomy abrogates both survival of the kidney and its protective effect on the heart (163). In euthymic recipients, there is an initial cellular infiltration in the renal graft that, between 30-60 days, diminishes and is associated with T cell apoptosis, associated with tolerance induction, whereas in thymectomized recipients the infiltrate persists with activation and proliferation, resulting in rejection (165,166). Tx of vascularized donor thymic tissue with the kidney in a thymectomized host is followed by graft survival and tolerance (164). In view of the involution of the the thymus relatively early in life, any approach that involves the Tx of thymic tissue may have a limited clinical role.

Of relevance to this thesis is that the induction of hematopoietic cell chimerism by a nonmyeloablative regimen and BM transplantation allowed subsequent (5-12 months later) kidney (167,168) or cardiac (169) Tx to be performed successfully over a minor antigen barrier in the absence of immunosuppressive therapy. Cardiac transplantation perfomed across this barrier in untreated recipients resulted in rejection within 44 days (21-44 days), whereas survival was >153 days (>153 - >362 days) in the chimeric recipients. No CAV or GVHD was seen.

If immunosuppression is with CyA for 12 days, a renal allograft across a two-haplotype full mismatch is rejected (170). Kidney graft survival can be achieved across this barrier if tacrolimus is continuously infused (for 12 days) to maintain trough levels of >35 ng/ml (170), but this level may not be clinically applicable. When a level of even 20-26 ng/ml is maintained, chronic allograft glomerulopathy develops. *In vitro* assays demonstrated that PBL from tolerant pigs

produce inhibitory cytokines, suggesting the involvement of regulatory mechanisms. It is not known whether a fully-mismatched kidney allograft is tolerogenic to a donor-specific or donor-matched heart graft. It is not yet known whether an isolated heart allograft across a full mismatch will be rejected if immunosuppression is with CyA for 12 days, but the fact that CyA does not prevent graft failure over even a MHC class I barrier indicates that it would not protect against a full MHC barrier. Studies are continuing to investigate the efficacy of tacrolimus over a full MHC barrier; preliminary data suggest that it will be inadequate to prevent rejection.

The above studies in MGH miniature swine previously performed at the Transplantation Biology Research Center of the Massachusetts General Hospital / Harvard Medical School, Boston, USA, will act for comparison in our studies of spleen allotransplantation.

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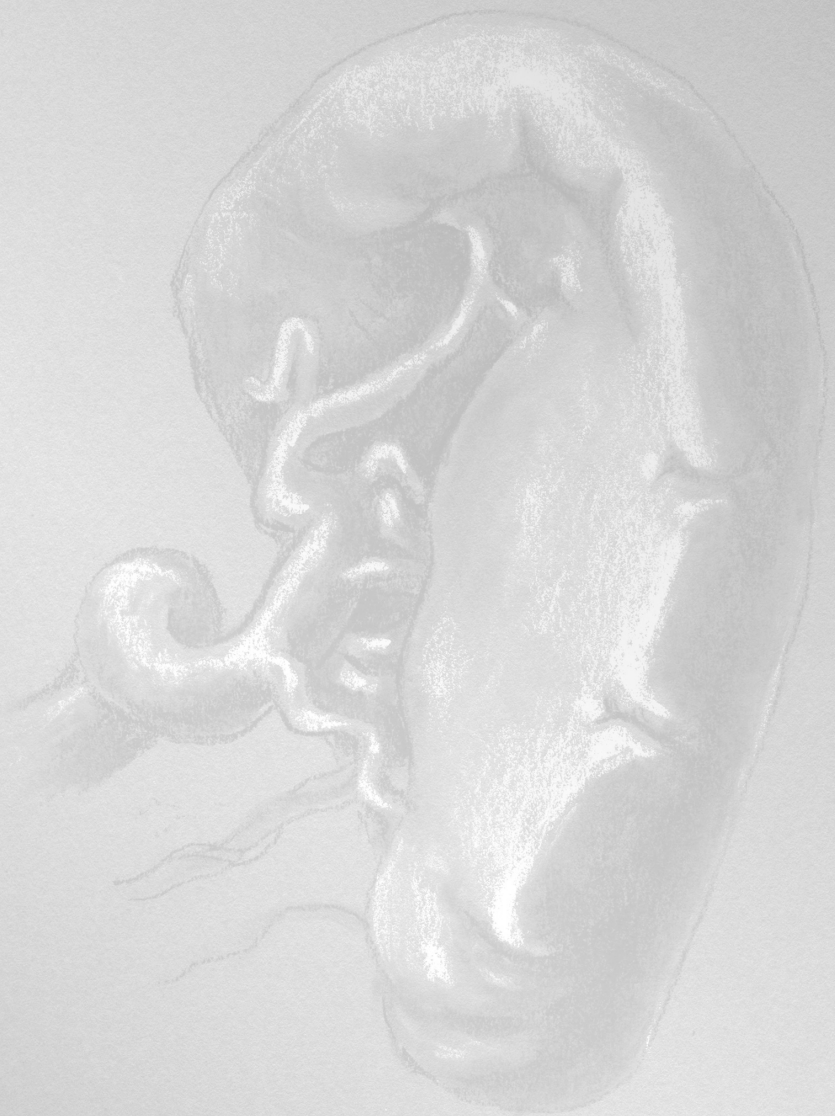
Aims of this Dissertation



2

The three ways of success: persistence, persistence, persistence.
T.R. Roosevelt (1858-1919)

AIMS OF THIS DISSERTATION

The ultimate goal in clinical allotransplantation and xenotransplantation is to achieve donor-specific immunological tolerance or long-term unresponsiveness to the transplanted organ in the absence of continuing immunosuppressive therapy. This would allow long-term graft survival of the organ without the risks and complications of pharmacologic immunosuppressive therapy. A novel approach to achieving this aim may be through spleen transplantation.

In **Aim #1**, we shall attempt to establish a relevant pre-clinical large animal model for spleen transplantation in Massachusetts General Hospital MHC-defined miniature swine.

In **Aim #2,** the pathobiology of spleen graft rejection and acceptance will be determined in the miniature swine model.

By spleen transplantation in MHC-matched (minor-mismatched), MHC-Class I-mismatched, or fully-MHC-mismatched donor-recipient combinations carried out with a short course of immunosuppressive therapy, we shall:
- attempt to induce prolonged hematopoietic cell chimerism and monitor recipients for possible graft-versus-host-disease (**Aim #3**).
- investigate whether recipients of spleen allografts exhibit *in vitro* cellular donor-specific unresponsiveness and/or long-term spleen allograft survival (**Aim #4**).
- determine which immunosuppressive regimen would be optimal for tolerance induction using spleen transplantation in miniature swine (**Aim #5).**

In **Aim #6** we shall assess whether successful MHC-mismatched spleen transplantation, with or without the induction of mixed chimerism and/or *in vitro* cellular donor-specific unresponsiveness, is tolerogenic and allows subsequent successful transplantation of a donor MHC-matched kidney without further immunosuppressive therapy, and by which mechanism such tolerance or unresponsiveness is induced.

In **Aim #7,** the spleen shall be investigated as a source of hematopoietic progenitor cells or stem cells.

In **Aim #8**, preliminary information will be obtained on whether there is likely to be a role for spleen transplantation in the induction of tolerance in discordant xenotransplantation in a pig-to-baboon transplantation model.

In **Aim #9,** possible complications of spleen transplantation or the immunosuppressive regimen will be identified and described.

Can Spleen Transplantation induce Tolerance? A Review of the Literature

3

Frank JMF Dor*, Bernd Gollackner*, David KC Cooper. Can spleen transplantation induce tolerance? A review of the literature.

Transplant International 2003;16:451-460.

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ABSTRACT

In some rat and guinea pig strain combinations, transplantation of an allogeneic spleen has been demonstrated to induce tolerance spontaneously to itself and to another donor-specific organ; prior irradiation of the recipient results in graft-versus-host disease (GVHD). In other combinations, following spleen transplantation (SpTx), a state of tolerance has been achieved when the recipient's immune system has been weakened, e.g., by a pharmacologic agent, irradiation, or native splenectomy. The data indicate that, if a balance can be achieved between the host-versus-graft and graft-versus-host responses, tolerance develops, possibly due to the development of suppressor/regulatory cells. There have been a number of unsuccessful studies in large animals, but none in which knowledge of the major histocompatibility complex of the donor and recipient has been known. SpTx has been performed in approximately 50 humans for a number of reasons; no clear immunologic advantage has been reported. GVHD has been documented in at least three patients, and was lethal in one despite excision of the donor spleen. In 23 of 30 patients whose blood was exposed extracorporeally to a pig spleen for a matter of hours in the treatment of sepsis, porcine microchimerism has been detected at follow-up two months to eight years later. The advantages of tolerance over chronic immunosuppressive therapy are so great that an approach that has tolerogenic potential, such as SpTx, would seem worthy of further investigation in a suitable large animal model.

INTRODUCTION

The induction of donor-specific transplantation (Tx) tolerance without long-term immunosuppressive therapy is the ultimate goal in both alloTx and xenoTx. The spleen has long been recognized as potentially tolerogenic and, in some species, can act as a source of hematopoietic cell restoration.

Carrel was probably the first to explore experimental spleen autoTx in 1910 (1). The possibility of the transplanted spleen contributing to a state of neutral reactivity, a balance between the host-versus-graft reaction and the graft-versus-host (GVH) reaction, was emphasized by Simonsen in 1953 (2). The concept of hematopoietic restoration by the intact spleen following whole body irradiation (WBI) finds its basis in the work of Jacobsen (3,4), in which shielding of the spleen from lethal WBI resulted in hematopoietic cell restoration and clinical survival of rodents.

SPLEEN TRANSPLANTATION IN RODENTS

Rats

Spontaneous development of tolerance (Table 1A)

With the development of the techniques for SpTx in rodents (5-10), Bitter-Suermann described spontaneous survival of vascularized spleen allografts in certain major histocompatibility complex-incompatible rat strain combinations (11). The spleen conferred a state of specific tolerance upon the recipient, as judged by subsequent skin and pancreas allograft survival (12-14). Spleen grafts, which were usually accepted, were rejected if the donor had been sub-lethally-irradiated prior to Tx. The graft induced lethal graft-versus-host disease (GVHD) if the recipient had received a similar dose of WBI. This observation pointed to immunological competence of both graft and host as a prerequisite for the successful induction of this type of Tx tolerance (12). The recipient would die of GVHD if the donor had been actively pre-sensitized by injections of spleen and thymus cells from the recipient before SpTx, or if the recipient had undergone native splenectomy immediately before SpTx (14).

Although prolongation of survival of a transplanted pancreas occurred when the spleen was included *en bloc* with the pancreas, 50% of the recipients developed GVHD and died (15,16). Prior donor-specific blood transfusion and a 26-day course of cyclosporine therapy prevented GVHD and was followed by indefinite pancreas-spleen graft survival (16). If a spontaneously-accepted WAG-to-AGUS rat spleen allograft was removed after 3-5 months, and only at that time was a pancreas allograft inserted, 68% of these grafts survived.

Subsequent transplanted organs or tissues from the same strain as the spleen donor survived indefinitely if transplanted 6 weeks to 5 months after SpTx or together with the spleen graft**.** This proved to be the case even if the spleen graft had atrophied or had been surgically removed by this time (11,17).

In studies by others, however, although the transplanted spleen was not rejected, its presence did not lead to prolonged survival (or tolerance) of a donor-specific skin graft and, indeed, in some studies there was evidence of sensitisation (18). Early studies by Bitter-Suermann et al. showed no effect on solid organ survival by the injection of donor-strain splenocytes into the recipient (19). This discrepancy with the results obtained following SpTx has been explained by the observation that the preparation of a single cell suspension not only disrupts the architecture of the organ but also alters the proportion of cellular elements to be transferred (7,11). There have been several studies by others with varying outcome (20-22), but these will not be reviewed here.

Table 1A. Organ transplant survival after spleen transplantation in rats without immunosuppression

First Author	Rat strains	Organ Tx with spleen	Organ survival	Reference
Bitter-Suermann	PVG->AGUS	skin	8d	Nature 1974;247: 465-466
	AGUS->VG	skin	>3m	
	Wistar->AGUS	skin	>4-5m	
Taub	(LewisxBN)F1	kidney	5m	Proc Eur Dial Transplant Assoc 1976;12:423-428
Tilney	Aug->(As x BN)F1	skin with presensitized spleen isografts	7d	J Surg Res 1977;22:54-58
	Aug->(LewisxBN)F1	skin with presensitized spleen isografts	7d	
Bramis	(LewisxBN)F1->Lewis	kidney	>90d	Transplant Proc 1977;9;1:341-346
		skin	>72d	
Bitter Suermann	WAG->AGUS	pancreas	indefinite	Transplantation 1978;26:28-34
Blanchard	ACI-> Lewis	heart	9d	J Microsurg 1980;1:381-386
	Lewis -> ACI	heart	20d	
Shah	WAG -> AGUS	pancreas	4-10m	Transplantation 1980;30: 83-89
Gugenheim	(LewisxBN)F1->Lewis	skin	9d	Transplantation 1983;36:470-471
Blanchard	ACI -> Lewis	kidney	17d	J Microsurg 1985;6:26-31
	Lewis -> ACI	kidney	93d	
Gray	Lewis->DA	islets	<14d (3)	J Surg Res 1986;40:77-84
			>100 (3)	
Schulak	Fischer->Lewis	pancreas	18d	Transplant Proc 1987;29:1013-1014
	LBN->Lewis	pancreas	11d	
	ACI->Lewis	pancreas	7d	
Duncan	(WAGxAGUS)F1->AGUS	heart (WAG)	>100d	Transplantation 1987;44:553-558
Wakely	Lewis->BN	pancreas	>12d	Transplantation 1990;49:241-245
	BN->Lewis	pancreas	11d	
Pirenne	BN->Lewis	small bowel	23d	Transplant Proc 1993;25:1206
Suzuki	ACI->DA	skin	indefinite	Transplantation 1997;64:650-654
Takano	Wistar (P->F1)	skin (P)	80% 1-2w	Transplant Proc 1998;30:2685-2686

d=days, w=weeks, m=months, P=parent, F1=first offspring

Induction of tolerance by immune manipulation (Table 1B)

When AGUS recipients were pre-treated with a mixture of spleen, thymus, liver, and blood cells from PVG donors, administered intravenously 8-22 days prior to SpTx, 72% of the spleen grafts survived longer than four months, in contrast to spleen allografts in non-prepared recipients where survival was approximately three weeks (23,24). Skin allografts in naïve recipients survived approximately eight days, whereas AGUS rats bearing successful long-term spleen allografts accepted subsequent skin allografts permanently. Even after removal or late atrophy ('absorption') of accepted spleen allografts, the rats remained tolerant to skin allografts. Two rats accepted skin that had been grafted four months after the spleens had been 'absorbed'

Table 1B. Organ transplant survival after spleen transplantation in rats with host immunosuppression or immunomodulation

First Author	Rat strains	Organ Tx with spleen	Immunosuppression/ Immunomodulation	Organ survival	Reference
Tauber	Lewis->ACI	heart	Delayed SpX d3	indefinite	J Microsurg 1981;2:261-268
Gray	Lewis->DA	islets	CyA 10 mg/kg for 14d	Indefinite	J Surg Res 1986;40:77-84
Pollak	Lewis->ACI	heart	Delayed SpX d3	indefinite	Transplantation 1986;42:528-531
Wakely	Lewis->BN	pancreas	DST on d-7	10d	Transplantation 1990;49:241-245
	Lewis->BN	pancreas	DST on d-7 CyA for 26d	>143d	
Westra	BN->Lewis	heart	CyA 25 on d2	>100 d	Transplantation 1991;52:952-955
Pirenne	BN->Lewis	small bowel	CyA on d2	52d	Transplant Proc 1993;25:1206
Suzuki	Lewis->Fischer344	skin	Tacrolimus for 14d	indefinite	Transplantation 1997;64:650-654
Hakamata	Wistar->Lewis	pancreas	Tacrolimus for 14d	21d	Biochem Biophys Res Commun 2001; 286:779-785

CyA= Cyclosporine A, SpX= host splenectomy, WBI=whole body irradiation, DST=donor-specific blood transfusion

(after remaining viable for five months after grafting), demonstrating that persistence of a viable spleen graft was not essential for skin graft survival in this model. The mechanism of the prolonged survival of the spleens in these pretreated rats was uncertain.

Although confirmatory studies have been reported, not all subsequent investigators have confirmed the ability of a splenic allograft to induce tolerance or prolonged hyporesponsiveness.

Mice

In mice, the age of the spleen donor was not found to be a major influence on outcome of SpTx (25). SpTx has also been utilised to study the anemia of chronic inflammation (26).

Guinea pigs

Spontaneous development of tolerance

Based on Billingham's previous conclusions regarding bone marrow Tx, Bitter-Suermann et al. concluded from their rat studies that a spleen graft could launch and sustain GVH reactivity

when (a) graft and host differed at major or minor histocompatibility loci, (b) the graft contained immunocompetent cells, and (c) the host was immunoincompetent, either for a genetic reason, from age, or because of exogenous immunosuppression (19,27). Bitter-Suermann and Shevach, however, observed that a majority of untreated guinea pigs (of strain 2) undergoing SpTx (from strain 13) succumbed to GVHD approximately six weeks after Tx, which was not in accordance with the third prerequisite above (19). The recipients were immunocompetent and not immunosuppressed but, after receiving a spleen allograft, they behaved as if exogenously immunosuppressed. These authors believed this to be the only animal model in which an allograft induced lethal GVHD in a healthy immunocompetent host quite capable of rejecting an organ transplant from a third party donor.

Mechanism of tolerance induction

In guinea pigs with long-term surviving spleen grafts, the response against the donor was markedly suppressed in the mixed lymphocyte reaction (MLR). Furthermore, cells were present in the tolerant spleen recipients that were capable of suppressing the MLR response of normal host-strain cells towards donor-strain cells (28), although the period of MLR hyporesponsiveness was rather short-lived. Donor-strain cardiac allografts survived indefinitely in immunologically-uncompromised recipients that had received passive transfer of cells from guinea pigs with long-standing spleen transplants. The presence of the cardiac graft further enhanced MLR suppression, whereas most cardiac-allografted controls had vigorous MLR responses to donor-stimulated cells after rejection of their cardiac transplants.

There was some discrepancy between the *in vivo* and *in vitro* data. A number of recipients of spleen allografts had markedly suppressed MLR responses, and their cells were uniformly capable of transferring tolerance *in vivo* but did not show adoptive MLR suppression *in vitro. In vivo* tolerance could be transferred by cells taken from animals four weeks after spleen allografting, a time when the activity of the MLR 'suppressor' cells was not prominent *in vitro* (29,30).

These experiments suggested the presence of regulatory cells capable of transferring MLR unresponsiveness and Tx tolerance to normal hosts, although it was not determined whether the cells involved were of recipient or graft origin. The state of unresponsiveness, favouring the survival of second donor-strain allografts, appeared to be weak during the first few weeks after SpTx. Skin allografts, for example, did not survive if placed earlier than 6-8 weeks after SpTx (13,31).

Following SpTx in rats, unresponsiveness developed as early as one week (32-35). T cells obtained from the recipient's lymph nodes and native spleen exhibited reduced MLR to donor-

strain cells, but responded normally to third party cells. In contrast, T cells obtained from the spleen graft were unresponsive to both donor and third-party stimulators. Therefore, although donor-specific T suppressor cells appeared in the recipient's lymph nodes and spleen within one week of SpTx, at this time antigen-nonspecific suppressor cells predominated in the spleen graft. Only minimal cytotoxic T cell activity could be detected in the spleen graft, with the host spleen and lymph nodes being devoid of cytotoxic T lymphocytes. Sera obtained one or two weeks following SpTx did not contain cytotoxic antibodies, and only a very weak response could be detected at one month.

SPLEEN TRANSPLANTATION IN LARGE ANIMALS

Apart from one early report of implantation of an allogeneic spleen in the peritoneal cavity in chickens (36), all reports have been in dogs or pigs.

Allotransplantation in dogs (Table 2A)

In the early 1960s, several groups performed SpTx in dogs, with comparable results (Table 2A). Spleens transplanted into untreated recipients underwent rejection within a few days. Marchioro et al documented a viable spleen graft after 200 days in a dog treated with azathioprine for only the first 125 days (37). Prior treatment of the recipient with WBI (150-300 cGy) protected the spleen from rejection (37), but produced a more pronounced GVH response, with anemia, thrombocytopenia, and lymphocytopenia (38). Protecting the spleen from lethal WBI did not result in survival, but an intravenous (unirradiated) homogenate of the native spleen did (39).

Effects of SpTx on a concomitantly-transplanted donor-specific organ (Table 2B)
In untreated dogs, the simultaneous Tx of spleen and kidney from the same donor prolonged survival to 20 days, compared with 11 days when the kidney was transplanted alone (37). Azathioprine therapy could prolong graft survival for from 2 to 7.5 months at which time, although the kidneys showed signs of chronic rejection, the splenic architecture remained normal (37). Administering WBI (100-450 cGy) to the recipient prior to SpTx resulted in hemolytic anemia in 8 of 12 dogs, which required repeated blood transfusion, and yet provided no protection to the renal allografts (37). Although the survival of renal allografts was prolonged when combined with simultaneous native splenectomy and SpTx, these animals developed a wasting disease, characterized by anorexia, weight loss, occasionally bloody diarrhea, and anemia, suggesting a GVH reaction, several of them dying from infection. Others have not found SpTx to be beneficial to survival of a second donor-specific organ.

Table 2A. Spleen allotransplantation in dogs

First Author	(n)	Immunosuppression	Outcome	Reference
Moore	29	none	19/29 early infarction	Ann Surg 1960;152:374-387
			1 spleen not rejected d65	
Fisher	20	SpX	rejection <10d	Surg Gyn Obst 1961;112:455-462
	24	WBI (300-500 cGy) at Tx	22/24 died	
	16	WBI (100-300 cGy) at Tx	1/16 died	
	12	WBI (300-500 cGy) prior to Tx	9/12 spleens grossly normal at d14	
Dammin	43	none	rejection 7-9d	Ann NY Acad Sci 1962;99:861-869
	?	WBI (250 cGy)	poor survival ?d	
	14	nitrogen mustard	6 died early <5d	
			7 died <8d	
			1 survived 32d	
			no difference with untreated	
Montague	18	nitrogen mustard	2/18 died (hematopoietic depression)	J Surg Res 1962;2:130-135
		SpX	4/18 died (early thrombosis spleen)	
			7/8 died 5-8d	
			No protection to spleen	
			rejection	
Wheeler	43	none	21/43 operative failure	J Surg Res 1962;2:114-123
			rejection <1w	
Fiscus	7	WBI (300 cGy)	death (6/7) < 10d	Surg For 1963;14:182-185
Jordan	36	none	11 died (early thrombosis)	Ann NY Acad Sci 1964;120;612-625
			25 rejection 5-12d	
	7	WBI (300 cGy)+SpX	GVHD, died <10d	
Marchioro	4	none	2-8 d survival	Ann NY Acad Sci 1964;120:626-651
	4	WBI (150 cGy) on d-8	4-7 d survival	
		WBI (300 cGy) on d-1	5-7 d survival	
	7	AZA	2-11d survival	
Mahajan	57		26/57 successful	J Surg Res 1965;5:413-427
	-12	none	rejection 5-8d	
	-14	6-mercaptopurine	25% spleen graft survival 13-28d	

AZA=Azathioprine, y=years

Hoffman et al (40) reported that immunization of the donor with horse red blood cells prior to SpTx led to the development of anti-horse red blood cell antibodies in the recipient. Furthermore, immunization of the recipient (after SpTx) with sheep red blood cells resulted in the production of anti-sheep red blood cell antibodies in the original donor after autologous retransplantation of the spleen. These observations demonstrated a transfer of sensitized lymphocytes in the transplanted spleens.

Allotransplantation for hemophilia A (Table 2C)

Experimental SpTx with the intention to cure hemophilia A has been carried out in a variety

Table 2B. Spleen + kidney allotransplantation in dogs

First Author	(n)	Immunosuppression	Outcome	Reference
Simonsen	1	none	spleen rejected on d4 accelerated kidney rejection	Acta Pathol Microbiol Scand 1953;32:36-84
Kountz	9	none	kidney no prolongation	Surg Forum 1962;13:59-62
	9	SpX	kidney 7 days prolongation wasting disease	
Marchioro	7	none	3 died (pneumonia) 4 survived 25d prolonged kidney survival 9d	Ann NY Acad Sci 1964;120:626-651
	12	WBI (100-450 cGy)	survival 6-32 d hemolysis (9) spleen rejection<10d accelerated kidney rejection	
	11	AZA	survival 6d-7.5m kidneys protected from acute rejection	
Dubois	29	none	spleen rejection <10d kidney no prolongation	Biomedicine 1976;25:221-223
Hoffman	29	none +autologous retransplantation	15/29 successful kidney no prolongation	Eur Surg Res 1984;16:40-46

Table 2C. Spleen allotransplantation for hemophilia in dogs

First Author	(n)	Immunosuppression	Outcome	Reference
Webster	2	none	52-72h survival correction of hemostasis	North Carolina Med J 1967;28:505-507
Norman	2	none	factor VIII 0% <24d	Surgery 1968;64:1-16
	4	AZA+CS+ALS +actinomycin C	prolonged factor VIII synthesis	
Marchioro	1 matched	AZA+CS	viable on d93, no factor VIII increase	Transplant Proc 1969;1:316-320
	1 mismatched	AZA+CS	rejection <15d	
Marchioro	1	AZA+CS	viable 47d, no factor VIII increase	Science 1969;10:188-190
McKee	8	?	7-8m(2), no factor VIII increase	J Lab Clin Med 1970;75:391-402
Groth	4	AZA+ALS	survival 3-8d	Surgery 1974;75:725-733

h=hours, CS= corticosteroids, ALS=-anti- lymphocyte serum

of dog models. Although it could not be clearly demonstrated that the level of factor VIII increased, satisfactory graft perfusion and survival were demonstrated for more than 8 months (41). Webster et al described successfully functioning spleen allografts in severe classical hemophilia; immediate correction of the hemostatic defect and improvement in blood coagulation was achieved (42,43). Others have had more mixed results.

Combined pancreas-spleen allotransplantation in pigs (Table 3)

Two groups (44,45) have investigated the immunologic effect of transplanting the spleen with the pancreas in pigs. None of the pigs undergoing pancreas Tx alone showed signs of rejection while receiving cyclosporine for 28 days, but several combined pancreas-spleen graft recipients rejected their grafts before this time, some as early as one week (44,45), suggesting that the presence of the spleen was immunogenic; in no case did signs of GVHD develop.

Human spleen xenotransplantation in pigs (Table 3)

There has been one brief and limited report in which pigs were treated with a single dose of cyclophosphamide (30mg/kg i.v.) four days before Tx of a human spleen (46). Two of five pigs developed transient GVHD-like signs two to three weeks later. The number of human hematopoietic cells detected in these pigs, particularly in the bone marrow, was reported to be higher than in pigs that had undergone injection of human splenocytes only, but details were not reported. The human spleens, however, eventually appeared to be rejected.

Table 3. Spleen allo- and xeno transplantation in pigs

First Author	(n)	Procedure	Immunosuppression	Outcome	Reference
Dafoe	8	Pancreaticoduodenal graft +spleen	CyA+CS	rejection 7-16d	Transplantation 1985;40:579-584
Dafoe	5	Pancreaticoduodenal graft +irradiated spleen graft	CyA+CS	no effect no rejection d28	Transplantation 1986;42;686-687
Gänger	7	Pancreaticoduodenal graft +spleen	none	rejection <1w	Eur Surg Res 1987;19:323-328
Li	5	Human spleen graft	CPP	stable chimerism GVHD rejection <2w	Transplant Proc 2000; 32:1103-1104

CPP=cyclophosphamide,GVHD=graft-versus-host disease

SPTX IN HUMANS

Human spleen autoTx has been reported, but will not be reviewed here.

Allotransplantation

Clinical experience with spleen alloTx began in the early 1960s.

For hypogammaglobulinemia, hemophilia, or Gaucher's disease (Table 4A)
In one patient with hypogammaglobulinemia, it was hoped that the transplanted spleen, donated by the patient's mother, would alleviate a state of immune deficiency (37), but no rise in serum antibody levels was observed during the three months for which the spleen survived. One spleen was transplanted from father to son for hemophilia A (47); factor VIII levels did not rise and, because of severe bleeding, the graft had to be excised after 4 days. The transplanted spleen was found to be markedly swollen and had ruptured. More recently, successful SpTx has been described (48, 49); in one case, factor VIII was increased and no spontaneous hemorrhage occurred during two years of follow-up (48). In a case report of SpTx for Gaucher's disease, a spleen graft from an unrelated living donor resulted in a temporary improvement in the patient's biochemical parameters, but was finally rejected by day 40 (50,51); chimerism of erythrocytes was documented 3-6 weeks after SpTx, and the patient developed *in vitro* donor-specific hyporesponsiveness (52).

Table 4A. Clinical spleen allotransplantation for hypogammaglobulinemia, hemophilia, and Gaucher's disease

First Author	(n)	Indication	Outcome/Complications	Graft Survival	Reference
Marchioro	1	hypogammaglobulinemia	no gammaglobulin production	3m	Ann NY Acad Sci 1964;120:626-651
Hathaway	1	hemophilia	hemorrhage	7d	Transplantation 1969;7:73-75
Groth	1	Gaucher's disease (irradiated spleen 300 cGy)	hemolysis & cachexia temporary benefit Chimerism erythrocytes Donor-specific hyporesponsiveness	40d	Lancet 1971;1:1260-1264 Clin Exp Immunol 1972;10:359-365.
Xia	1	hemophilia (mother-to-son)	clinical improvement	>2y	Chin Med J (Engl) 1992;105:609-611
Liu	3	hemophilia	clinical improvement (2)	<5y (2)	Arch Surg 1995;130:33-39
Xiang	1	hemophilia	clinical improvement	>5 m	Transplant Proc 2002;34:1929-1931

For malignant disease (Table 4B)
In four patients with terminal malignancies, the purpose of SpTx was to superimpose a state of altered immunologic reactivity, and thus suppress the growth of the neoplasms (37). In an effort to generate a GVH reaction against the tumor, potential spleen donors, who also had a terminal malignancy, were sensitized with tumor cell suspensions from the patient 12 to 56 days before Tx. Recipient splenectomy was performed in all patients, and immunosuppression with azathioprine was initiated several days before the transplant. If rejection of the spleen

were suspected, prednisone and actinomycin C were added. None of these patients demonstrated cessation or regression of tumor growth, and hypersplenism was reported in 3 out of 4 patients. This was transient in two but sustained in one, when it was associated with hemolytic anemia, leukopenia, and thrombocytopenia. Bitter-Suermann suggested that the failure of the donor spleen to generate an immunologic response to the malignant disease (a graft-versus-tumor effect) may have been due to the use of immunosuppressive drug therapy that may have affected both graft and host reactions. Thus, the results "may have been masked, without invalidating the underlying concept of a transfer of cancer immunity" (13).
In a leukaemic patient, a spleen was transplanted from his healthy identical twin (53), and remained viable for six months without immunosuppression, but without beneficial effect on the leukaemia. Others, however, have reported shrinkage of hepatocellular carcinomas and reduced serum alpha-fetoprotein levels after SpTx (54).

Table 4B. Clinical spleen allotransplantation for malignant disease

First Author	(n)	Indication	Outcome/Complications	Graft Survival	Reference
Marchioro	4	terminal carcinoma	hemolytic anemia (1) leukopenia (1)	10d-8m	Ann NY Acad Sci 1964;120:626-651
Raccuglia	1	leukemia	No GVL effect	5m, viable	Clin Exp Immunol 1973;14:1-18
Liu	3	hepatocellular carcinoma	shrinkage of tumors, decrease AFP	5-11m	Arch Surg 1995;130:33-39

AFP = alpha-fetoprotein, GVL= graft-versus-leukemia

Combined pancreas-SpTx (Table 4C)
In the 1980s, several groups included the donor spleen to improve the perfusion of the pancreas graft when performing pancreas Tx. Starzl et al. reported the first four cases (55), immunosuppression consisting of cyclosporine and corticosteroids. No immunologic benefit appeared to result from the presence of the donor spleen in any patient. In a blood group A recipient of a composite graft from a donor of group O, urgent splenectomy was necessary after 6 days because of a severe hemolytic anemia associated with a positive Coomb's test and the presence of anti-A isoagglutinins. After removal of the transplanted spleen, the isoagglutinins disappeared over nine weeks.

Deierhoi et al. (56) reported one case of lethal GVHD after HLA-mismatched composite pancreas-SpTx. One week after Tx, the patient developed a progressive leukopenia, which persisted after discontinuing azathioprine therapy and irradiating the spleen (1000 cGy over three days). Twenty days after the transplant, donor splenectomy was performed. Over the next two weeks, severe cutaneous GVHD was followed by multi-organ failure. Human leukocyte antigen-typing of blood lymphocytes revealed 80-100% to be of donor type. Cyclosporine was

discontinued and high-dose corticosteroids begun but the patient succumbed from systemic candidiasis.

Table 4C. clinical combined pancreas-spleen allotransplantation

First Author	(n)	Indication	Outcome/Complications	Graft Survival	Reference
Starzl	4	Diabetes	hemolytic anemia (1)	6d-11m	Surg Gynec Obstet 1984;159:265-272
Gonwa	2	Diabetes	GVHD (2) hemolytic anemia (1)	16-24d	Transplantation 1985;40:299-304
Sollinger	7	Diabetes	less complications	?	Transplant Proc 1985;27:360-362
Munda	4	Diabetes	no side effects	12d-8m	Transplant Proc 1985;27:353-357
Deierhoi	1	Diabetes	lethal GVHD	19d	Transplantation 1986;41:544-546
Peltenburg	1	Diabetes	spleen graft rupture accelerated rejection	<3d	Transplantation 1992;53:226-228
Booster	12	Diabetes	accelerated rejection (1)	2d-9m	Transplantation 1993;56:1098-110.

Two additional cases of GVHD after pancreas-SpTx have been reported by Gonwa et al. (57). Both patients developed leukopenia and thrombocytopenia, which in one patient did not respond to therapy, necessitating excision of the spleen graft. Mixed hematopoietic cell chimerism was present, although the extent was not reported. The second patient, who also developed skin GVHD, responded well to irradiation of the spleen.

The largest and most recent study on composite pancreas-SpTx was reported by Booster et al (58). In order to prevent GVHD, in 11 of 12 grafts, during cold storage of the donor organs before Tx, the spleen alone was irradiated (600 cGy). No GVHD was observed, but no immunologic advantage of SpTx was identified. In four patients, a search was made for chimerism, and lymphocyte chimerism of 14-20% was detected in two of them.

Xenotransplantation

Although clinical xenogeneic SpTx does not appear to have been performed, several hundred patients, with a variety of disease states, have had their blood or plasma perfused through pig (or other animal) spleens (59-62). The clinical response to extracorporeal spleen perfusion in patients with septic conditions has been reported to be good. Details of this form of therapy are limited, but a careful investigation by Paradis et al (63) (into the possibility that these patients had become infected with porcine endogenous retrovirus) documented that 23 of the 30 patients tested demonstrated pig microchimerism. None of these patients had been exogenously immunosuppressed at the time of the procedure (which lasted only hours) or since, and yet microchimerism had persisted for between two months and eight years since spleen perfusion had taken place.

COMMENT

There is convincing evidence of a tolerogenic effect by the transplanted spleen under various conditions in some rodent models. Several studies have indicated that a balance between host-versus-graft and GVH responses can be achieved after SpTx, leading to long-term survival of both the spleen and a second donor-specific organ. There is evidence to suggest that the induction of tolerance is associated with the development of regulatory cells. A donor-specific organ transplant can be protected from rejection by the transfer of cells from an animal with a long-surviving spleen graft.

In large animals and humans there has been no definite evidence that SpTx results in tolerance to itself or improves survival of a concomitantly-transplanted second organ. Indeed, the prevailing evidence is that the transplanted spleen is rejected, as is any other organ. However, no study in large animals has investigated the potential of this approach in a comprehensive way in a model in which knowledge of the major histocompatibility complex of both donor and recipient was known. What is clear from clinical SpTx is that a GVH response can result and can be severe enough to cause lethal GVHD, indicating the spleen's potential in counteracting the usual host response that occurs after organ Tx.

The benefits of inducing a state of tolerance in a recipient of an organ allograft, and the advantages of this state over chronic pharmacologic immunosuppressive therapy, are considerable. It would seem worth investigating SpTx in a large animal model, such as the Massachusetts General Hospital major histocompatibility complex-defined miniature swine Tx model, in which there is considerable experience of the Tx of other organs. We are currently undertaking such a study (64).

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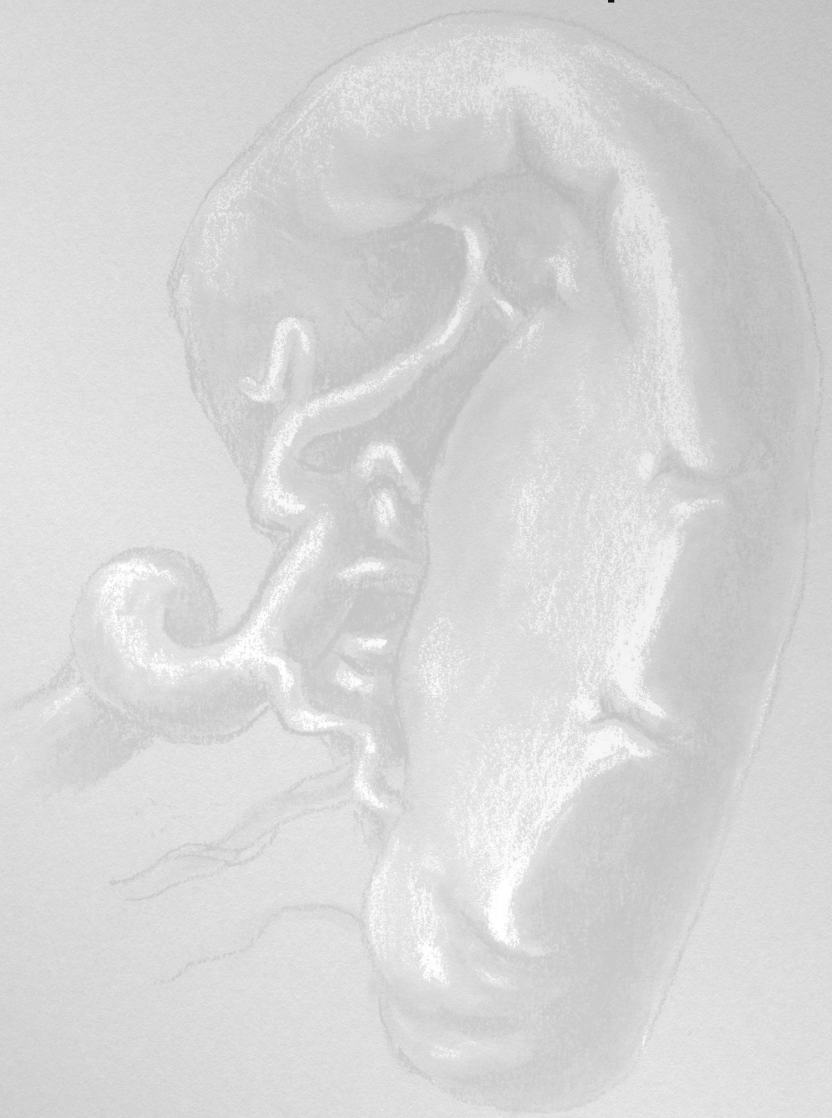
Spleen Transplantation in Miniature Swine: Surgical Technique and Results in MHC-matched Donor and Recipient Pairs



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Bernd Gollackner*, Frank JMF Dor*, Christoph Knosalla, Leo H Buhler, Michael Duggan, Christene A Huang, Stuart L Houser, David H Sachs, Tatsuo Kawai, Dicken SC Ko, David KC Cooper. Spleen transplantation in miniature swine: surgical technique and results in MHC-matched donor and recipient pairs.

Transplantation 2003;75:1799-1806.

*authors contributed equally

ABSTRACT

Background: Spleen transplantation (SpTx) between some strains of rodents can lead to donor-specific tolerance either spontaneously or following a short course of immunosuppression. The aim of the present study was to develop a surgical technique for SpTx in miniature swine to investigate its immunological impact in a large animal model.

Methods: The preferred surgical technique of SpTx (n=8) involved excision of the donor spleen with its vascular pedicle to the aorta and portal vein. Carrel patches of donor aorta and portal vein were respectively anastomosed to the abdominal aorta and inferior vena cava of the (splenectomized) recipient. The results in four MHC-matched pairs that were mismatched for the porcine allelic antigen are reported. Two recipients were untreated, one received a 12-day course of cyclosporine (CyA) alone, and one received thymic irradiation (TI; 700 cGy) and CyA. Hematopoietic cell chimerism was followed by FACS, and graft survival was assessed by histology.

Results: SpTx was technically successful. In two untreated pigs, chimerism was detected in the blood (max. 5% for 17 and 25 days, respectively) and lymph nodes (max. 6% for 28 and 56 days, respectively), but both grafts showed histological rejection by day 28. In two treated pigs, chimerism was present in the blood for 47 and 57 days, respectively, and rejection was prevented, with follow-up for 57 and 217 days, respectively.

Conclusion: SpTx in MHC-matched pairs treated with CyA +/- TI results in prolonged chimerism, associated with the development of tolerance to the transplanted spleen.

INTRODUCTION

In some rodent models, transplantation (Tx) of the spleen has resulted in the spontaneous development of tolerance to the graft and to a subsequently transplanted donor-specific organ (reviewed in 1). In other models, the same result was achieved if a short course of immunosuppressive therapy was administered at the time of SpTx. There is some evidence that the induction of tolerance was associated with the development of regulatory cells in the recipient. If the recipient's immune response was suppressed further, graft-versus-host disease (GVHD) resulted. Importantly, a state of tolerance was not induced by the Tx of spleen cells rather than of the intact whole organ.

Studies carried out in large animal models have not been able to duplicate the results achieved in rodents (1). However, in none of these investigations have large animals been used in which the major histocompatibility complex (MHC) of the donor and recipient was known. SpTx has been reported in humans (1), but in none has the recipient clearly benefited from the procedure. Hemolytic anemia, often combined with thrombocytopenia and leukopenia, and GVHD

(which has been fatal in one patient) have been reported, demonstrating the potential of the spleen's graft-versus-host immune response. Hematopoietic cell chimerism has also been reported.

It seems realistic to anticipate that, if the immune responses of both host and donor spleen are balanced, a state of tolerance could be achieved that might allow the concomitant or subsequent Tx of another donor-specific organ. This approach might also have potential in achieving T cell tolerance to a discordant xenograft. Before further clinical investigations are undertaken, which carry some risk to the patient, this approach requires exploration in a suitable large animal model. Since there have been no studies in large animals in which the MHC of the donor and recipient was known, we have initiated a series of studies in Massachusetts General Hospital MHC-inbred miniature swine. We here report the development of the surgical technique of SpTx, and the results in four MHC-matched pairs. In view of the beneficial immunosuppressive effect associated with native splenectomy reported in rodent studies (1), the recipient spleen was excised in all cases.

MATERIALS AND METHODS

Animals

Animals were selected from our herd of MHC-inbred miniature swine (2). Spleen donors were 3-11 months old, weighed 14-72 kg and were positive for the pig allelic antigen (PAA), an allelic non-histocompatibility marker of no known function (3). Recipients were 4-13 months old, weighed 12-72 kg, and were negative for PAA. Donor and recipient were matched for weight, blood group (all of type O), and MHC antigens, and mismatched for minor antigens. Eight spleen transplants have been performed. All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996).

Operative procedures

Catheters were inserted into the right carotid artery and internal and external jugular veins in both donor and recipient pigs. In the recipient, the carotid artery catheter was removed at the end of the operation, the venous catheters remaining *in situ* for 28 days.

Our initial technique (n=2) involved donor splenectomy and end-to-side anastomoses of the donor suprapancreatic splenic artery to the recipient infrarenal abdominal aorta and of the

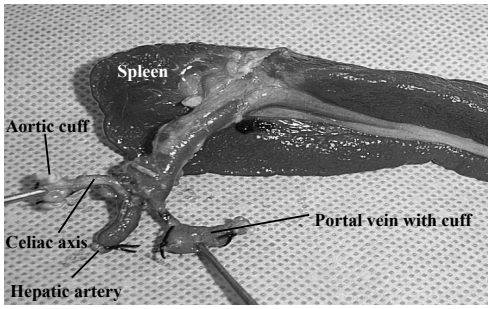
donor splenic vein to the infrarenal inferior vena cava (IVC). The diameter of the splenic vessels in the donor pigs used was small (<5 mm), and this approach was complicated by stenosis and/or thrombosis of these vessels at the anastomotic sites. We therefore developed a technique in the donor (n=6) where the splenic artery and celiac axis were mobilized to the aorta to enable a Carrel patch of aorta to be taken with the graft. The splenic vein was dissected to its confluence with the superior mesenteric vein to enable a cuff of portal vein to be included.

Donor operation (Figure 1A)

After midline laparotomy, the splenic mesentery was divided. In the pig, at least one accessory splenic artery, arising from the left gastroepiploic artery with sometimes an additional small branch from the short gastric arteries, contributes a blood supply to the distal spleen, and this was ligated and divided with its accompanying vein. The pancreas was mobilized from around the splenic vessels. The main splenic artery was mobilized to its origin from the celiac axis. The common hepatic artery was identified and preserved to maintain perfusion of the liver during the dissection. The celiac axis was traced and dissected down to its origin from the aorta. The left gastric artery and a small branch to the diaphragm were ligated and divided. After division or resection of the tail of the pancreas, the splenic vein was dissected to its confluence with the superior mesenteric vein where it forms the portal vein. After heparinization (300 units/kg), the aorta and portal vein were sequentially doubly clamped (to prevent congestion of the splenic graft), and portal venous and aortic segments were excised with the splenic vessels to provide arterial and venous cuffs (Carrel patches) to facilitate insertion into the recipient (Figure 1A). (Blood was withdrawn from the donor before the clamping of the aorta and IVC for subsequent transfusion into the recipient). The donor pig was then euthanized. The donor spleen was weighed (average weight 8g/kg bodyweight) and biopsied. The spleen was then placed in a tray of cold (4°C) saline, and the splenic artery was flushed with approximately 40 ml/kg bodyweight of cold (4°C) electrolyte solution for kidney preservation (Baxter, Deerfield, IL) until the venous effluent was clear (Figure 1B). The spleen was then reweighed (demonstrating that the flushing procedure resulted in spleen weight loss of 30-50%) and biopsied again. The spleen was then implanted into the recipient.

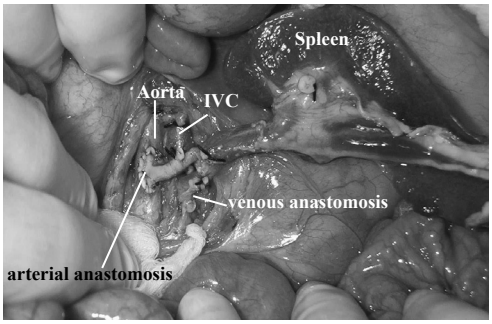
Recipient operation (Figure 1C)

After midline laparotomy and native splenectomy, the infrarenal aorta and IVC were mobilized, and heparin (100 units/kg) administered. The donor spleen was implanted by anastomosing the cuff of donor aorta (from which the celiac axis and splenic artery arise) end-to-side to the recipient abdominal aorta, and the cuff of portal vein (into which drains the splenic vein) end-to-side to the IVC, using continuous sutures of 5-0 polypropylene (Figure 1C). Reperfusion of the donor spleen resulted in a fall in hematocrit of approximately 25% (from approximately 40% to 30%) accompanied by a modest fall in platelets, and so (unirradiated)



A

B



C

Figure 1. Surgical technique of spleen transplantation in miniature swine.

(A) In the donor, excision of the spleen involves dissection of the splenic artery and celiac axis to the aorta and of the splenic vein to its confluence with the superior mesenteric vein to form the portal vein, which necessitates total pancreatectomy. Before transplantation into the recipient, the hepatic artery stump will be shortened.

(B) Flushing of the spleen graft with cold (4°C) electrolyte solution for kidney preservation trough the splenic artery, while placed in a tray of cold (4°C) saline, until the effluent from the splenic vein is clear.

(C) In the recipient, end-to-side anastomoses are performed between the cuff of the donor aorta to the distal abdominal aorta and between the cuff of the donor portal vein to the IVC.

See addendum for colour version

packed red blood cells, drawn from the spleen donor before euthanasia, were infused. At the end of the operative procedure, the donor spleen was positioned superficial to the bowel, immediately under the anterior abdominal wall in the left or right flank.

Split-thickness skin harvesting from donor and recipient
After euthanasia of the donor, split-thickness skin was aseptically harvested with a Zimmer dermatome. For cryopreservation, segments of skin were spread on nylon gauze and placed in petri dishes containing RPMI 1640 and an equal volume of balanced salt solution containing 15% dimethylsulfoxide (cryopreservation medium; Microbiological Associates, Walkersville, MD). The skin was frozen at 1°C/min in a Planer freezing apparatus (G.V. Planer, Subury-on-Thames, Middlesex, UK) and stored at -80°C. In the recipient, skin was harvested as described above before laparotomy.

Skin grafting in the recipient
Split skin grafts of self, donor, and third party MHC-matched and MHC-mismatched pigs were performed by techniques published previously (4) approximately two months after SpTx. Before use, the skin was rapidly thawed and washed three times with RPMI 1640 medium. Grafts were placed on a deep split-thickness bed on the recipient's dorso-lateral thorax. Grafts were examined daily until macroscopic rejection occurred, and rejection was confirmed by histological examination of sequential biopsies.

Biopsies
Biopsies of the cervical thymus, mesenteric lymph nodes, bone marrow, and splenic graft were taken immediately pre-reperfusion, and subsequently on post-Tx days 14, 28, and then at increasing intervals. A biopsy of the transplanted spleen was taken two hours after reperfusion. Splenic graft (wedge) biopsies and mesenteric lymph node biopsies were performed through an abdominal flank incision. All biopsies were examined histologically and by flow cytometric analysis for the presence of donor (PAA-positive) cells. Whenever a skin rash developed or there was diarrhea, skin and/or rectal biopsies were taken and examined for histopathologic features of GVHD. Biopsies for histological examination were fixed in 10% formalin, processed overnight, and paraffin-embedded. Five-micron sections were stained with hematoxylin and eosin, and examined by light microscopy, with systematic attention being paid to the following anatomic structures - red pulp, white pulp, stroma, and blood vessels. A full autopsy was performed at the time of death or euthanasia.

Thymic irradiation

With the aim of reducing the number of mature T lymphocytes, thymic irradiation (TI) (700 cGy) was administered from a cobalt irradiator on day –1, as described previously (5).

Immunosuppressive therapy

Cyclosporine (CyA) (Sandimmune, generously provided by Novartis Pharmaceutical Corporation, East Hanover, NJ) was administered intravenously as a single daily infusion at a dose of 10-30 mg/kg (adjusted to maintain a whole blood trough level of 400-800 ng/mL) for 12 consecutive days, starting on the day of the SpTx. CyA levels were determined by a monoclonal fluorescence polarization immunoassay (Abbott Laboratories, Dallas, TX), which measures the parent compound, but not metabolites.

Monitoring and supportive therapy

Blood cell count, serum chemistry, and CyA levels were determined daily for 28 days by routine methods. All pigs received daily prophylactic enrofloxacin (5 mg/kg/d i.v.) while indwelling venous catheters were present, which was usually for 28 days. During this period, dalteparin (Fragmin, Pharmacia & Upjohn, Kalamazoo, MI) 100 IU/kg was administered subcutaneously daily as prophylaxis to prevent thrombosis in the catheters and in the vessels of the transplanted spleen.

Preparation of cell suspensions and flow cytometry

These procedures and the antibodies used have been described previously (5-7). Flow cytometry was carried out on blood, lymph nodes, thymus and bone marrow at intervals to determine the presence of hematopoietic cell chimerism.

RESULTS

Immediate posttransplant outcome

Since this surgical technique has been adopted, there have been no anastomotic or thrombotic complications. Biopsies taken after cold flushing of the spleen (pre-reperfusion) revealed normal splenic architecture but with a reduced number of red blood cells in the sinusoids (Figure 2A). In two of the six cases, reperfusion injury was documented on splenic biopsies taken two hours after reperfusion. In one case, for uncertain reason, the donor developed cardiac arrest during mobilization of the spleen, and there was a period of warm ischemia before the spleen could be excised. In the other, the donor suffered a prolonged period of hypotension during mobilization of the spleen. In both cases, biopsies showed disruption of the splenic architecture with increased polymorphonuclear cell infiltration and, in one case, congestion and hemorrhage (not shown). Both animals died suddenly several hours after operation. Autopsy showed no other abnormalities.

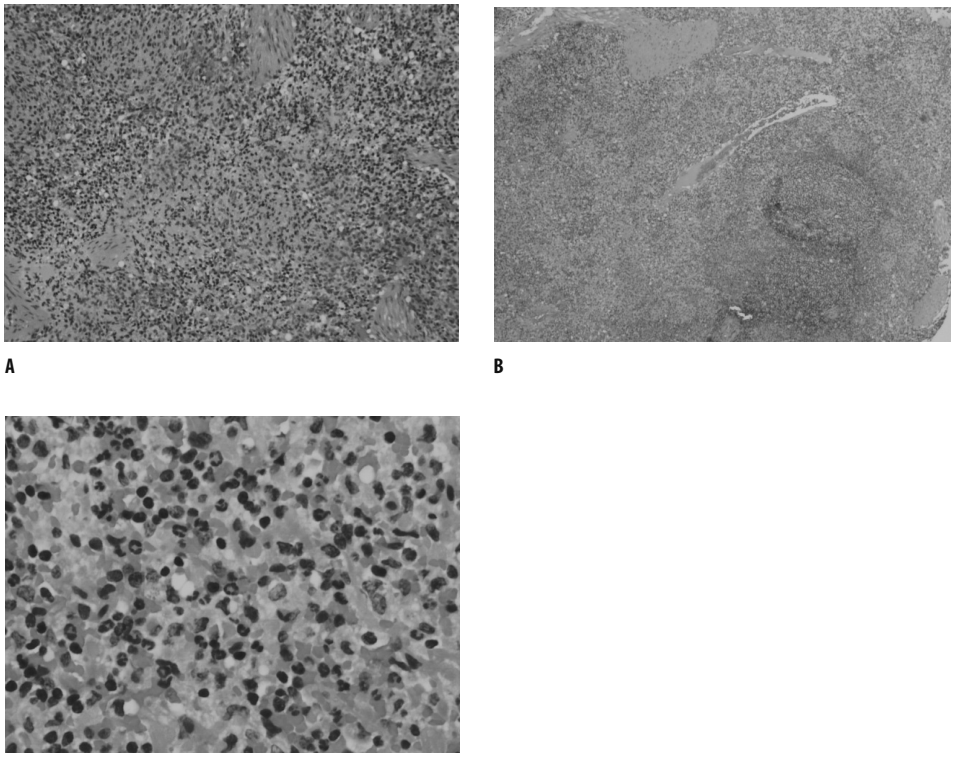


Figure 2. Histology of spleen biopsies taken after cold flushing (pre-reperfusion) (A) and two hours after reperfusion (B, C).
(A) Normal splenic architecture but with a reduced number of red blood cells in the sinusoids.
(B) Normal splenic architecture following uneventful spleen transplantation.
(C) polymorphonuclear cell infiltration two hours post-reperfusion after uneventful spleen transplantation.

In the remaining four cases, in which no insult to the spleen was documented, the post-reperfusion biopsies demonstrated normal splenic architecture (Figure 2B) with some polymorphonuclear cell infiltration (Figure 2C). Post-Tx recovery was uneventful. There were slight increases in serum amylase and lipase between days 1-4, which were possibly related to handling of the pancreas at the time of native splenectomy; these findings were not associated with clinical features of pancreatitis.

Long-term posttransplant outcome

By 28 days, one of the grafts was frankly rejected, and the other revealed evidence of imminent rejection (Figure 3). Neither spleen was excised at this stage, and yet neither pig demonstrated any clinical or biochemical abnormalities during the period of follow-up, which

extended for 88 and 160 days, respectively. The pig that received CyA alone did not reject its graft, but it died suddenly on day 57 from an unrelated volvulus (Figure 3). Between post-Tx days 6 and 10, this pig developed transient features of cutaneous GVHD (grade 1) on biopsy, that resolved spontaneously. The recipient that received both CyA and TI did not reject its splenic graft and was euthanized on day 217 (Figure 3).

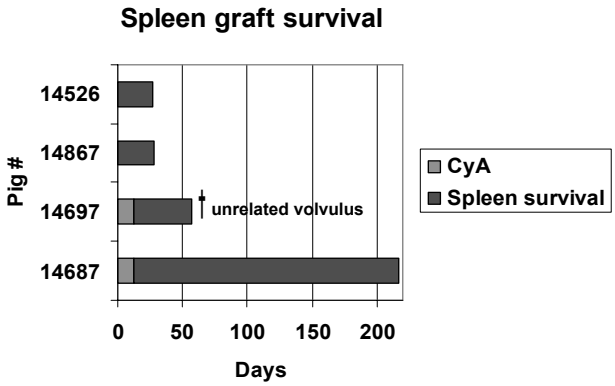


Figure 3. Period of CyA therapy and survival of spleen grafts (assessed by histology) in MHC-matched minor-mismatched miniature swine (n=4). Pigs #14526 and #14867 received no immunosuppressive therapy, whereas pigs #14697 and #14687 received a 12-day course of CyA. In addition, TI (700 cGy) was administered to pig #14687. Pig #14697 died on day 57 from an unrelated volvulus, with a viable spleen graft.

Histological examination of spleen biopsies

At the time of splenic biopsy, the macroscopic appearance correlated well with the histological findings. A spleen of aubergine (dark purple) color and soft texture, with good pulsation of the splenic artery, and bleeding from the biopsy site, indicated a viable graft.

Splenic biopsies from the two untreated recipients demonstrated normal histology on day 14 in one case and an atypical lymphocytic proliferation with cellular pleomorphism and an increase in mitotic activity (resembling posttransplant lymphoproliferative disease) in the other, perhaps a harbinger of early rejection (not shown). The stroma in the latter graft had foci of atypical lymphocytic pleomorphic infiltration. Identifiable blood vessels looked normal. By day 28, there was multifocal hemorrhage (Figure 4A) which, by day 56, was associated with focal areas of fibrinoid necrosis, a reduction in the extent of the white pulp, and vessel wall necrosis. These features were consistent with acute rejection. Moreover, by day 28, the graft which had revealed a proliferation of lymphoid tissue previously, now showed frank necrosis and hemorrhage on biopsy. Despite evidence of rejection, both spleens were left *in situ*. On day 88, a biopsy of the more necrotic of the two grafts revealed a loss of architecture, large areas of necrosis surrounded by

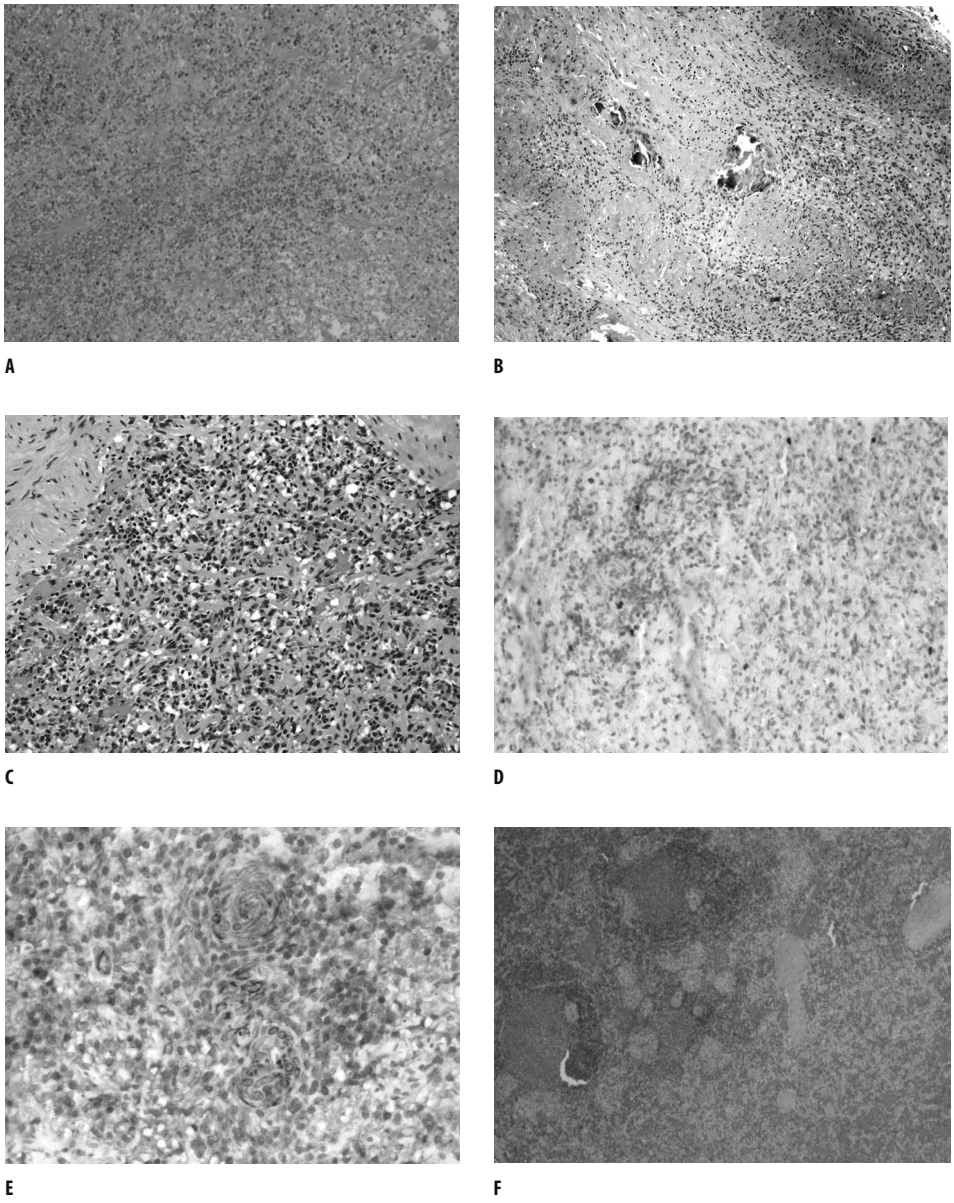


Figure 4. Histology of spleen grafts.

(A) Rejected graft on day 28 in an untreated pig, showing hemorrhage and fibrinoid necrosis.

(B) Rejected graft on day 90, showing large areas of necrosis surrounded by palisading histiocytes, with some fibrous replacement of the red and white pulp.

(C+D+E) Spleen graft on day 160 (130 days after rejection) in an untreated pig, indicating features suggestive of stromal regeneration and cellular repopulation.

(C) H&E staining showing the red pulp,

(D) vimentin staining showing a central artery in an atrophic Malpighian corpuscle.
(E) atrophic white pulp on a PCNA stain with counterstained lymphocytes using hematoxylin.
(F) Normal architecture on day 217 in a pig treated with a 12-day course of CyA and TI.

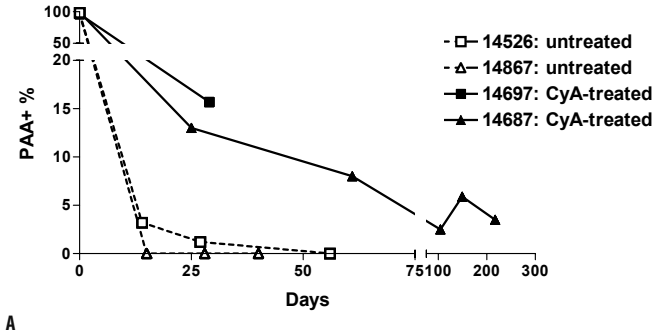
palisading histiocytes and some fibrous replacement of the red and white pulp (Figure 4B). A few remnants of Malpighian corpuscles could be identified in the white pulp. The vessels were entirely necrotic, whereas the stroma still showed some viable cells. The other graft, on day 160, looked surprisingly normal macroscopically. Histologically, no normal white pulp could be identified, but there was a fibroblast-like proliferation occupying most of the red pulp, associated with an inflammatory component of histiocytes and lymphocytes (Figure 4C). The stroma was intact and viable. Compared with a normal spleen, few blood vessels could be identified, but those present were grossly normal. Our impression was that diffuse necrosis had been avoided, significant stromal recovery had taken place, and some repopulation of the spleen with viable cells had occurred (Figure 4D&E).

The transplanted spleens of the two pigs receiving CyA showed no features of rejection up to days 57 (death) and 217 (euthanized), respectively (Figure 4F).

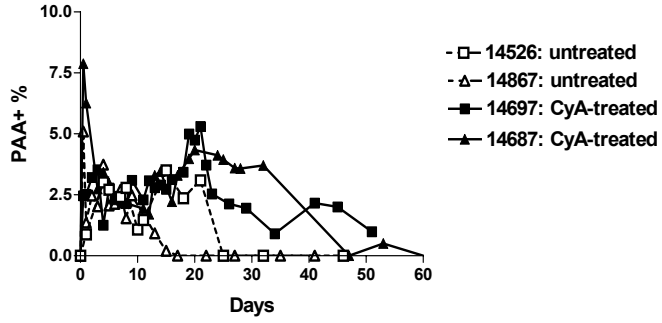
Flow cytometry for hematopoietic cell chimerism

Replacement in the spleen of donor splenocytes by recipient lymphocytes (mainly CD3+/CD8+ T cells and CD16+ cells, not shown) occurred over the course of the first 2-4 weeks in all four pigs, but was more rapid in the two animals in which the spleen was rejected (Figure 5A)**.** All recipient pigs received a transfusion of packed red blood cells from the spleen donor at the time of SpTx. These packed red blood cells contained small numbers of leukocytes. Transfusion of these packed red blood cells alone (without SpTx) has been demonstrated to lead to chimerism of <1% for <2 days (not shown).

The two untreated pigs remained chimeric (1-5%) for PAA+ donor cells in the blood until days 17 and 25, respectively (Figure 5B), and donor cells were found in the lymph nodes until day 56. Adding a 12 day-course of CyA to the regimen (without TI) resulted in a prolongation of chimerism in the blood (1-6%) until day 53 (Figure 5B). Four days later the pig was found dead, and autopsy demonstrated a volvulus with secondary peritonitis. Because of autolysis of the tissues, FACS could not be successfully performed. Chimerism had been documented in the lymph nodes and spleen graft on day 29. In the pig that received a 12-day course of CyA and TI, chimerism (1-8%) persisted in the blood until day 54 (Figure 5B), the donor cells consisting mostly of T lymphocytes (CD3+, CD4+, CD8+, not shown). Chimerism was detectable in the lymph nodes on days 28 and 217, the day of euthanasia (but not on days 12, 104 and 149) and in the thymus on days 60, 69 and 217 (Figure 6). Both CD1+ and CD3+ cells were detected in the thymus, suggesting the presence of immature cells. In none of the four recipients was chimerism detected in the bone marrow on any occasion.



A



B

Figure 5. Chimerism in the transplanted spleen (A) and recipient blood (B) after spleen transplantation (n=4). Spleens were transplanted from PAA+ donors into PAA- recipients.

(A) Percentage of PAA+ donor cells in the transplanted spleen in untreated (n=2, dashed lines) and CyA-treated (n=2, straight lines) recipients.

(B) Percentage of PAA+ donor cells in the blood in untreated (n=2, dashed lines) and CyA-treated (n=2, straight lines) recipients.

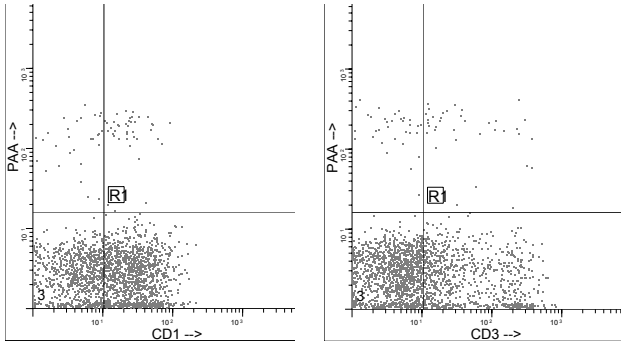


Figure 6. CD1+ (left) and CD3+ (right) populations of PAA+ donor cells in the recipient thymus on day 69 in a pig treated with a 12-day course of CyA and TI. PAA+ donor cells made up 3% of the total number of thymocytes.

Skin grafting

Grafting of frozen self, donor, donor-type, and fully-mismatched skin was performed on day 65 in the two untreated pigs and on day 73 in the pig treated with CyA and TI. In all three pigs, all skin grafts except self underwent rejection within 8-23 days, confirmed by histology**.**

DISCUSSION

Our study demonstrates that, using the surgical technique we have developed, SpTx in miniature swine is feasible. Several previous groups, using different anastomotic techniques, have reported a high incidence of technical failure in large animals (9-13). We believe that the inclusion of Carrel patches in the donor preparation is an important feature contributing to the success of the technique.

The splenic artery and vein, particularly in young pigs of <25 kg, are of small diameter and, without the use of microsurgical techniques, are difficult to anastomose to the recipient vessels. This has proved a problematic aspect of the technique in other animal models, first noted by Carrel in 1910 (9). By dissecting the splenic vessels down to the abdominal aorta and portal vein, respectively, and taking a cuff of these vessels, anastomoses can be performed without risk of stenosis or thrombosis. We have, however, taken the precaution of administering dalteparin during the early post-Tx period, partly to prevent thrombosis in these vessels, and partly to maintain patency of the indwelling venous lines in the neck (10).

Using the technique described here, the donor pig has been euthanized to obviate the need to perform reconstructive surgery on the aorta and portal vein. In a clinical setting, however, anastomoses of the splenic artery and vein directly to the recipient aorta and IVC would be possible using microsurgical techniques. Furthermore, if required, reconstructive procedures on the donor aorta and portal vein would be feasible using synthetic vascular prostheses or autologous vein grafts.

In two experiments, hemodynamic instability in the donor resulted in injury to the spleen following reperfusion. This injury appeared to be associated with the sudden deaths of these two animals, possibly as a result of cytokine release. No cause for the deaths could be determined at autopsy. This experience, however, has provided evidence that technically satisfactory Tx of a well-preserved spleen can be confirmed by biopsy taken two hours after reperfusion.

From our initial experience, it would appear that the spleen in the pig represents 8g/kg body weight, which is much more than its weight in humans, in whom the normal spleen repre-

sents only 2g/kg (8). Whether the relative masses of the spleens may be associated with different immunological effects following SpTx remains unknown, but it is possible that observations made in miniature swine may not be representative of those in other animals, including the human. Nevertheless, the MHC-inbred miniature swine represents the best large animal model in which to explore the phenomenon of tolerance associated with SpTx reported in numerous rodent models (1).

Our study indicates that donor lymphoid cells in the spleen are uniformly replaced by cells of recipient origin, but that this occurs more rapidly when rejection is occurring. The distribution of PAA in our herd has been well described for several lymphoid tissues and blood cells (3), but not previously for the spleen. In the PAA+ donor pigs in the present study and in subsequent experiments not reported here (Chapter 5), 95-100% of splenocytes were PAA+. The percentage of donor (PAA+) cells remaining in the spleen in the two untreated, rejecting pigs was 0-4% by day 15, whereas it remained at >15% on day 29 in both treated pigs and at 4% on day 215 in the long-term survivor. In subsequent studies not reported here, we have confirmed that, when rejection is occuring in the absence of immunosuppression, host (PAA-) cells largely replace donor cells in the spleen within 12 days (Chapter 5). However, when rejection is prevented, donor cells are only slowly replaced, and persist for several weeks or months.

We also document the histopathologic appearances of acute rejection in the spleen transplanted across a minor-antigen barrier. (In contrast to some reported studies in large animals (1), complete rejection of the spleen graft was tolerated without obvious clinical detriment to the recipient pig.) Hemorrhage and necrosis developed, followed by some fibrous replacement of the splenic tissue, similar but not identical to the changes previously described in the rodent and canine models (1). In one spleen graft that was followed for 130 days after rejection, significant regeneration of stroma and repopulation with cells took place.

Successful SpTx was accompanied by chimerism in the blood. As chimerism was lost earlier in those pigs that rejected the spleen, we conclude that immunosuppressive therapy prolonged the period of chimerism, though not necessarily the extent of chimerism (which remained at 1-8% in all cases). Previous work by others at our center (7) has demonstrated that untreated MHC-matched kidney recipients achieved chimerism of only 0.2-0.7%, and this was lost within 7 days, whereas the percentage of donor cells in the blood of our untreated spleen recipients was 1-6% and was detectable for 17-25 days. Chimerism in the blood after MHC-matched kidney transplantation in recipients given a 12-day course of CyA has also been reported to be a maximum of only 0.7%, but persisted for 40-60 days (7). In contrast, in the present study, chimerism after SpTx was sometimes nearly 10 times higher than this, although it lasted for about the same period of time (54-57 days). Only following bone marrow Tx have greater levels of chimerism been detected in MHC-matched pairs, but this has been in association with

regimens that involved myeloablation (e.g., with whole body irradiation or cytotoxic drug therapy) and/or specific T cell depletion with a potent immunotoxin (14).

In addition, all spleen recipients demonstrated chimerism on at least one occasion in mesenteric lymph nodes and, in the CyA/TI-treated pig, donor cells were detected in the thymus on days 61, 69 and 217. Chimerism in the thymus has not been reported following kidney Tx in MHC-matched miniature swine (7). We did not detect donor cells in the bone marrow on any occasion, suggesting that engraftment in the bone marrow did not occur.

In the limited experience reported in the present paper, tolerance to the transplanted spleen would appear to have been induced by a regimen of 12 days CyA and TI and probably by CyA alone (although the death of the pig from a volvulus precludes certainty in this respect). In comparison, a 12-day course of CyA leads to 100% survival of MHC-matched kidney transplants in miniature swine (7). In untreated recipients, two thirds of MHC-matched kidney grafts survived indefinitely (15). Although the numbers in our study are small, tolerance to the spleen graft did not appear to be achieved in the absence of CyA therapy.

In the present study, tolerance to donor-specific skin grafts was not achieved, although graft survival was possibly slightly prolonged at 8-23 days (compared to a mean of 12 days reported previously from our center (2,16)). Pig skin expresses immunogenic alloantigens that can lead to skin graft rejection despite tolerance to other donor tissue and donor-specific unresponsiveness *in vitro* (17). Our findings are therefore consistent with rejection due to the presence of skin-specific antigens.

We are currently investigating the outcome of SpTx across class I and full MHC barriers.

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Histopathology of Spleen Allograft Rejection in Miniature Swine

5

Frank JMF Dor, Bernd Gollackner, Kenji Kuwaki, Dicken SC Ko, David KC Cooper, Stuart L Houser. Histopathology of spleen allograft rejection in miniature swine.
International Journal of Experimental Pathology 2005;86:57-66.

ABSTRACT

Spleen transplantation has established donor-specific tolerance in rodents**,** but not in large animals or humans. We report the histopathology of rejection in an established model of spleen transplantation in MHC-defined miniature swine. Of 17 spleen transplants, rejection was observed in 2 grafts transplanted into untreated MHC-matched, minor antigen-disparate recipients (Group 1) and in 5 grafts transplanted into fully MHC-disparate recipients (Group 2), one of which was untreated and 4 of which received some form of immunosuppressive therapy. Following biopsy and/or necropsy, fixed allograft tissue sections were treated with multiple stains, immunohistochemical markers, and TUNEL assay. Common features of rejection occurred in grafts from both groups, but with varying time courses. Necrosis developed as early as day 8 in Group 2 and day 28 in Group 1, ranging from focal fibrinoid necrosis of arteriolar walls and sinusoids to diffuse liquefactive necrosis, usually associated with hemorrhage. Other features of rejection included white pulp expansion by atypical cells and decreased staining of basement membranes and reticular fibers. A doubling of the baseline TUNEL index preceded histologically identifiable rejection. This study establishes histologic guidelines for diagnosing and, perhaps, predicting acute rejection of splenic allografts transplanted across known histocompatibility barriers in a large animal model.

INTRODUCTION

Since 1910, spleen allotransplantation (SpTx) has been performed for various reasons in rodents, large animals, and humans (1). In some rodent models, there is clear evidence that a transplanted spleen can induce a state of donor-specific tolerance, and allow long-term survival of both the transplanted spleen and another donor-specific organ without exogenous immunosuppressive therapy (1-3); however, in large animals and humans, its tolerogenic capacity has not been demonstrated (1,4,5). We are currently investigating whether SpTx can induce tolerance in MHC-defined miniature swine, a clinically-relevant large animal model. No study of SpTx has been made previously in any large animal model in which information on the major histocompatibility complex (MHC) of both recipient and donor was known.

Bitter-Suermann (6) described the morphologic features of SpTx in rats, and there have been several reports in outbred dogs (4,7-12). Most reports on human spleen allograft rejection have not included detailed histologic descriptions. In our current studies in miniature swine, histologic and immunohistochemical studies were performed to determine the course after SpTx with or without various immunosuppressive regimens. We here characterize previously unreported histologic features of spleen allograft rejection, and suggest a systematic grad-

ing system based on histopathologic features, similar to those used to evaluate rejection of transplanted hearts and kidneys.

As the transplanted spleen can induce a state of tolerance to itself and to a second donor-specific organ (13), the histopathological interpretation of spleen biopsies may become clinically relevant.

METHODS

Animals

Pigs were selected from our herd of MHC-inbred miniature swine, of blood group O (14). Spleen donors were 5.1 ± 3.3 months old, weighed 31.9 ± 20 kg, and were positive for the pig allelic antigen (PAA), an allelic non-histocompatibility marker with no known function (15). Weight-matched recipients were 5.2 ± 4.5 months old, weighed 31.2 ± 28.7 kg, and were negative for PAA. SpTx has been carried out over a minor antigen (Group 1; n=4), MHC class I (n=1), or full MHC (Group 2; n=12) histocompatibility barrier (Table 1).

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996), and were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical procedures

The technique of SpTx has been described previously (16). Wedge biopsies of the donor spleen were taken immediately before explantation from the donor, after flushing, two hours after reperfusion in the recipient, and subsequently through an abdominal flank incision on post-Tx days 14, 28, and then at increasing intervals. In one untreated recipient (#14534) in Group 2, additional spleen biopsies were taken on days 4, 8, and 11. Biopsies were taken from different sites in the spleen on each occasion, working from distal to proximal. Bleeding resulted from biopsy of a viable spleen graft, but not from a totally-rejected graft (Grade 3, see below). The bleeding could be readily controlled by cautery coagulation, and the application of pressure to the biopsy site for 10 minutes, sometimes with the use of Surgicel (Ethicon, Somerville, NJ). Examination and interpretation of a total of 106 biopsies form the basis of this study. The grafts were evaluated macroscopically with regards to size, color, consistency/texture, arterial

Table 1 Immunosuppressive regimens and outcome.

Group	Animal #	Immunosuppression			Outcome (day of rejection [a])
		WBI	TI	CyA	
1: MHC- matched	14526	-	-	-	Rejection [b] (28)
	14867	-	-	-	Rejection (27)
	14697	-	-	12 days	No rejection
	14687	-	+	12 days	No rejection
MHC Class I- mismatch	15111	-	+	12 days	No rejection
2: MHC-mismatched	14534	-	-	-	Rejection (8)
	14646	-	+	- (CPP)	Rejection (16)
	14840	-	+	28 days	Rejection (62)
	14832	-	+	12 days	Rejection (47)
	15029	-	+	23 days	No rejection [c]
	15051	+	+	45 days	No rejection
	15242	+	+	45 days	No rejection
	15311	+	+	45 days	Rejection (61)
	15328	+	+	45 days	No rejection
	15399	+	+	30 days	No rejection
	15410	+	+	45 days	No rejection
	15446	+	+	45 days	No rejection

CyA = cyclosporine at 10-30 mg/kg i.v. daily to maintain a whole blood trough level of 400-800 ng/mL; CPP = cyclophosphamide (3x 20 mg/kg iv on days -5,-4 and -3); WBI = whole body irradiation (100 cGy on day -2); TI =thymic irradiation (700 cGy on day-1).(a) Based on histological examination of the spleen graft; (b) Defined as the development of Grade 3 changes (coagulative necrosis, see Table 2); (c) Died from infection.

pulsation, and bleeding at the biopsy sites. All biopsies were examined histologically and by flow cytometric analysis for the presence of donor (PAA-positive) cells.

Immunosuppressive therapy

The immunosuppressive regimen for each recipient pig is summarized in Table 1. Cyclosporine (CyA) was administered daily by an intravenous (i.v.) infusion for 12-45 days. Whole body and/or thymic irradiation were administered from a cobalt irradiator (17,18).

Histological examination

Tissues were fixed in 10% formalin and embedded in paraffin, or frozen in tissue medium (Tissue-Tek OCT 4583, Miles, Elkhart, IN) and stored at -80°C. Five-micron histologic sections were cut from paraffin blocks, stained with hematoxylin and eosin (H&E) and studied by light microscopy. To assess possible changes in elastic tissue, basement membrane integrity, and collagen deposition, special stains included periodic acid-Schiff (PAS), trichrome, Verhoeff elastic,

and reticulin stains. Immunohistochemical study of sections included the use of monoclonal antibodies to α-smooth muscle actin (SMA; clone 1A4, Sigma, St Louis, MO), vimentin (Santa Cruz Biotechnology, Santa Cruz, CA), and proliferating cell nuclear antigen (PCNA; clone PC10, Dako, Carpinteria, CA) on fixed tissue. Macrophage/L1 protein/calprotectin Ab-1 (clone Mac 387, NeoMarkers, Fremont, CA) was used to label macrophages on 5-micron cryosections of tissue. Binding was visualized by using horseradish peroxidase-conjugated secondary antibodies (Vector, Burlingame, CA). Bound antibodies were revealed by incubation of formalin-fixed and cryopreserved sections in aminoethylcarbazole (Dako). Sections were counterstained with hematoxylin solution (Richard-Allan Scientific, Kalamazoo, MI). Negative controls omitted the primary antibodies.

Apoptosis was determined by a terminal deoxyribonucleotidyl transferase-mediated, dUTP nick end labeling (TUNEL) assay on paraffin sections. After deparaffinization, treatment with proteinase K (Fisher Scientific, Fairlawn, NJ) for 15min at room temperature, and quenching in 2% H_2O_2, tissue sections were incubated with biotinylated dUTP (Boehringer, Indianapolis, IN) and terminal deoxyribonucleotidyl transferase (Promega, Madison, WI) for 1h at 37°C. Slides were then blocked, washed with phosphate-buffered saline, and incubated with avidin-biotin complex horseradish peroxidase (Dako). Following development with diaminobenzidine (Zymed Laboratories, South San Francisco, CA), slides were counterstained with methyl green. Positive controls included sections of human adenocarcinoma of the colon, in which apoptosis is common. For negative controls, distilled water was substituted for deoxyribonucleotidyl transferase. The TUNEL index (TuI) was calculated as the mean number of TUNEL-positive cells counted in 10 random microscopic fields at x200 magnification.

Flow cytometry

Spleen biopsies were processed into single cell suspensions, and red blood cells were lysed. The concentration was adjusted to approximately $1x10^7$ cells/mL, and 100µL ($1x10^6$ cells) were distributed into each staining tube. The following swine-specific antibodies were used: anti-CD1 (76-7-4, mouse IgG2aK), anti-CD3 (898H2-6-15, mouse IgG2aK), anti-CD4 (74-12-4, mouse IgG2bK), anti-CD8α (76-2-11, mouse IgG2aK), anti-CD16 (G7, mouse IgG1), and anti-PAA (1038H-10-9, mouse IgMK). Two-color staining using fluorescein isothiocyanate-conjugated CD1, CD3, CD4, CD8, CD16 and biotinylated PAA antibody was used to determine the proportion of donor versus recipient lymphocytes, with appropriate negative isotype controls. As a second-step antibody, phycoerythrin streptavidin (Pharmingen, San Diego, CA) was used. Data were acquired using a Becton Dickinson FACScan (San Jose, CA) and analyzed with WinList mode analysis software (Verity Software House, Topsham, ME).

Fluorescence in situ hybridization (FISH)

FISH was performed on gender-mismatched grafts using a paraffin pretreatment reagent kit as recommended (Vysis, Downers Grove, IL). Five-micron fixed graft tissue sections were deparaffinized, digested with proteinase K, and hybridized overnight in a Hybrite chamber with a porcine combined XF/YCy3 paint (CA-1685, Cambio, Cambridge, UK), with X labeled with fluorescein isothiocyanate (FITC) and Y labeled with cyanine 3 (Cy3). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). Sections were examined with a fluorescence microscope (Olympus, Model Bx60F-3, Tokyo, Japan), using appropriate filters to detect the green, red and blue fluorescence of the FITC, Cy3, and DAPI, respectively. Images were photographed with an enhanced digital camera (Spot, Model 130, Diagnostic Instruments, Sterling Heights, MI), captured to a desktop computer (Compaq Deskpro DPENM P700 CCP, Hewlett Packard Co., Palo Alto, CA) and merged. Cells with intranuclear red signal were considered to have originated in a male animal; cells with two intranuclear green signals were female-derived. Sections of lymph nodes from male and female pigs served as controls.

Identification of Howell-Jolly bodies in the blood

Blood was drawn into a K2-EDTA tube before SpTx and on a weekly basis thereafter. Blood smears were Wright-stained and examined microscopically for Howell-Jolly bodies, which indicate a level of splenic dysfunction (19).

RESULTS

Outcome of spleen transplantation

In Group 1 (n=4), grafts in two untreated recipients were rejected by days 27 and 28 (Table 1), whereas grafts survived long-term in two treated recipients. The one spleen graft transplanted across a MHC class I barrier survived long-term (Table 1). In Group 2 (n=12), grafts in two pigs that received either no treatment or pre-Tx cyclophosphamide alone showed acute rejection by days 8 and 16, respectively, while two that received thymic irradiation and CyA rejected their grafts by days 47 and 62 (Table 1). When whole body irradiation was added to the regimen, only one of six pigs rejected its graft (day 61). Our histological study therefore is focused on the 7 rejected grafts (n=2 in Group 1; n=5 in Group 2).

A review of the Table 1 data suggests that, when no or minimal immunosuppressive therapy was administered, rejection progressed faster after MHC-mismatched SpTx (Group 2, when it occurred in 8 and 16 days) than in MHC-matched minor-mismatched pairs (Group 1, 27

or 28 days). Furthermore, greater pre- and post-Tx therapy was required to protect the graft from rejection and to induce tolerance in the MHC-mismatched pairs of Group 2 than in the MHC-matched pairs of Group 1. When the results of experiments within Group 2 are compared, there was increased graft survival and a greater incidence of tolerance induction when conditioning and therapy were increased. Whole body and thymic irradiation combined with 45-days of CyA prevented spleen rejection in 6 of 7 cases. A regimen that did not contain all of these elements resulted in rejection in 4 of 5 cases; in the single case in which rejection did not occur, follow-up was limited to 23 days by the death of the recipient from infection.

Macroscopic appearance of spleen grafts

Within 2 weeks of SpTx, spleens not undergoing rejection were enlarged to twice their normal size and demonstrated normal color, a soft texture, good vascular pulsation and bleeding from the biopsy site. Long-term surviving grafts became dark purple in color, but remained soft. Some capsular fibrosis and adhesions were identified. When rejection was occurring, early changes included those in color (from aubergine to yellow-brown), texture (soft to firm), arterial pulsation (diminished or absent), and a lack of bleeding from biopsy sites. Advanced rejection was usually associated with shrinkage in spleen size to less than one-third of that at Tx.

Microscopic appearance of spleen grafts undergoing rejection

Figure 1 illustrates the normal histological appearance of a pig spleen before explantation from the donor. Two hours after reperfusion, a slight increase in neutrophils in sinusoids of the red pulp was seen (not shown) (16). The speed of development of rejection depended on (a) the extent of MHC disparity between donor and recipient and (b) the intensity of the conditioning/immunosuppressive therapy administered. Although the speed of development of rejection was variable, except for this time sequence, the histopathological features of rejected grafts were similar.

Initially, a marked expansion of periarteriolar lymphatic sheaths (PALS) of the white pulp by PCNA-positive, pleomorphic, large, mitotically-active lymphocytes developed and was associated with an increased density of reticulin staining in the PALS, consistent with a reactive increase of collagen fibers in the white pulp (Figure 2). Similarly, increased SMA and vimentin staining in the peripheral aspects of the PALS suggested a concomitant vascular proliferation or possible angiogenesis. Alternatively, this finding may reflect a proliferation of T cell-zone fibroblasts, which are known to express SMA (20). The increase in proliferative activity was demonstrated by PCNA staining (Figure 2).

As necrosis developed, there was a loss of even baseline staining with reticulin, SMA, vimentin, and PCNA (data not shown). More subtle histological changes, which correlated with early,

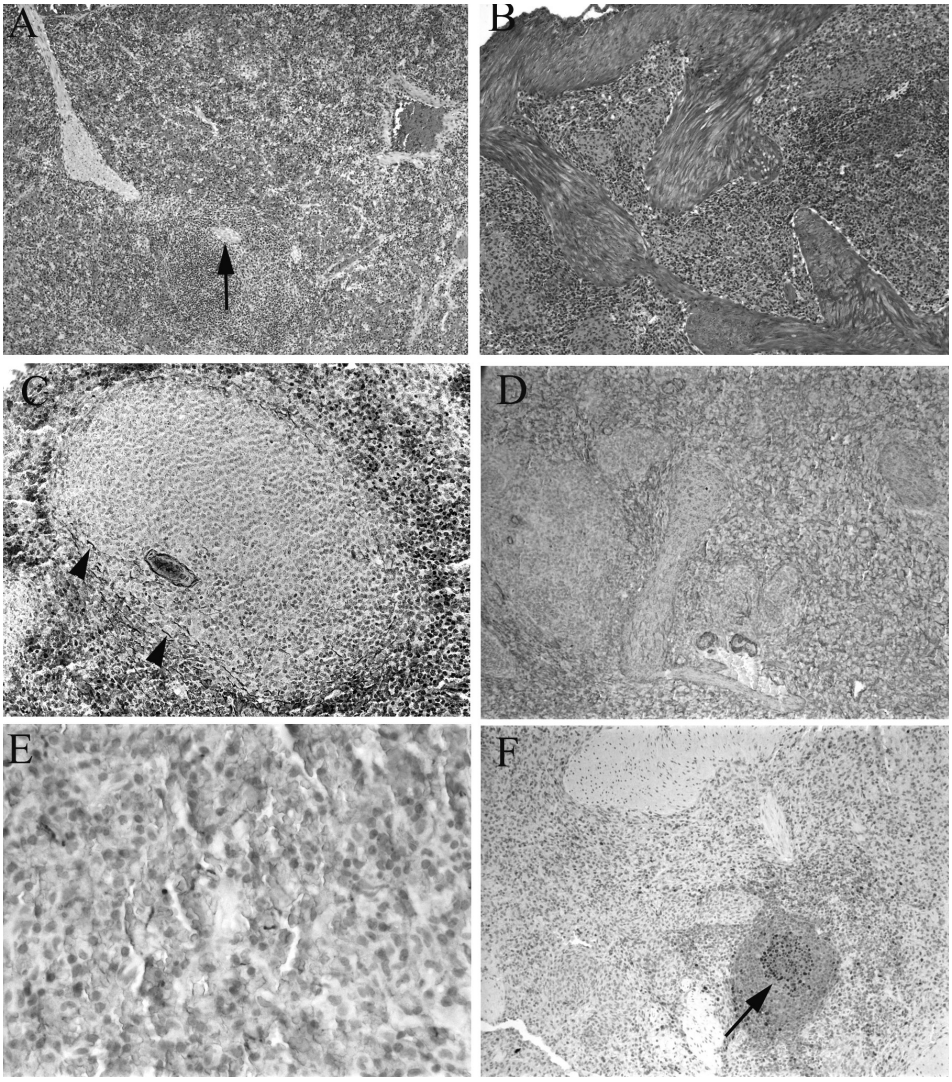

Figure 1. Normal histology of porcine spleen is illustrated with various staining techniques.
(A) Red pulp is predominant, and white pulp contains a small central artery (arrow), H&E
(B) Capsule and trabeculae, containing abundant blue-stained collagen, trichrome stain
(C) Scant collagen fibers (arrow heads) in periarteriolar lymphatic sheaths (PALS), reticulin stain
(D) Smooth muscle cells of trabeculae and of vessels and sinusoids in the white and red pulp express actin, SMA stain
(E) Red pulp, with delicately stained sinusoidal endothelial and/or smooth muscle cells, vimentin stain
(F) Normal cellular proliferative activity in red pulp and lymphoid follicle, with prevalence of staining in a germinal center (arrow), PCNA stain
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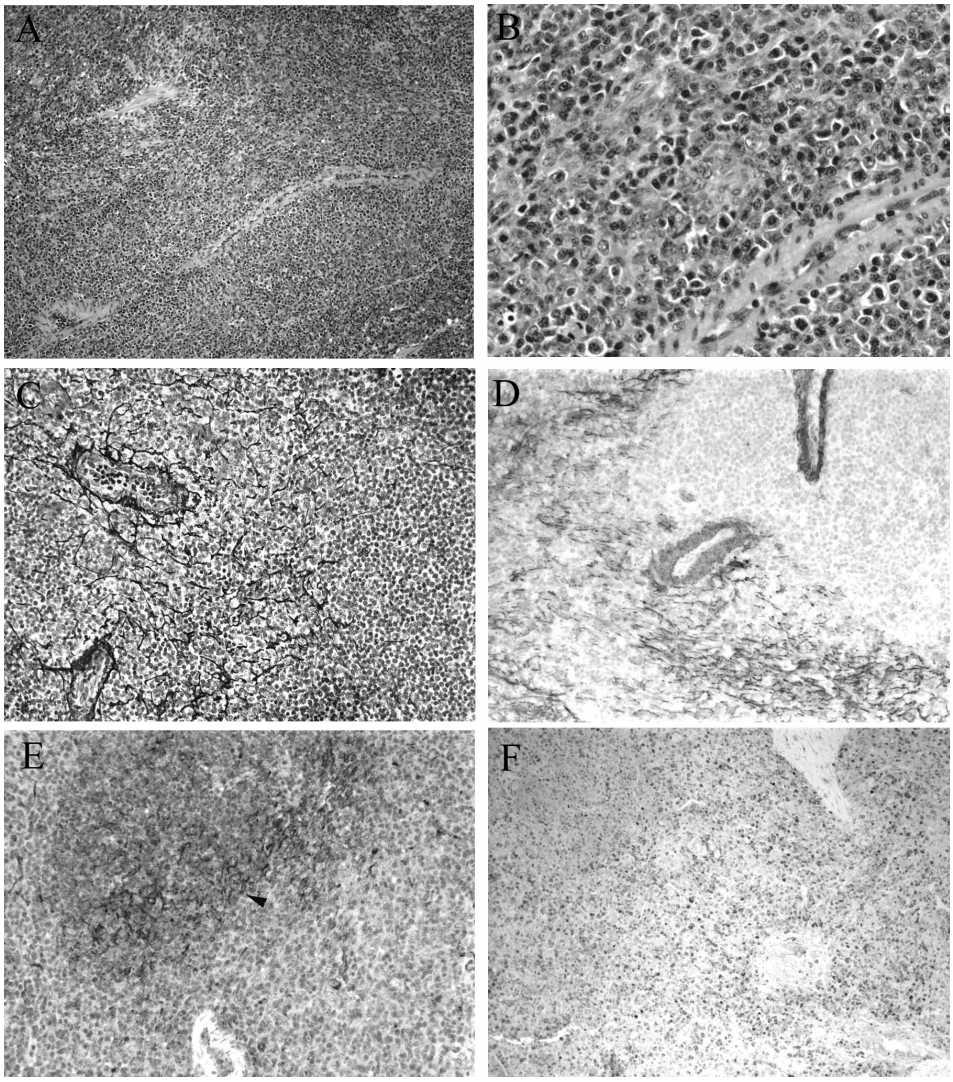

Figure 2. Histology associated with expanded PALS (#14867, Group 1, 15 days after SpTx) can be compared to baseline findings seen in Figure 1.
(A) Marked expansion of PALS around central artery, H&E
(B) High power view of pleomorphic, atypical lymphoid cells in the PALS, H&E
(C) Increased density of collagen fibers in PALS, reticulin stain
(D) Increased smooth muscle cell staining in red pulp adjacent to PALS, SMA stain
(E) Similar increase in staining of smooth muscle-like cells and/or endothelial cells (arrowhead) in expanded PALS, vimentin stain
(F) Increased proliferative activity associated with expanded PALS, PCNA stain
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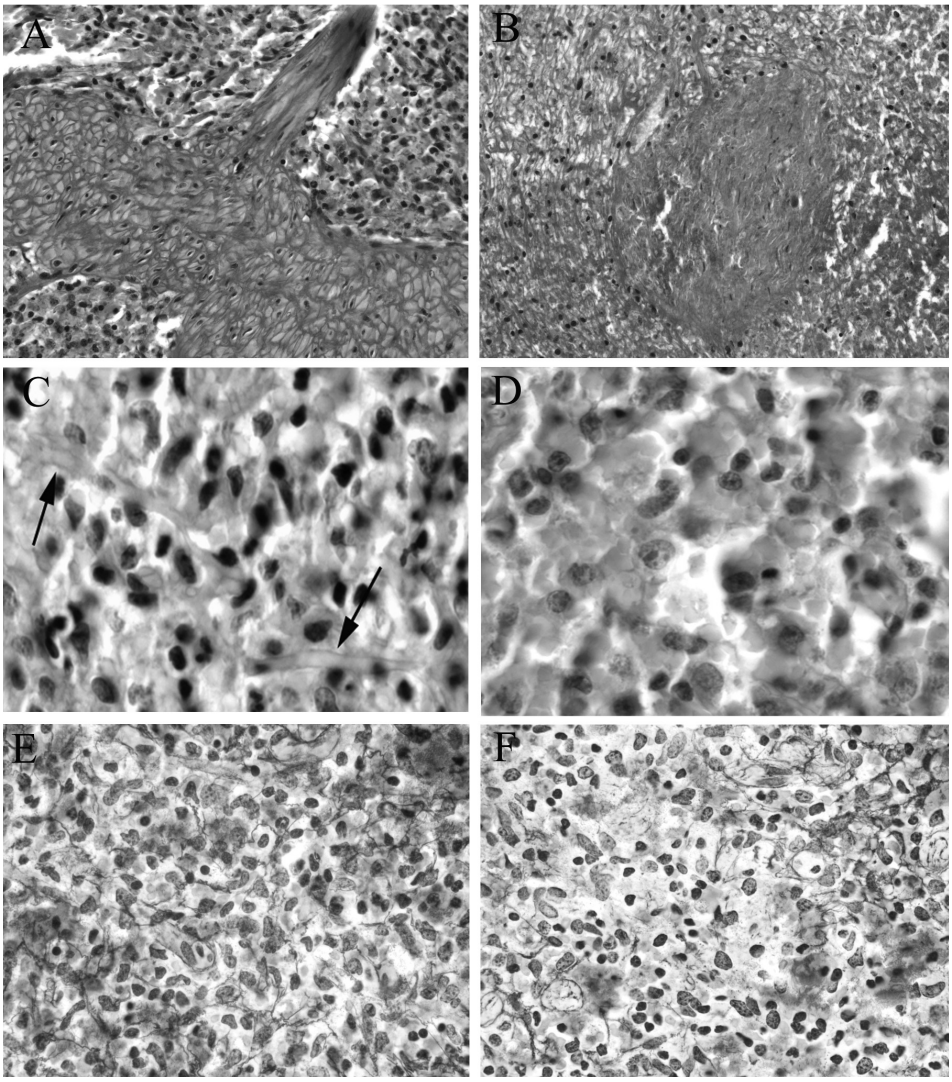

Figure 3. Normal histology (A,C,E) is compared with subtle histological changes which are associated with early, focal graft necrosis (B, D, F). Normal staining of basement membranes of trabecular cells, PAS stain (A) is degraded in early rejection (B) (#14840, Group 2, 104 days after SpTx)**,** PAS stain. The regular network of reticular fibers of the red pulp (C) is degraded in (D) (#14534, Group 2, 4 days after SpTx), PAS stain. The normal (E) and centrally disrupted (F) (#14526, Group 1, 57 days after SpTx) patterns of reticular fibers are again observed with further staining, reticulin stain.

focal necrosis included a decrease of staining of normally PAS-positive basement membranes of trabecular cells and a diminished PAS and reticulin staining of reticular fibers in the red pulp (Figure 3).

Prior to the appearance of histological features of rejection, there was at least a doubling of the TuI when compared to that of baseline (Figure 4A, B). The onset of rejection, ranging from focal fibrinoid necrosis of arteriolar walls and sinusoids to diffuse liquefactive necrosis, was usually associated with variable degrees of hemorrhage (Figure 4C-F). Acute vasculitis accompanied the process of necrosis in one graft, and, in another, extramedullary hematopoiesis was associated with patchy, liquefactive necrosis (Figure 5A-C). Diffuse necrosis was the end-result of rejection, and developed as early as day 8 in untreated pigs in Group 2 and by day 28 in Group 1. Necrosis was accompanied by an increase in collagen and by either the first appearance or an increased density of elastic fibers in the capsule and trabeculae. Replacement fibrosis adjacent to geographic areas of necrosis was seen in the spleen in one untreated Group 1 recipient on day 88 and in one treated Group 2 recipient on day 138. These areas of fibrous replacement consisted of a variably dense deposition of collagen and a diffuse population of SMA- and vimentin-positive spindle cells, most of which were PCNA-negative (not shown). This end-stage scarring corresponded to shrinkage in allograft size.

Microscopic appearance of spleen grafts surviving long-term

Eight grafts (Group 1 n=3, Group 2 n=4, and the one MHC class I-mismatched) remained viable 40-367 days after SpTx. Five of them showed atrophy of the white pulp from days 28-160, i.e., a decreased number of lymphoid cells, giving the histiocyte-rich sheaths of cells around central arteries a pale look on H&E stain. Accompanying the depletion of white pulp was replacement of normal red pulp by vacuolated histiocytes and SMA-positive cells, presumably smooth muscle cells migrating in from adjacent trabeculae (Figure 5D-F).

Correlation of histopathology with spleen cell chimerism on flow cytometry

In both Groups, a significant loss of PAA+ cells in the spleen either preceded or occurred concomitantly with the development of histological features of rejection. In long-term surviving grafts, recipient cells slowly replaced donor cells almost entirely.

Before explantation from the donor and immediately after reperfusion in the recipient, the donor spleen demonstrated 96.6 ± 2.0% PAA-positivity (Figure 6), which was comparable to the PAA-positivity of leukocytes in the blood of the donor animals (96.2 ± 2.2%). Some PAA- recipient cells (~6%) could be seen 30min post-reperfusion. In untreated pigs of Group 1, the percentage of donor (PAA+) cells remaining in the spleen had fallen to 0-4% by day 15, being

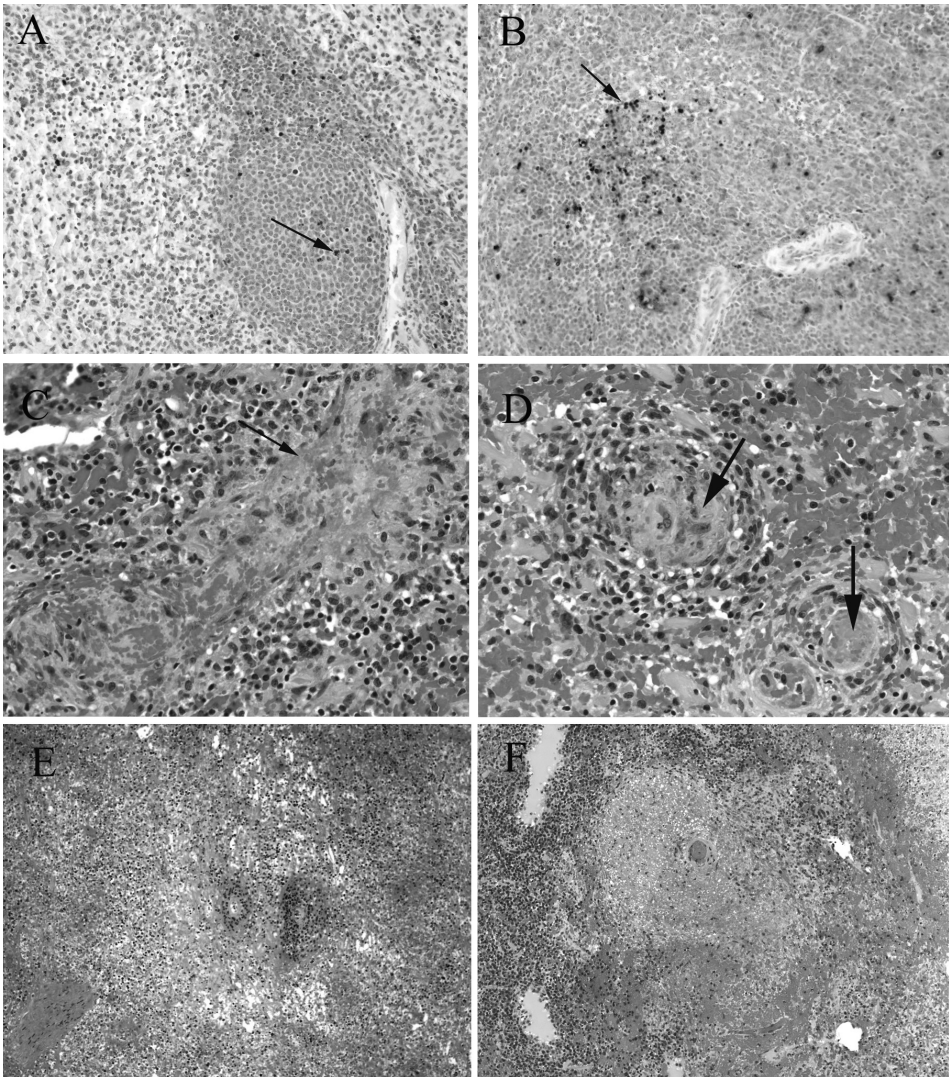

Figure 4.

A TUNEL index at baseline on the day of SpTx (A) (#14867, Group 1) is increased more than two-fold 15 days after SpTx (B) (#14867, Group 1) before a subsequent biopsy showed histologic evidence of rejection (arrows mark TUNEL-positive cells). Spleen allograft necrosis varied morphologically from focal, fibrinoid necrosis of sinusoidal walls (C) (#14526, Group 1, 57 days after SpTx) (arrow), associated with fibrin thrombi in small vessels (D) (#14526, Group 1, 57 days after SpTx) (arrows), to diffuse liquefactive necrosis (E) (#14867, Group 1, 28 days after SpTx). Necrosis was usually associated with some hemorrhage (F) (#14867, Group 1, 28 days after SpTx).

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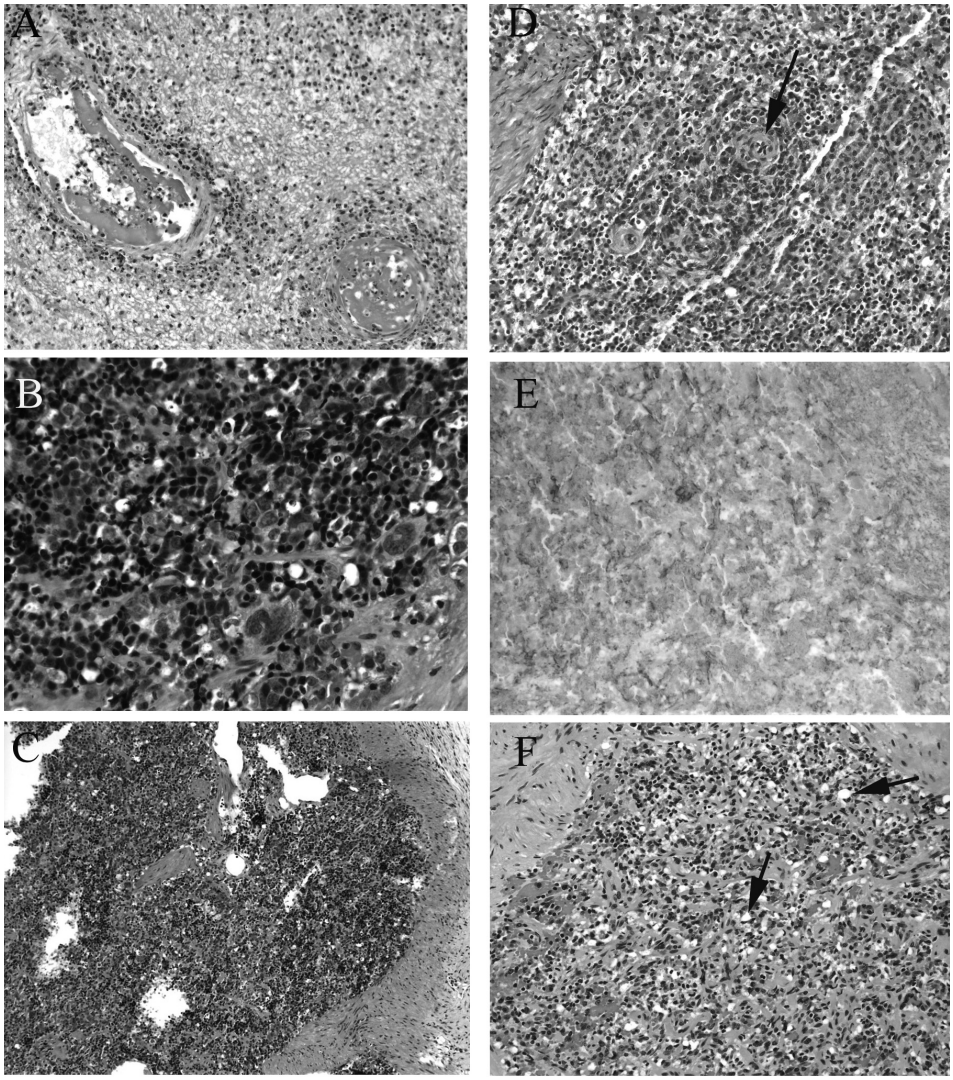

Figure 5.

Other features of late rejection included acute vasculitis accompanying vessel wall necrosis (A) (#14646, Group 2, 28 days after SpTx), and, in one graft, extramedullary hematopoiesis (#14832, Group 2, 47 days after SpTx) (B and C). Histological changes associated with prolonged graft survival included atrophic white pulp (D) (#15051, Group 2, 57 days after SpTx) (arrow points to a central artery); increased SMA expression in red pulp (E) (#14526, Group 1, 160 days after SpTx), SMA stain; and an increase in vacuolated histiocytes (arrows) in red pulp (F) (#14526, Group 1, 160 days after SpTx).

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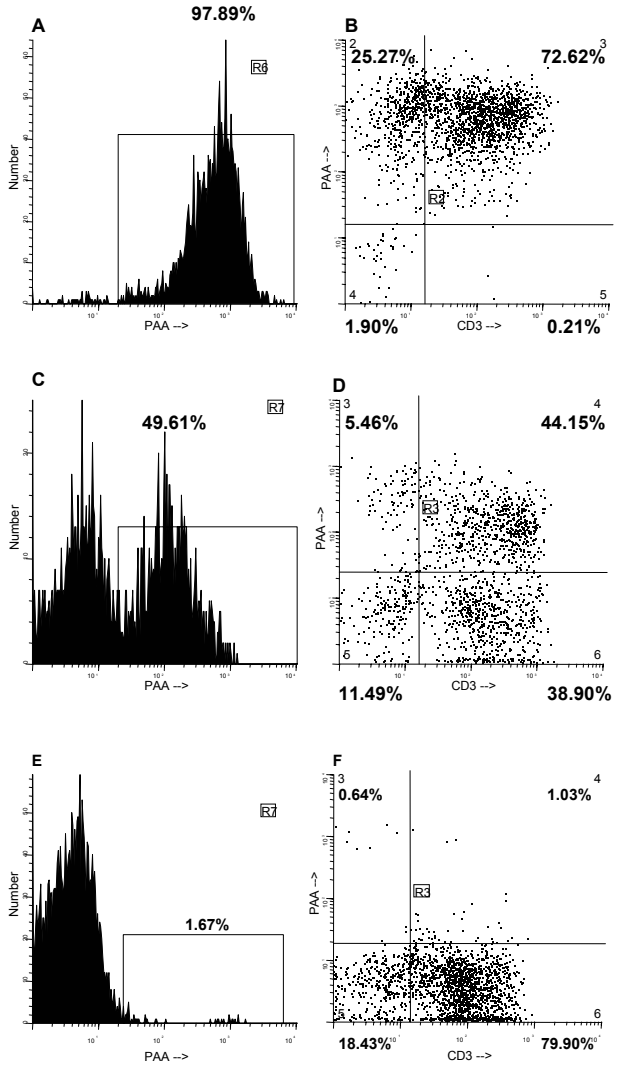

Figure 6.

(A and B) Flow cytometry of a donor (PAA+) spleen (donor to #15410) before explantation, demonstrating the percentage of PAA+cells (97.89%) (A), and the profile of CD3+PAA+ cells (B). (C and D) Flow cytometry of an accepted spleen graft after cessation of CyA (#15051, Group 2). Replacement with recipient cells is indicated by the presence of PAA- cells. The spleen graft still consists of 49.61% donor cells (C), but some CD3+PAA- recipient cells can be seen (D). (E and F) Almost complete repopulation of the spleen graft by recipient cells occurred (#15410, Group 2, 183 days after SpTx). Only 1.67% of the cells are still of donor origin (E). Virtually all CD3+ cells (F) are recipient type (PAA-).

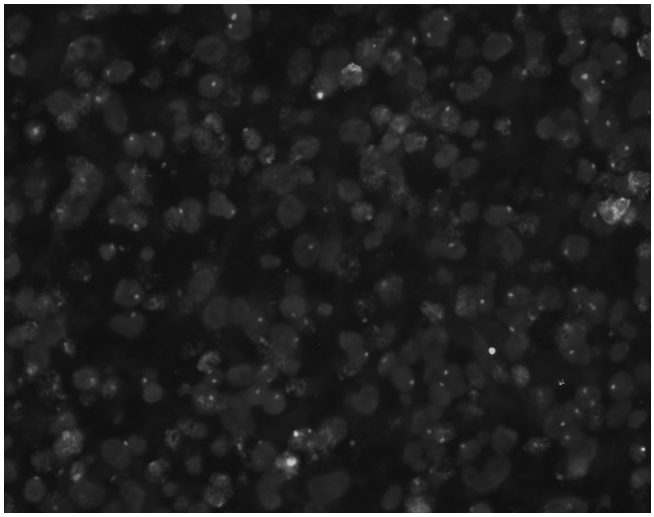

Figure 7.
Section of spleen which was transplanted from a female donor into a male recipient (#14840, Group 2, 43 days after SpTx). A notable number of recipient-derived cells with Y chromosomes (red signal) has populated the graft at the time of expanded white pulp (day 43), FISH technique, x400.
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replaced by host (PAA-) cells (CD3+, CD8+ and CD16+). In immunosuppressed pigs in Group 1, the percentage of PAA+ cells remained at >15% on day 29, and at 4% on day 215. The FISH technique demonstrated recipient-derived cells populating a gender-mismatched graft (Figure 7), supporting the flow cytometry data.

When necrosis of a graft developed, the overall cellularity of the spleen was markedly diminished. In untreated recipients in Group 2, PAA+ donor cells were completely replaced by recipient cells within 8 days. In treated Group 2 recipients that eventually rejected their grafts**,** 30% of cells in the graft were PAA+ during CyA therapy but, when rejection occurred 2-3 weeks later, all PAA+ cells were rapidly lost from the graft. In Group 2 recipients that did not reject their grafts, PAA+ donor cells decreased by about 50% within the first post-Tx month, after which the spleen graft was slowly replaced almost entirely with recipient cells, which usually occurred within 100 days post-Tx. The chimerism in the spleen graft reflected the extent of lymphoid chimerism in blood (13).

Presence of Howell-Jolly bodies

After combined native splenectomy and survival of a viable spleen transplant,
Howell-Jolly bodies were never seen in the blood, indicating good splenic graft function. When rejection developed, however, Howell-Jolly bodies appeared in the blood within a week (Figure 8).

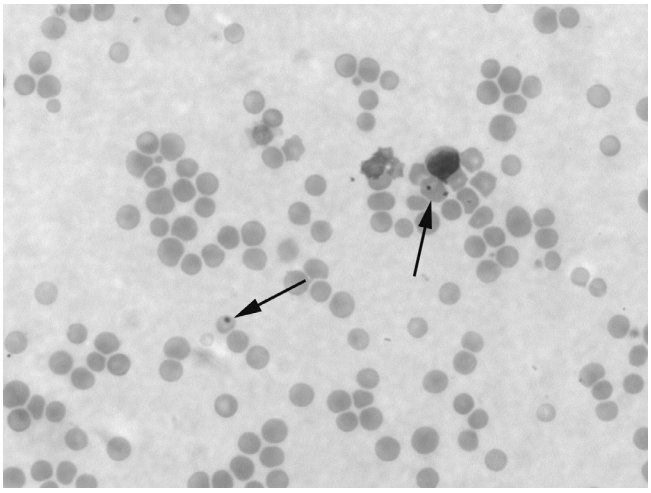

Figure 8. Red blood cells (arrows) contain Howell-Jolly bodies in a peripheral blood smear of a recipient of a rejected (Grade 3) spleen allograft (#15311, Group 2, 75 days after SpTx, two weeks after spleen graft rejection (characterized by coagulative necrosis), Wright stain x640.
See addendum for colour version

DISCUSSION

To our knowledge, this is the first report that describes the histopathology of spleen allograft rejection in a large animal model with known MHC barriers. In miniature swine, the course of histopathological change in the transplanted spleen varied with respect to the extent of donor-recipient MHC-mismatch and the immunosuppressive regimen administered to the recipient. The sequence of histopathologic events was delayed in MHC-matched pairs and in immunosuppressed recipients. However, our observation was that common features of rejection occurred in minor-mismatched and fully MHC-disparate grafts, but with varying time courses. This correlates with the course of rejection in other organ transplants, where the pattern of rejection is similar yet the rapidity of its development varies depending on the extent of MHC incompatibility between donor and recipient (21).

An early indicator that rejection was occurring was expansion of white pulp, as described in other studies (6,10,22). Correlation with flow cytometry indicated that expansion of the white pulp was due to infiltration by recipient cells. Others (6, 22) have attributed the expansion of PALS to an increase in the number of donor cells. In our model, this finding has not been the case, and the lymphoid cellular expansion was recipient-derived. Atrophy of the white pulp, on the other hand, perhaps reflects a "burned out" immune response to the allograft, allowing relatively long-term graft survival.

Based on our histologic findings and on data reported by others previously (see below), a histologic grading system for acute cellular rejection in spleen allografts is proposed (Table 2). Focal or multifocal fibrinoid necrosis of sinusoidal and/or arteriolar walls (Grade 1) did not preclude long-term graft survival. Expansion of white pulp by large, atypical, and mitotically-active lymphoid cells (Grade 2) was invariably followed by borderline-severe or severe rejection (Grades 3A and B), manifested by focal or diffuse coagulative necrosis, respectively. The TuI may be a helpful indicator of spleen graft survival; in one untreated Group 2 spleen, although the graft looked normal on day 4, the TuI had increased almost 4-fold (12.6 to 40.0) from baseline; by day 8, the graft demonstrated diffuse necrosis. In future studies, these histological changes may have a predictive significance and aid in the treatment or prevention of rejection.

Table 2 Grading scheme for splenic allograft rejection.

Grade	Rejection	Histological Features
0	None	Normal splenic architecture
1	Mild	Focal or multifocal fibrinoid necrosis of sinusoidal and/or arteriolar walls, with or without hemorrhage
2	Moderate	Expansion of white pulp by large, atypical, and mitotically-active lymphoid cells
3A	Borderline severe	Focal* coagulative necrosis
3B	Severe	Diffuse coagulative necrosis

*Focal is involvement of a small fraction of tissue in a high-power field.

It is unclear why extramedullary hematopoiesis occurred in one of our pig spleen grafts (6,11). The spleen is known, however, to be an organ capable of hematopoiesis in certain disease states (23,24) and, in other studies, we have observed that the naive spleen has hematopoietic stem cell activity (Chapter 7).

In rodents, spleen grafts from untreated PVG rats to untreated AGUS rat recipients were rejected within 2 to 3 weeks (6). The grafts enlarged to six times normal size**,** and became firm and white. The circulation through the graft ceased within days. Histologically, a slight expansion of the PALS occurred as early as 2 days after SpTx, approximately 50% of the lymphocytes becoming enlarged with pyroninophilic cytoplasm (6)**;** the authors do not specify whether these lymphocytes were of donor or recipient origin. There was also an increase in the cellularity of the red pulp. In the PALS, most of the lymphocytes and large pyroninophilic cells disappeared within 7 days, after which the sheaths consisted almost entirely of pale elongated cells (reticular cells, macrophages, fibroblasts), which increased rapidly in number, and multinucleate giant cells occasionally appeared. The central arteries of Malpighian corpuscles gradually became surrounded by an enlarging mass of pale tissue which expanded into, and replaced part of, the red pulp, at some stages resembling "epithelioid cells" seen in certain forms of chronic

inflammation. Red pulp cellularity was gradually diminished and, after 12 days, foci of necrosis enlarged and coalesced until the whole spleen became necrotic.

In large animals, most histological observations have been made in dogs (4,7-12). Multiple subcapsular hemorrhages developed, with diffuse infiltration of polymorphonuclear leukocytes into red pulp. Subsequently, generalized hemorrhage was seen, with many pyroninophilic cells in the red pulp, followed by complete hemorrhagic infarction and necrosis.

In the few human SpTx studies, the histological picture of rejection remains uncertain (1,4,5,22,25-28). Within days, the graft may contain an increased number of lymphocytes, histiocytes, and reticulum cells. Red pulp congestion, immunoblast proliferation, and intimal swelling may develop, with lymphoid expansion predominantly in the marginal zones. Rupture may occur, the spleen showing extensive edema, focal hemorrhage, and cellular depletion in the PALS, with a generalized and massive infiltrate of large lymphoid cells. Extensive necrosis follows. Others have reported that, within 1-2 months, marked fibrosis, focal necrosis, and intimal proliferation (causing arterial occlusion) were present and, after 7-9 months, complete atrophy had occurred (5). Raccuglia et al. (27) reported SpTx in a patient with leukemia; after almost a year, the graft appeared histologically viable, but showed congestion, diffuse deposits of hemosiderin and, although lymphatic tissue was abundant, the small follicular lymphocytes were decreased.

The sequence of histopathological events leading to allograft rejection in our study is similar to those described in rodents and dogs. With the availability of special staining techniques and flow cytometry, however, we have been able to detect subtle morphologic changes that may help predict the course of the transplanted spleen and the ultimate outcome. Our observations should prove valuable if SpTx, for the purpose of inducing tolerance, becomes a clinical reality.

ACKNOWLEDGEMENTS

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Immunological Unresponsiveness in Chimeric Miniature Swine following MHC-mismatched Spleen Transplantation

6

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ABSTRACT

Background: In rodents, spleen allotransplantation (SpTx) induces tolerance. We investigated the induction of chimerism and donor-specific unresponsiveness following pig SpTx.

Methods: Thirteen pigs underwent splenectomy (day 0); all received a blood transfusion. In 11/13 pigs, SpTx was performed across a MHC class I (n=1) or full (n=10) barrier; two control pigs received no SpTx. All pigs were monitored for chimerism, and anti-donor immune responses, including suppressor assays. Four pigs (2 asplenic controls, and 2 with SpTx) underwent delayed donor-matched kidney Tx without immunosuppression.

Results: Six of the 11 spleen grafts were lost from rejection (n=5) or splenic vein thrombosis (n=1), and 5 remained viable. All 11 SpTx recipients developed multilineage chimerism, but chimerism was rapidly lost if the graft failed. Two control pigs showed <6% blood chimerism for 4 and 11 days only. Pigs with functioning spleen grafts had multilineage chimerism in blood, thymus and bone marrow for at least 2-6 months, without graft-versus-host disease. These pigs developed *in vitro* donor-specific hyporesponsiveness and suppression. In 2 pigs tolerant to the spleen graft, donor MHC-matched kidney grafts survived for >4 and >7 months in the absence of exogenous immunosuppression; in 2 asplenic pigs, kidney grafts were rejected on days 4 and 15.

Conclusions: Successful SpTx can result in hematopoietic cell engraftment and *in vitro* donor-specific unresponsiveness, enabling prolonged survival of subsequent donor-matched kidney grafts without immunosuppression.

INTRODUCTION

Immunological tolerance would eliminate the need for, and avoid complications of, chronic immunosuppressive drug therapy. Successful spleen allotransplantation (SpTx) in rodents can result in specific tolerance towards donor-matched organ allografts in the absence of exogenous immunosuppressive therapy (reviewed in (1)). Studies investigating SpTx in large animals have been hampered by technical complications and an absence of knowledge of the major histocompatibility complex (MHC) of donor or recipient, and have not demonstrated evidence for tolerogenicity, although specific tolerance-inducing immunosuppressive regimens were not employed. In humans, however, hematopoietic cell chimerism has been reported following SpTx (1). Indeed, the era of hematopoietic progenitor cell (HPC) transplantation (Tx) began in 1949 when Jacobsen demonstrated that HPCs present in the lead-shielded spleen reconstituted the bone marrow (BM) of mice following lethal irradiation (2). The mechanism by which the spleen induces tolerance in rodents remains uncertain.

Massachusetts General Hospital partially-inbred, MHC-defined miniature swine constitute a suitable large animal model in which to investigate the effects of SpTx. We have previously

reported that SpTx in miniature swine is technically feasible (3), and that, in MHC-matched, minor-mismatched donor and recipient pairs, it results in hematopoietic cell chimerism; when a 12-day course of cyclosporine was administered to the recipient, tolerance to the transplanted spleen could be achieved (3). The induction of tolerance was associated with a longer period of mixed chimerism in blood, lymph nodes, and thymus, but this was not permanent, and no engraftment in BM was observed.

If a tolerance-inducing protocol is to be practicable for human organ Tx, then it must at least be successful in large animal models across a full MHC barrier, since there are differences in the response to vascularized organ allografts between small and large animal species with regard to tolerance induction. Markedly different tissue expression patterns of MHC class II antigens between rodents and large animals could account for this discrepancy in response to vascularized grafts. These antigens, which are potent stimulators of the T helper pathway in the immune response, are notably absent from the vascular endothelial cells of rodents, but expressed in all large animals, including humans. In view of this and the potential risk of graft-versus-host disease (GVHD), it would not seem wise or ethical to initiate a clinical trial of SpTx on the basis of rodent studies alone (1).

We have therefore investigated SpTx in miniature swine using an immunosuppressive regimen that is mildly myelosuppressive but non-myeloablative.

METHODS

Animals

SpTx and kidney Tx donors and recipients were selected from our herd of partially-inbred, MHC-defined miniature swine (4). Donors were aged 5.1±3.6 months and weighed 31.5±19.0kg, recipients 4.9±4.5 months and 30.8±22.4kg. In order to detect donor hematopoietic cells in the recipient, donor and recipient pairs were mismatched for the pig allelic antigen (PAA), a non-MHC cell surface marker of unknown function (5), donors being PAA+ and recipients PAA-. Donors and recipients had a two-haplotype mismatch for both MHC class I and II ('full mismatch'), except for pig 15111, which was matched for MHC class II, but two-haplotype mismatched for MHC class I.

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996).

Surgical procedures

All recipient pigs had intravascular catheters inserted into jugular veins for blood draw and drug infusion. The technique of SpTx in miniature swine has been described previously (3). All pigs (including control pigs) underwent native splenectomy and received a transfusion of donor unirradiated packed red blood cells on day 0 (the day of splenectomy +/- SpTx) (3). The technique of kidney Tx has been described previously (6,7), at which time a unilateral right nephrectomy was performed. Once good kidney graft function was assured intra-operatively, the native contralateral ureter was ligated; native nephrectomy was performed when the kidney graft had shown good function for a prolonged period.

Recipient irradiation

Whole body (WBI; 100cGy on day -2) and thymic (TI; 700cGy on day -1) irradiation were administered from a cobalt irradiator (8).

Immunosuppressive / supportive therapy

Cyclosporine (CyA; Sandimmune, generously provided by Novartis Pharmaceutical Corporation, East Hanover, NJ) was administered as a single daily infusion of 10-30mg/kg i.v. (adjusted to maintain a whole blood trough level of 400-800ng/mL) for 12, 28 or 45 days through an indwelling catheter, starting on the day of SpTx. All pigs received daily prophylactic enrofloxacin (5mg/kg/d i.v.) and dalteparin (100IU/kg/d subcutaneously) while indwelling venous catheters were present.

Monitoring

Blood cell count, serum chemistry, and CyA levels were determined daily or x3 weekly by routine methods. Blood was drawn daily to monitor chimerism during the first 2 weeks, and then at increasing intervals until chimerism was lost. Spleen rejection was monitored by sequential biopsies. Kidney rejection was monitored by serum creatinine and by sequential biopsies. Monitoring for graft-versus-host-disease (GVHD) included daily inspection of the skin, stools, regular determination of liver enzymes, and biopsy of any skin rash.

Histology of spleen and kidney allografts, host BM, thymus, and lymph nodes

Biopsies of the donor spleen were taken before excision from the donor and 2 hours after reperfusion on the day of SpTx (day 0). Biopsies of the spleen, mesenteric lymph node, and BM were taken approximately on days 14, 28, 45 and 60, and at increasing intervals thereaf-

ter. The thymus was biopsied on the same days as the BM, except on day 14. Biopsies of the spleen and of the donor-matched kidney grafts (on approximate days 18, 30, 60, and 100 or on indication) were prepared as described previously (9). Spleen (9) and kidney (10) rejection were diagnosed according to standardized grading systems.

Flow cytometry

T cell count in recipient blood

T cell depletion (as a result of irradiation) was assessed by flow cytometry between day -3 and day 0 (before and after WBI and TI, respectively) by staining with a FITC-conjugated mouse anti-pig-CD3 antibody (898H2-6-15, mouse IgG2aK) (11), with F36.7.5 (IgG2a) as a negative isotype control.

Chimerism in donor spleen and in host blood, BM, thymus, and lymph nodes.

The following swine-specific antibodies were used to follow the presence of donor cells and their phenotypes in the spleen graft, and in recipient blood, lymph node, thymus and BM (12): Anti-CD1 (76-7-4, mouse IgG2aK) (13), anti-CD3 (898H2-6-15, mouse IgG2aK) (14), anti-CD4 (74-12-4, mouse IgG2bK), anti-CD8α (76-2-11, mouse IgG2aK) (15,16), anti-CD9 (1038H-4-6, mouse IgMK) (17), anti-CD16 (G7, mouse IgG1), and anti-PAA (1038H-10-9, mouse IgMK) (5). Data were acquired using a Becton Dickinson FACScan (Becton Dickinson Biosciences), and analyzed with WinList mode analysis software (Verity Software House, Topsham, ME).

Anti-donor antibodies

The development of anti-donor antibodies in the serum before and after SpTx and kidney Tx was investigated by FACS (18). Both naïve pig serum and pre-Tx serum from each animal were used as controls.

Colony-forming unit (CFU) assay of cells from spleen graft and host BM

Single cell suspensions of spleen graft and BM were made, and *in vitro* progenitor cell assays were performed (19-21).

Polymerase chain reaction (PCR) assay for functional engraftment of donor cells in host BM

These assays were performed as reported previously (22).

Fluorescence in situ hybridization (FISH) of spleen biopsies

FISH was performed on gender-mismatched grafts that were either rejected (14840, recipient male/donor female) or tolerated (15051, recipient female/donor male), as described (9). Sections of lymph nodes from male and female pigs served as controls.

Isolation of peripheral blood lymphocytes

Peripheral blood lymphocytes were isolated from freshly drawn, heparinized whole blood (8), and stored at 4°C until used.

Mixed lymphocyte reaction (MLR) and cell-mediated lympholysis (CML) assays

MLR and CML cocultures, to test for proliferative and cytotoxic responses to alloantigen, respectively, have been described previously (8,16).

Suppressor CML (sCML) and suppressor- mlr (sMLR) assays

sCML and sMLR assays were performed as described (14,15) in Group B pigs (see below) after all exogenous immunosuppressive therapy had been discontinued and when donor-specific hyporesponsiveness was observed in CML and MLR assays. In Group C pigs, these assays were performed at similar time-points, even when no hyporesponsiveness was seen.

In sCML assays, cells from tolerant or naïve animals were stimulated for 6 days with irradiated (2,500cGy) donor or third-party MHC cells (responder and stimulator each at 4×10^6 cells/mL). After one day of rest, the cells were co-cultured with (a) naive unstimulated recipient MHC-matched responders (0.5mL at 4×10^6 primed cells/well and 0.5mL at 4×10^6 naive cells) plus (b) the same antigen present in the original stimulation (1mL at 4×10^6 cells/mL of either donor or third-party MHC stimulators). The co-cultures were incubated for 6 days, after which CML was measured using standard techniques.
sMLR assays were set up in two phases: In phase 1, the priming phase, cells from tolerant or naive animals were stimulated with donor-matched or third-party cells. In phase 2, the co-culture phase, the putatively activated regulatory cells were co-cultured with recipient-matched naive responders, and stimulated with donor-type or third-party PBL stimulators. Briefly, for primary cultures, responder cells at 4×10^6 cells/mL from either tolerant or naive MHC-matched animals were incubated with either donor- or third-party-type cells for 5 days. Secondary co-cultures were performed as follows: pre-stimulated cells were rested for 24h, and then either (i) cultured again with irradiated stimulators (each at 2×10^6 cells/mL or 4×10^6 cells/mL to test

for a crowding effect) or (ii) placed in co-culture with recipient-matched naive cells (each at 2×10^6 cells/mL) and stimulator cells (at 4×10^6 cells/mL) for an additional 5 days.

Experimental groups (Table 1)

All pigs in all groups underwent native splenectomy and received a transfusion of washed red blood cells (~50mL) on day 0 (day of splenectomy +/- SpTx); in those that underwent SpTx, the red blood cells were taken from the spleen donor, and in those that underwent splenectomy alone, the cells were from a MHC-mismatched donor pig.

Eleven pigs underwent full (n=10) or class 1 (n=1) MHC-mismatched SpTx (day 0), and received either no treatment or varying immunosuppressive regimens (Table 1). Six were tested after loss of their grafts (Table 1, Group A), and 5 were studied when the graft remained viable (histologically normal) after discontinuation of immunosuppressive therapy and they remained unresponsive to the graft (Group B), although 2 of these pigs subsequently needed to be euthanized due to an outbreak of porcine circovirus in the facility (that affected other pigs also) (Table 1)**.** Two pigs received the identical conditioning and immunosuppressive regimen as the majority of pigs in Group B, including blood transfusion, but did not undergo SpTx (Group C). A total of 13 pigs were therefore available for study.

To test for the induction of tolerance to other donor-specific organs after discontinuation of immunosuppressive therapy, kidney Tx (from a donor MHC-matched to the spleen and/or blood donor) was performed in 4 pigs, 2 of which were asplenic and 2 of which were tolerant to their grafts. Kidney Tx was carried out in pigs from each of Groups A (15733), B (15111, 15410), and C (15330) two or more months after SpTx (Groups A and B) or after native splenectomy and blood transfusion (Group C).

Statistics

Statistical differences between Groups A, B, and C were determined by the Students' t test. A p-value of <0.05 was considered statistically significant.

RESULTS

Clinical course after SpTx (Table 1)

Six recipients lost their spleen grafts, and constitute Group A (Table 1). Four, that did not receive the full regimen, rejected their grafts between days 8-62 (9); one pig that rejected its

Table 1. Immunosuppressive therapy and clinical course in pigs in groups A, B and C

	Immunosuppression					Spleen graft survival		Chimerism blood		
Pig #	WBI	TI	CyA(d)	Other	GVHD	(days)	% T cell depletion	(days)	In vitro DSH (f)	Complication (g)
Group A: (n=6)										
14534	-	-	0	-	+ (e)	8	ND	5	ND	-
14646	-	+	0	CPP	+ (e)	16	ND	10	ND	-
14832	-	+	12	-	-	47	ND	32	ND	-
14840	-	-	28	-	+ (e)	62	ND	48	ND	-
15311(a)	+	+	45	-	-	61	64.5	49	ND	-
15733(b)	+	+	45	-	-	3	78.3	50	ND	-
Group B: (n=5)										
15111 (c)	-	+	12	-	-	>363	ND	79	Y	-
15051	+	+	45	-	-	>59	82.4	>59	Y	PCV-2 (59)
15242	+	+	45	-	-	>57	71.4	>57	Y	PCV-2 (57)
15410	+	+	45	-	-	>183	77.7	178	Y	-
15842	+	+	45	-	-	>100	73.9	>100	Y	-
Group C: (n=2)										
15312	+	+	45	-	-	N/A	72.5	4	N	-
15330	+	+	45	-	-	N/A	76.1	11	N	-

WBI = whole body irradiation (100cGy on day -2); TI = thymic irradiation (700cGy on day -1); CyA = cyclosporine (administered i.v. as a single daily infusion at 10-30mg/kg to maintain a whole blood trough level of 400-800ng/mL for 12-45 days, starting on the day of SpTx); CPP = cyclophosphamide (3x 20 mg/kg i.v. on days -5,-4 and -3). ND=not determined; N/A=not applicable.

(a) Poor pre-Tx T cell depletion.

(b) Spleen graft excised on day 3 for splenic vein thrombosis.

(c) MHC-Class I-mismatched donor-recipient combination; all others were full MHC-mismatched pairs.

(d) CyA-treatment in days.

(e) Mild cutaneous GVHD, from approximately 1-2 weeks post-Tx.

(f) DSH = donor-specific hyporesponsiveness; N=no; Y=yes;

(g) Complications: PCV-2 = infection with porcine circovirus type 2; figures in parentheses indicate day of euthanasia of the pig.

graft (on day 61) after the full regimen (15311) had poor T cell depletion (64% versus an overall mean of 75%, Table 1). In the sixth pig (15733), the spleen underwent rupture following compression and thrombosis of the splenic vein, and was excised on day 3, but CyA was continued until day 45; this pig developed features of asplenia (see below).

No rejection occurred in the remaining 5 pigs that had undergone SpTx and in which the full course of CyA had been completed (Table 1) (Group B), allowing data to be obtained of unresponsiveness to the graft in the absence of exogenous immunosuppression. Three of these pigs with functioning spleen grafts were followed for prolonged periods (>100 days), but 2 pigs had to be euthanized on days 57 and 59, respectively, because of an outbreak of porcine circovirus type 2 infection.

The 2 pigs that underwent native splenectomy and a donor blood transfusion (but no SpTx) constitute Group C (Table 1). Both pigs developed features of asplenia, namely thrombocytosis (>1,000k/μL) and Howell-Jolly bodies in the blood, but clinically remained well.

In summary, therefore, we were able to follow 6 pigs that lost their grafts within 2 months (Group A), 5 that remained unresponsive to their grafts (3 for >100 days) (Group B), and 2 that never received SpTx but did receive immunosuppression and blood transfusion (Group C).

Graft-versus-host-disease (gvhd)

In 3 pigs in Group A, a mild form of transient, self-limiting cutaneous GVHD (lasting from days 7-13) was observed (Table 1), confirmed by skin biopsy histology. Although the Group B pigs developed higher levels of chimerism, none developed any clinical or histological features of GVHD.

White blood cell, platelet, and T cell counts

In all pigs that received the full regimen (Table 1), there were temporary reductions in white blood cell, platelet, and T cell counts during the first month, though the white blood counts never fell below 3K/μL.

Hematopoietic cell chimerism following SpTx

Spleen graft

Splenocytes and leukocytes in the blood of the donor pigs expressed PAA comparably (97.0±2.0% vs 96.1±2.2%). Immediately after donor spleen reperfusion, the number of PAA^+ cells in the spleen decreased by approximately 7-8%, indicating perfusion of the graft by PAA^- recipient cells. In grafts that were rejected, there was relatively rapid infiltration of the spleen by host cells, and all cells became PAA^- (mainly $CD3^+/CD8^+$ and $CD16^+$) (9). In nonrejected grafts, the number of PAA^+ donor cells decreased by approximately 50% within the first month after SpTx, after which there was a slower repopulation with PAA^- cells. Within 100-150 days, donor cells were replaced almost entirely (>98%) by recipient cells (9).

Blood

Multilineage chimerism in pigs that underwent SpTx (Groups A and B) was initially significantly higher than in control pigs (Group C) (Figure 1A-C)**.** In Group A, the significant difference with Group C was lost after 15-30 days. Chimerism in all lineages remained significantly higher in Group B than in Groups A and C until day 100-150. In the 2 control pigs of Group C, a low level of chimerism (1.9-5.9%) resulted from the transfusion of donor blood on the day of splenectomy (day 0), but this fell to almost undetectable levels by day 4, and was not measurable after day 11; the chimeric cells were mostly $CD3^+$ lymphocytes. In pigs that lost their spleen grafts (Group A), chimerism was completely lost a few days after rejection. When the graft was not lost, multilineage chimerism in the blood persisted for a prolonged period (Table 1 and

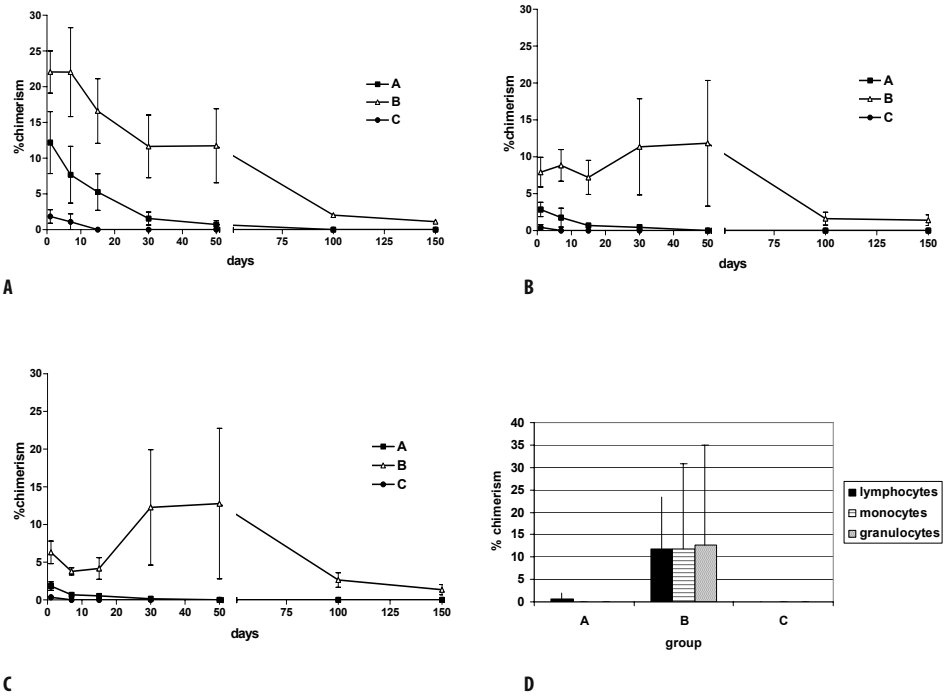


Figure 1. Average chimerism in the blood per lineage by flow cytometry.

(A) lymphocytes, (B) monocytes, (C) granulocytes. There was a low level of transient chimerism in the pigs of Group C that received the full immunosuppressive regimen and blood transfusion only, but no SpTx. Two hours after transfusion of donor packed red blood cells, the maximum levels of chimerism documented were lymphocytes 5.9%, monocytes 3.3%, and granulocytes 1.9% (not shown), after which chimerism in all lineages fell rapidly. Monocyte and granulocyte chimerism was undetectable by day 5, and lymphoid chimerism was present at levels of 0.5-1.0% for only 11 days, consisting largely of $CD3^+$ T cells. Group A pigs were chimeric until day 50, by which time most spleen grafts were rejected or were undergoing rejection (Table 1). Group A chimerism was significantly higher than Group C until day 15 (granulocytes) and 30 (lymphocytes and monocytes). Group B pigs had significantly higher chimerism in all lineages compared to Groups A and C at all time points. (D) At day 50 (5 days after cessation of CyA therapy) significantly greater chimerism in each lineage was seen in Group B compared to Groups A ($p<0.05$) and C ($p<0.05$). There were no significant differences between Groups A and C ($p=0.11$).

Figures 1A-C); donor T cells sometimes made up >50% of the total T cell pool in the absence of GVHD. At day 50, significant differences in chimerism existed between the 3 Groups; Group B had significantly higher chimerism in all lineages than Groups A and C (Figure 1D).

Bone marrow

In naïve PAA$^+$ pigs, although PAA is expressed on almost all cells in the blood and lymphoid tissues (5), it is expressed on only 50-60% of BM cells. If no spleen was transplanted (Group C) or after the spleen graft was lost (Group A), there were no PAA$^+$ cells demonstrable in the BM. Chimerism (varying from 2.4% to 26.9%) was detectable in 3 of 5 Group B pigs (except pig 15111, the class I-mismatched) (Figure 2A) from day 28. Following correction for low expression of PAA on BM cells in PAA$^+$ pigs, this chimerism can be interpreted as representing approximately 5-55% in the PAA$^-$ SpTx recipients. Chimerism in BM generally decreased at the same rate as in the blood, although it could be sustained at a higher level, presumably because the BM stroma offers an excellent environment for hematopoietic cells to thrive.

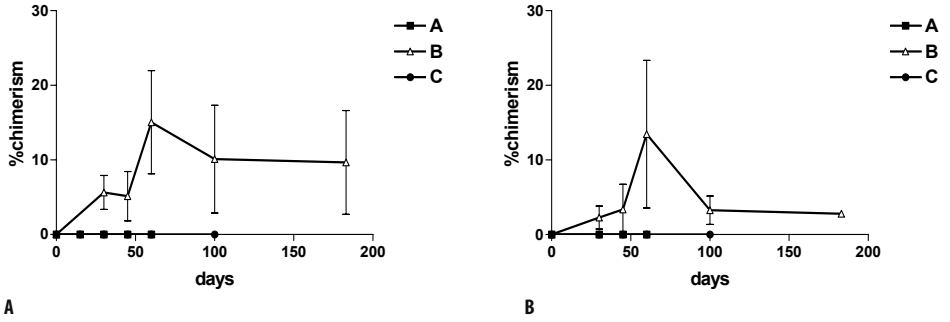


Figure 2.

(A) Mean chimerism in the BM in pigs from Groups A, B and C. Only pigs with a viable spleen graft (Group B) demonstrated chimerism in the BM; donor cells accounted for 2.4% to 27.0% of the BM cells. (In a naïve PAA$^+$ pig, PAA expression on BM cells is only 50-60%, as opposed to nearly 100% expression on cells in the blood). No pig from Group A or C demonstrated chimerism in BM at any time point during follow up (max. 100 days).
(B) Mean chimerism in the thymus in pigs from Groups A, B and C. Only pigs with a viable spleen graft (Group B) demonstrated chimerism in the thymus; donor cells accounted for 1.5% to 33% of the thymocytes. No pig from Group A or C demonstrated chimerism in the thymus at any time point during follow up (max. 100 days).

Thymus

As TI of 700cGy causes thymic atrophy (8), thymic biopsies were not taken until day 28. No thymic chimerism was seen in the Groups A or C pigs, but was documented in 3/5 Group B pigs; donor cells contributed between 2.5% and 33% of thymocytes (Figure 2B). A significant proportion of small CD1$^+$ immature thymocytes expressed PAA, indicative of donor thymopoiesis (23). Although CD1 is also expressed on a subpopulation of peripheral B cells, the majority of the PAA$^+$CD1$^+$ cells in the thymus were also CD4$^+$CD8$^+$ (not shown) and therefore were not donor CD1$^+$ B cells circulat-

ing through the thymus. The level of thymic chimerism was consistent with that of myeloid blood chimerism, and reflected the level of HPC engraftment, as observed by others after BM Tx (24). When the spleen remained viable, thymic chimerism was slowly lost, as in BM and blood.

Lymph nodes

Donor cells in the mesenteric lymph nodes could be demonstrated in all pigs with chimerism in the blood (except in Group C), but were lost when blood chimerism was lost (not shown); the extent of chimerism reflected the lymphoid chimerism in the blood, the predominant donor cell phenotype being $CD3^+$.

FISH in spleen grafts

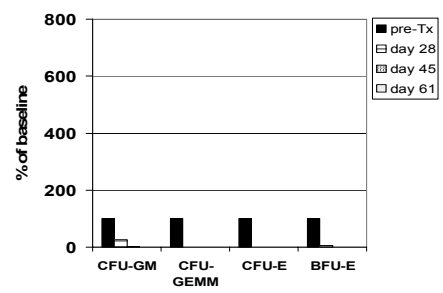
The observations made by flow cytometry for PAA^+ and PAA^- cells were confirmed by FISH in 2 cases. In the Group A pig 14840 (male), on day 42 the (female) spleen graft that was undergoing rejection consisted largely of male cells (not shown), indicating infiltration by recipient cells. In the Group B pig 15051 (female), FISH of the (male) spleen graft that was not undergoing rejection demonstrated the co-existence of donor (male) and recipient (female) cells on day 57, illustrating the dynamic interchange of cells between graft and host.

CFUs from transplanted spleens

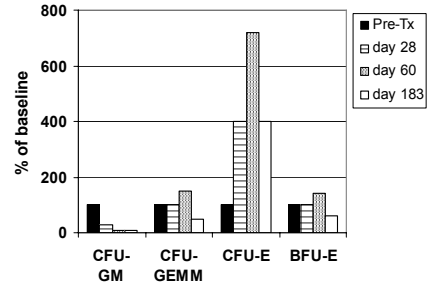
The CFU growth from donor spleen biopsies taken *before* SpTx were within the normal range of CFU growth from naïve pig spleens; primitive progenitor cells with long-term repopulating ability were found in naïve spleens at comparable frequencies to those in the BM of the same pigs (25). As early as 28 days after SpTx, all spleen grafts lost significant numbers of HPCs, as measured by the capacity of spleen cells to give rise to CFUs *in vitro*. At the time of histologically-proven rejection, no CFUs were detected (Figure 3A). Viable grafts, however, maintained significant numbers of hematopoietic progenitor cells (HPC) (Figure 3B). The data indicate that, in viable spleens, the frequencies of HPC were diminished but still present at significant levels.

Engraftment of donor spleen-derived HPC in host BM

Only a limited number of pigs (n=5) could be tested by PCR, since the assay required donors positive for MHC Class I/C and recipients negative. Pigs that lost their grafts (n=2) showed no donor cells in the BM. Colonies from 3 pigs with viable spleen grafts (obtained from CFU assays on recipient BM) were tested by PCR and confirmatory Southern Blot, and all had evidence for engraftment of donor spleen-derived HPC, at least of some lineages (Figure 3C). Evidence for engraftment persisted for several weeks to months, though donor HPC activity was eventually lost in all cases.



A



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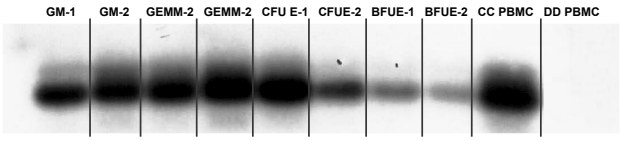


C

Figure 3.

(A) CFU growth from spleen grafts over time in a graft that went on to be rejected on day 61 in a pig that had received the full regimen (15311, Group A), and (B) a graft that survived long-term (>183 days) in pig 15410 (Group B) that was treated identically. (CFU-GM = CFU-granulocyte-monocyte; CFU-GEMM = CFU-granulocyte-erythrocyte-macrophage-megakaryocyte; CFU-E = CFU-erythroid; BFU-E = burst-forming unit-erythroid).

(C) Southern Blot from recipient BM-derived CFU-DNA (pig 15410 on day 60; Group B), demonstrating the presence of donor-derived colonies in all lineages, indicating donor hematopoiesis (functional engraftment). GM = CFU-GM (granulocyte-monocyte); GEMM = CFU-GEMM. (granulocyte-erythrocyte-macrophage-megakaryocyte); CFU-E = CFU-erythroid; BFU-E = burst-forming unit-erythroid; CC PBMC = PBMC from a miniature swine of SLA^{cc} haplotype (positive control); DD PBMC = PBMC from a miniature swine of SLA^{dd} haplotype (negative control).

CML assays

Within two weeks of graft rejection, the Group A pigs that rejected their grafts demonstrated high responses to donor cells (Figure 4A).

Whereas pre-SpTx, normal anti-donor responses had been observed, all Group B pigs demonstrated donor-specific hyporesponsiveness (Figure 4B), but remained immunocompetent to third party cells (Figure 4C). No donor cytotoxic T cell reactivity to host cells was documented in these mixed chimeras (Figure 4D). These pigs also showed post-SpTx hyporesponsiveness to all stimulator cells that were matched for MHC class I to the spleen donor (not shown), irrespective of their MHC Class II matching (to self, donor, or third party).

CML assays in Group C never showed donor-specific hyporesponsiveness (not shown). In pig 15330, anti-donor reactivity on the day of donor-matched kidney Tx (day 60) was equal to the

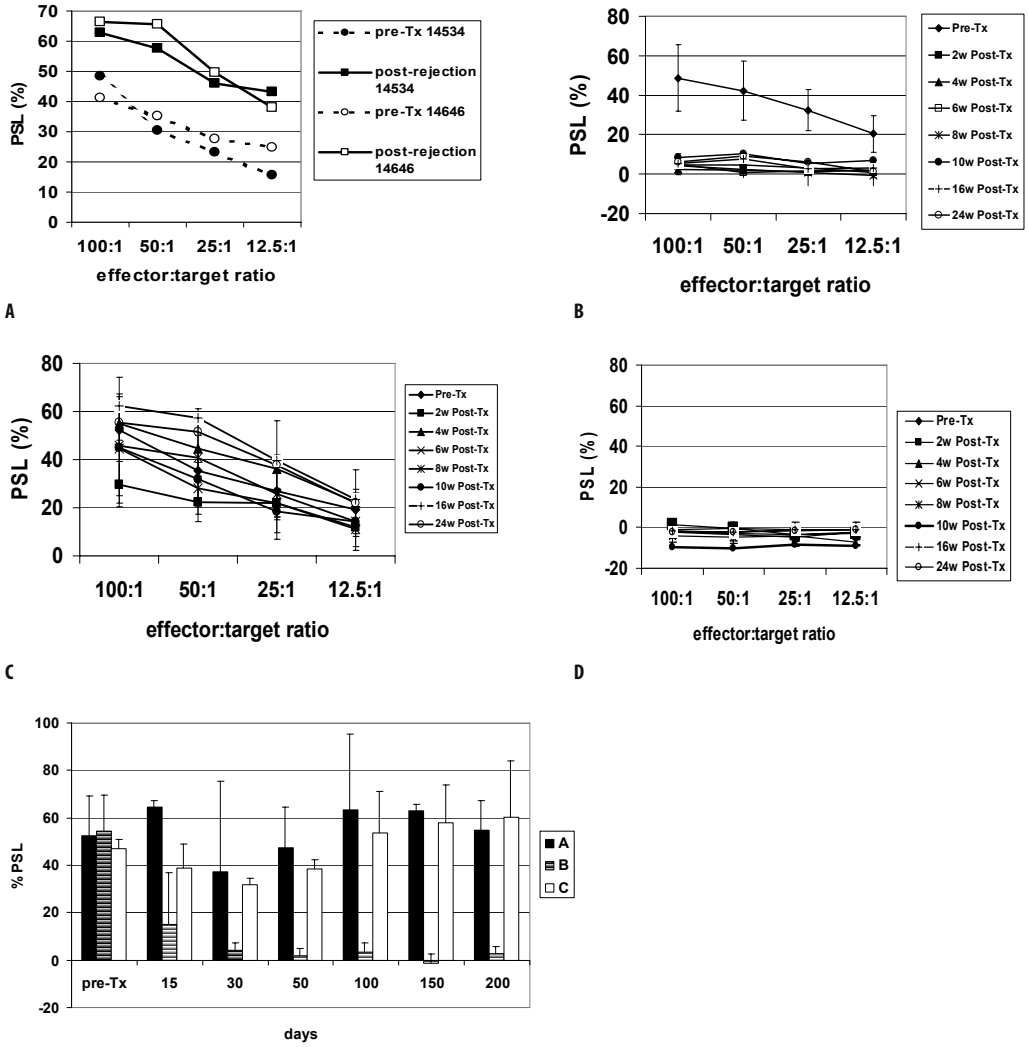


Figure 4E

Figure 4.

(A) In the two Group A pigs (14534 and 14646), after rejection of the spleen graft (solid lines), an increased anti-donor cytotoxic T cell response was documented by CML assays, expressed as percent specific lysis (PSL), in comparison with the pre-Tx values (dashed lines).

(B) Group B recipients became hyporesponsive to donor stimulators in CML assays for at least 6 months after succesful SpTx, whereas there was still a cytotoxic T cell response to third party antigens (C), an indicator of immunocompetence. Since these pigs were mixed hematopoietic cell chimeras, the donor-anti-self response was tested as well, but remained negative at all time points (D), indicating that the graft was not generating a cytotoxic anti-host response. (E) Donor-specific hyporesponsiveness in Group B pigs (in time post-Tx) was significantly different from Groups A (at all time points) and C (from day 30 onwards).

day of native splenectomy (day 0), but hyperresponsiveness to the donor developed after rejection of the kidney graft (day 4).

Figure 4E illustrates anti-donor cytotoxic lymphocyte reactivity (effector:target ratio 100:1) from Groups A, B, and C at different time points. Group B pigs had significantly lower percent specific lysis (PSL) than Group A pigs at all time points post-Tx, and from day 30 post-Tx compared to control pigs (Group C).

MLR assays

After rejection, in Group A proliferative T cell responses were increased to donor stimulators, but not to third party stimulators or self antigens (not shown).

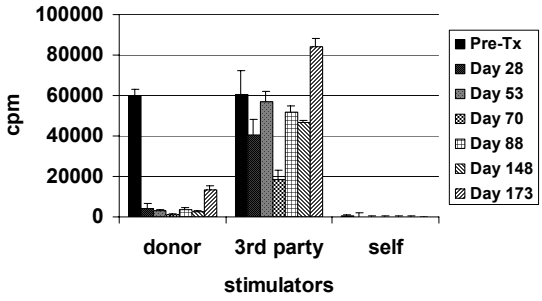
In Group B, as expected, since pig 15111 was a recipient of a MHC class I-mismatched spleen graft (and MLR assays mainly reflect MHC class II reactivity), this pig never demonstrated reactivity to donor cells on MLR. The other recipients in Group B showed a similar response pattern to their CML responses, with evidence for donor-specific hyporesponsiveness (Figure 5A). Donor MHC class II-matched stimulators were also not able to induce a proliferative T cell response in the tolerant recipients (not shown).

Group C pigs remained responsive to donor antigens. Pig 15330 demonstrated increased proliferation to donor stimulators after rejection of a donor-matched kidney graft (not shown).

sCML and sMLR assays

After discontinuation of immunosuppressive therapy, in the Group B pigs with donor-specific hyporesponsiveness, sCML (n=5) and sMLR (n=4; not performed in pig 15111) demonstrated that cells from these tolerant pigs were able to suppress the cytotoxic and proliferative T cell response from a naive recipient-matched pig to donor antigens, but not to third party antigens, at all time-points tested (Figures 5B-E).

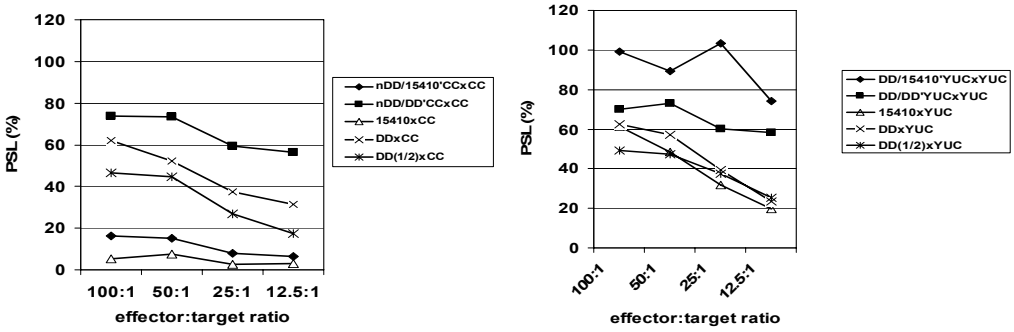
Although Group C pigs did not show hyporesponsiveness to donor cells in primary CML and MLR assays, they were tested for suppressive activity at similar time-points to those in Group B. No suppression was seen in either sCML or sMLR. In contrast, when mixed with naïve MHC-matched cells, the anti-donor responses of the pig's cells appeared somewhat stronger than either of them alone (Figures 5F-I).



A

Figure 5.

(A) MLR assays from pig 15410 (Group B, SLA I^D/II^D) at different time points after SpTx. Other Group B pigs behaved similarly. This pig was long-term tolerant to the spleen graft, and later had a long-surviving donor-matched kidney Tx in the absence of exogenous immunosuppression. The anti-donor proliferative T cell response to donor stimulators was very low (although on day 173 some reactivity may have returned), but remained normal to third party antigens. The anti-self response was absent, even though the pig had multilineage chimerism in the blood at all time-points.

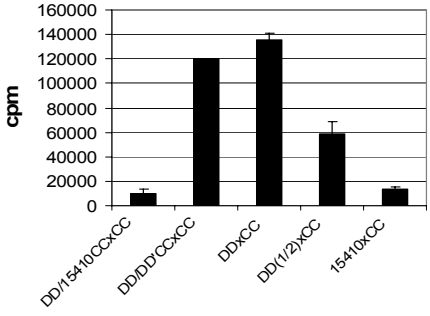


B C

Figure 5.

(B) Results of sCML assays from pig 15410 (DD) (Group B) that was tolerant following fully MHC-mismatched SpTx and subsequently received a CC kidney graft. Peripheral blood mononuclear cells taken from pig 15410 were unresponsive to CC (donor) antigens (triangles) and were able to suppress the anti-CC response from a naïve MHC-matched pig (DD) (diamonds), whereas mononuclear cells from a naïve MHC-matched pig (DD) could not suppress this response (squares). As controls, the response of the naïve DD MHC-matched pig was measured using the normal number of cells (crosses), but also with 50% of the cells (stars), which is the number of naïve DD cells when co-cultured with the cells of pig 15410. Other Group B pigs had similar responses.

(C) sCML assays indicated that suppression was donor-specific, as suppression was not seen after stimulation with third party MHC antigens.



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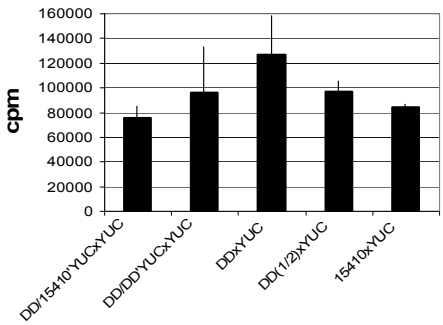
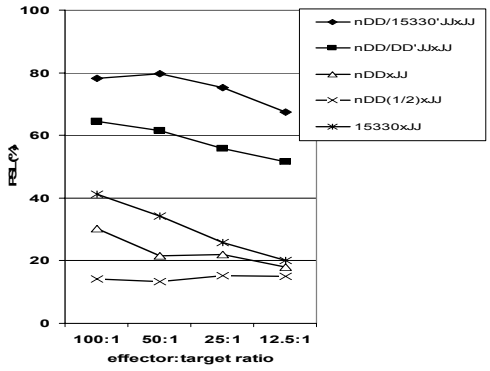


E

Figure 5.

(D and E) demonstrate suppression of T cell proliferation to donor antigens in sMLR assays (pig 15410, representative of all Group B pigs). Mononuclear cells from 15410 (DD) suppressed the anti-CC response from a naive DD pig in a donor-specific manner.



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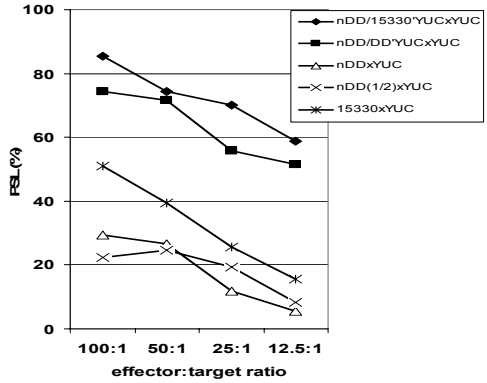

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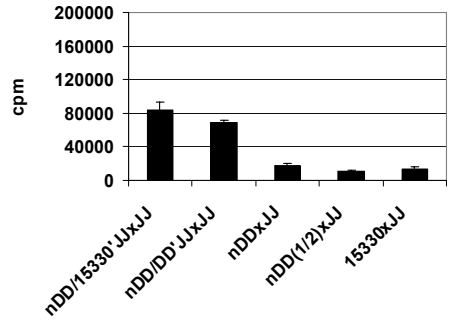

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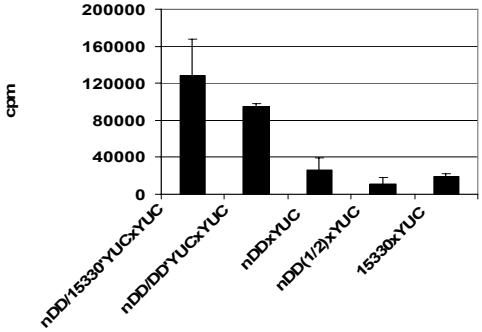

I

Figure 5.

(F and G) demonstrate that donor-specific suppression was not observed in the Group C pig 15330 (DD), in which there was almost no difference between anti-donor (JJ) and anti-third party (YUC) reactivity in sCML assays, as well as in sMLR assays (H and I).

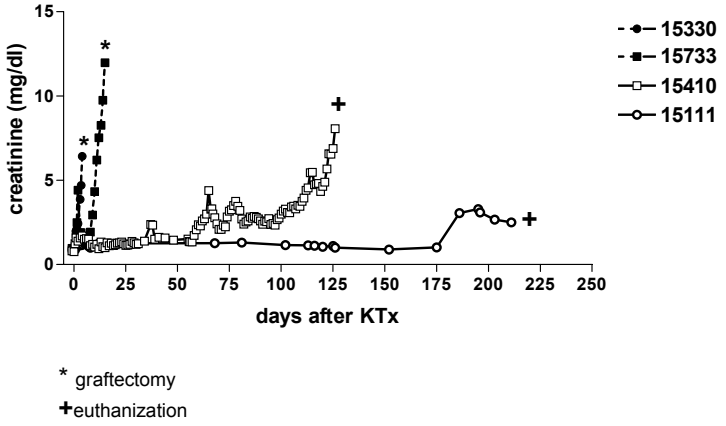

Figure 6.

Serum creatinine levels (mg/dL) in the two recipients of a donor-matched kidney allograft after SpTx (15111, 15410 [both Group B], straight lines) and in the two 'control' asplenic pigs (15733 [Group A], 15330 [Group C], dashed lines). All kidney transplants were performed in the absence of exogenous immunosuppression.

Humoral responses

The 5 rejectors in Group A developed a vigorous anti-donor antibody response (not shown). In contrast, in Group B, no anti-donor or anti-self IgM or IgG antibodies were induced (not shown). Group C pigs were only exposed to donor antigen on day 0, when they received an unirradiated packed red blood cell transfusion. Both pigs remained negative for anti-donor antibodies.

Clinical course after kidney Tx

Four pigs received donor-matched kidney grafts in the absence of exogenous immunosuppression. Two asplenic pigs rejected the kidneys on days 15 and 4, respectively (Figure 6). Two pigs that were unresponsive to their spleen grafts maintained kidney grafts for >7 and >4 months, respectively, and in neither was rejection clearly documented.

Kidney graft survival in asplenic pigs (n=2; Groups A and C)

In the Group A pig 15733, that lost its spleen graft from rupture on day 3, CyA was continued until day 45, and donor-matched kidney Tx was performed on day 60. The graft was rejected by day 15 (serum creatinine 12 mg/dL) (Figure 6); histologically, it showed diffuse interstitial edema and focal hemorrhage, diffuse infiltration with mononuclear cells and neutrophils, necrotizing glomerulitis with multiple microthrombi, and necrotizing arteritis with endothelialitis, indicating rejection from both humoral and cellular mechanisms (Figure 7E). The

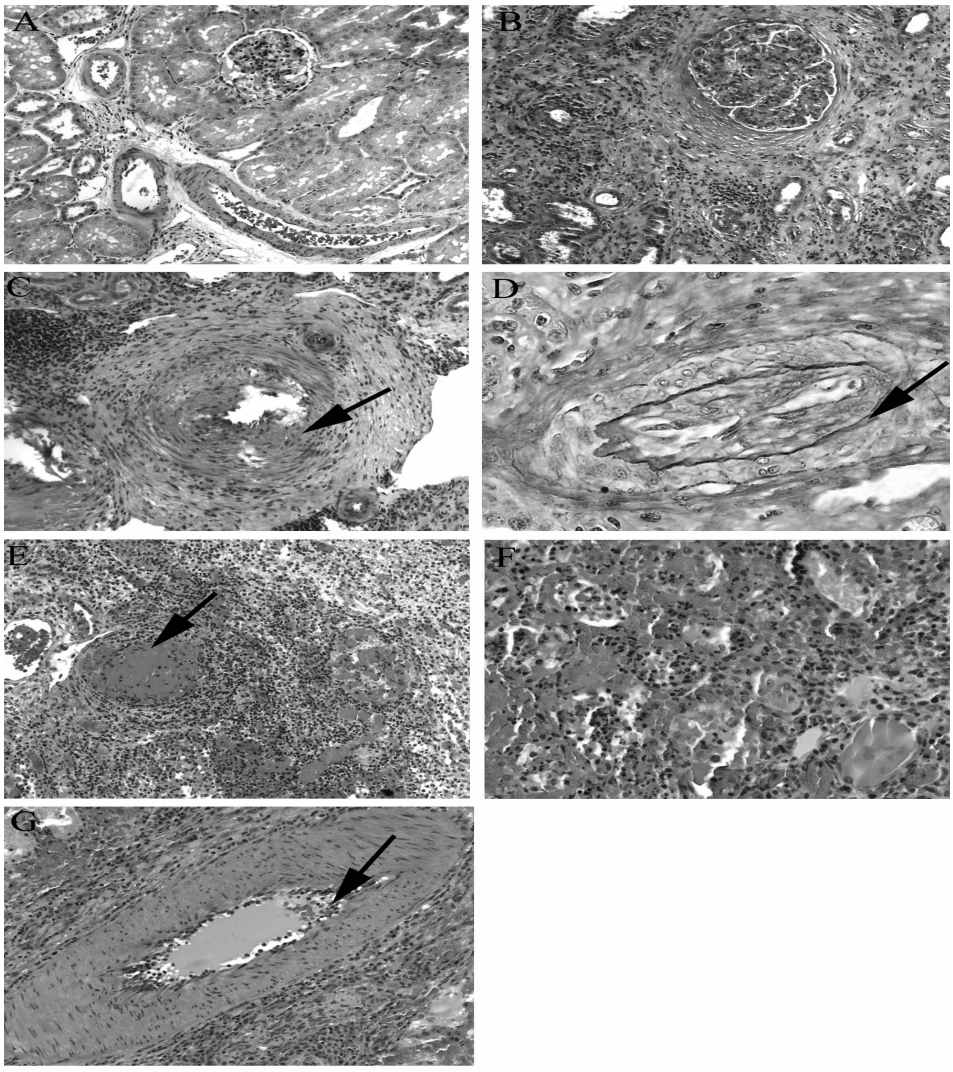

Figure 7. Histopathology of kidney allografts in two Group B pigs tolerant to their spleen grafts and in two 'control' pigs (Groups A and C). Pig 15111 (tolerant to MHC class I-mismatched spleen graft, Group B); the kidney (MHC-matched to the spleen donor) showed (A) no features of rejection after almost 3 months (H&E x160). Pig 15410 (tolerant to full MHC-mismatched spleen graft, Group B); after 129 days the kidney showed (B) type 1 acute cellular rejection with an interstitial mononuclear cell infiltrate, tubulitis, and glomerulopathy in the absence of vasculitis (H&E x160); evidence of chronic rejection was manifested by interstitial fibrosis and tubular atrophy, as well as (C and D) occlusive arteriopathy; arrows indicate internal elastic lamina (H&E x200 and Verhoeff elastic stain x 640, respectively). Pig 15733 (Group A, that lost its full MHC-mismatched spleen graft from vascular pedicle compression on day 3) developed (E) acute cellular rejection of the kidney graft by day 15; arrow indicates fibrinoid necrosis of arterial wall (H&E x160). Pig 15330 (Group C) received a kidney graft from a donor MHC-matched to the donor of a blood transfusion given on day 0, and developed severe acute cellular and humoral rejection within 4 days, marked by (F) tubular necrosis, proteinaceous casts, and interstitial hemorrhage (H&E x320), as well as (G) endothelialitis (arrow) (H&E x200).
See addendum for colour version

ligature on the native ureter was released, allowing the pig to survive. During rejection and after graftectomy, the pig showed increased MLR and CML responses to its donor, but did not develop anti-donor antibody.
In Group C, pig 15330 received a kidney graft (on day 60), that was MHC-matched to the donor of the red blood cell transfusion given at the time of native splenectomy (day 0). Although there was a good urine output initially, serum creatinine rose quickly to 6.4mg/dL by day 4 (Figure 6), and the graft showed histologic features of severe cellular and humoral rejection (Figure 7F and G). After graft excision, the native ureter was untied; two weeks later, the pig showed increased anti-donor responses on CML and MLR, but no anti-donor antibody was present in the serum.

Kidney graft survival in pigs with viable spleen grafts (n=2; Group B)
Pig 15111 received a kidney MHC-matched to the spleen donor on day 139, which survived >7 months before the pig was euthanized. Although the kidney graft developed hydronephrosis due to scarring of the ureter, associated with a rise in serum creatinine to 2.5mg/dL (Figure 6), there were no histopathologic features of acute rejection (Figure 7A).

Pig 15410 received a donor-matched kidney Tx 60 days after SpTx (by which time CyA was undetectable in the blood) without further immunosuppression. The serum creatinine was stable (<1.8mg/dL) for 60 days (Figure 6). The increase in creatinine to 4.4mg/dL that occurred between days 60 and 65 was associated with antibiotic treatment (gentamicin and vancomycin) for a wound infection, after which the creatinine fell spontaneously (to 2.0mg/dL). However, the creatinine continued to fluctuate after day 74, and steadily increased after day 100 to 8.1mg/dL on day 129 (Figure 6). Although the pig remained in good health and continued to produce urine, it was euthanized (see Discussion). Microscopic examination showed type I acute cellular rejection accompanied by arteriopathy, glomerulopathy, and interstitial fibrosis, which was compatible with either chronic rejection or the so-called "*de novo* collapsing glomerulopathy" (26) (Figure 7B-D). No vasculitis was observed in the graft at any time point. On the day of euthanasia, this pig still demonstrated *in vitro* donor-specific suppression, and had not developed anti-donor antibody. Furthermore, the spleen graft showed normal histological appearance.

DISCUSSION

The induction of Tx tolerance remains an ultimate goal in alloTx. One approach suggested by rodent studies (1) is offered by SpTx. The spleen is an organ with considerable HPC activity, and successful SpTx across full MHC barriers in miniature swine induces multilineage chimerism and engraftment in host BM in the absence of GVHD. The development of mixed chime-

rism has been associated with Tx tolerance in various animal models and clinical studies (27). Since the donor spleen is normally not used in clinical organ Tx, it would be available for Tx along with a life-supporting organ, such as a kidney or heart.

Even though the number of experiments is relatively limited, the present report provides the first data from a relevant large animal model. This study indicates that, with adequate pre-Tx conditioning of the host, SpTx can lead to multilineage chimerism in the blood, and temporary engraftment of donor HPC in the host BM, associated with the presence of donor cells in the thymus and lymph nodes. The chimerism is associated with donor-specific unresponsiveness and evidence for suppressor activity *in vitro*, and prolonged donor-specific kidney graft survival.

In contrast to SpTx between MHC-matched, minor antigen-mismatched miniature swine (3), a 12 to 28-day course of CyA and TI was insufficient to protect a fully MHC-mismatched spleen from rejection and induce a state of tolerance to this organ; the addition of a low dose of WBI and a more prolonged course of CyA therapy was required. This regimen, however, was non-myeloablative, minimally myelosuppressive and well-tolerated. One hundred cGy WBI had the benefit of reducing the host-versus-graft immune response without affecting the graft-versus-host response of the transplanted spleen, thus facilitating a balance in the two responses, as emphasized by Bitter-Suermann and others in rodent models of SpTx (1,28). Its effects, however, can probably be duplicated by pharmacologic agents administered pre-Tx (29). Eventhough WBI depleted host T cells, TI was probably of benefit in depleting residual T cells in the thymus (30) and in creating thymic 'space' (31), thus ensuring exposure of maturing thymocytes to donor antigen that would subsequently be recognized as "self". TI without WBI was not able to prevent rejection. Pre-Tx therapy with a T cell-depleting agent that is effective in pigs, e.g., the pCD3-CRM9 diphtheria immunotoxin (32), which was not available to us for this study, might well negate the need for TI for T cell depletion, but whether it would suffice in creating "space" for donor cell engraftment in the thymus is unclear. Co-stimulatory blockade with an anti-CD154 monoclonal antibody might also replace TI, as evidenced in mice (33,34).

After SpTx, donor cells (including HPC and antigen-presenting cells) emigrate from a viable spleen graft into the circulation over a prolonged period of time, and migrate to the host BM and thymus, where they engraft. The significance of their presence in host lymph nodes is uncertain, but indicates that they have become widely dispersed throughout the host's lymphoid organs. There is a dynamic exchange of immune cells between the graft and the host.

If the graft is lost, the source of donor HPC is gone, and therefore chimerism and the potential for engraftment is lost. It is not clear why chimerism should be slowly lost while the spleen graft remains histologically viable. Although we cannot exclude that donor elements would

still have been present in the recipient BM (below the detection limits of flow cytometry), we know that functional engraftment (i.e., donor-derived colonies from BM-CFUs as evidenced by PCR and Southern Blot) was eventually lost. Nevertheless, even though no chimerism could be detected, the spleen graft remained histologically viable.

A major complication following any form of HPC Tx, whether it be BM Tx or the Tx of mobilized peripheral blood HPC, is GVHD (35,36). This has also been reported after SpTx in some animal models and in humans (1,37-43) and, indeed, has been fatal in at least one patient following SpTx (42). One striking observation made in the present study of SpTx was the almost complete absence of GVHD, despite the presence of large numbers of mature T cells of donor origin. Furthermore, there was an absence in CML assays of donor cytotoxic T cell reactivity to host cells. Several differences between SpTx and HPC Tx may account for this discrepancy. First, the spleen releases donor cells into the host circulation over a matter of weeks or months rather than as a single infusion. Second, there are differences in phenotype between spleen and BM HPC that may be of importance in this respect (24).

The *in vitro* cellular and humoral responses demonstrated that successful SpTx across MHC class I or full MHC barriers was associated with *in vitro* cellular hyporesponsiveness to donor antigens and an absence of an antibody response. Tolerance to the transplanted spleen was clearly achieved. Suppressor cell assays strongly suggested that the hyporesponsiveness was at least partially a result of the development of regulatory cells. (There is also some evidence that tolerance induced by SpTx in rodent models is associated with the development of regulatory cells (1).) In the absence of a transplanted spleen, the same immunosuppressive regimen did not result in hyporesponsiveness and suppression. A detailed analysis of the suppressor mechanism after SpTx would preferably be done in a rodent model in future experiments to characterize cells mediating suppression, since in rodents more T cell antigens are characterized than in pigs and monoclonal antibodies to these antigens are commercially available. Furthermore, larger numbers of SpTx can be performed in rodents in a relatively short time.

We cannot completely rule out that the peroperative donor-specific red blood cell transfusion participated in tolerance induction, since we included it in all of the experiments. However, in Groups A and C there was no evidence of persisting donor cells in the recipient circulation or donor-specific hyporesponsiveness *in vitro*. Also, pigs in group C rejected the kidney grafts that were from the original red blood cell transfusion donor.

In the limited number of *in vivo* studies we have been able to carry out to date, in which a donor-matched kidney was transplanted in the absence of further immunosuppressive therapy, we demonstrated prolonged survival of the kidney graft but were unable to conclusively demonstrate tolerance to the kidney. Neither kidney transplanted into pigs with long-term

functioning spleen grafts and *in vitro* evidence for cellular hyporesponsiveness underwent classical rejection, but both showed features of impaired renal function. Whether this was due to non-immunological mechanisms leading to glomerulopathy and/or vasculopathy, as previously described (25, 44-45), to chronic rejection, or to other cause (26), remains uncertain. Even though evidence for regulatory cells was present until the day of euthanasia, this may not have been sufficient to protect against chronic rejection (46).

Gradual deterioration of kidney graft function has been reported previously in thymectomized pigs that became tolerant to their MHC class I-mismatched renal allografts following recovery from acute rejection (47). However, in contrast to our study, there was significant anti-donor cytotoxic T lymphocyte reactivity in these pigs. Tolerance was confirmed by the successful Tx of a second donor-matched kidney in the absence of immunosuppressive therapy. This approach was not available to us in the present case since, on humane grounds, no further surgical procedures were undertaken.

Nevertheless, the prolonged survival of these kidneys in the absence of exogenous immunosuppression is remarkable. In previous studies in miniature swine at our center, kidney allografts transplanted across a two-haplotype full MHC barrier, in the absence of any immunosuppressive therapy, uniformly rejected within 10-20 days (48) in contrast to >4 and >7 months in the present study. From studies on BM Tx, the presence of HPC chimerism alone in a full MHC-mismatched donor-recipient combination may not always be sufficient to induce tolerance to a subsequent donor-matched kidney allograft in the absence of further immunosuppression (49).

We have not yet performed simultaneous Tx of the spleen and kidney, but we hypothesize that tolerance to the kidney graft would be easier to achieve than when kidney Tx is delayed. This hypothesis is supported by experience with concurrent renal and BM Tx in pigs (50), non-human primates (51,52), and humans (53). These regimens, however, have generally involved a significant degree of myelosuppression, achieved by either irradiation or intensive cytotoxic drug therapy, whereas in several rodent models successful SpTx has been documented to induce tolerance to other donor-specific organs without the need for either myelosuppression or intensive immunosuppressive therapy (1).

SpTx-induced chimerism may lead to donor-specific hyporesponsiveness by a central mechanism, but our data suggest that peripheral tolerance is also induced; cells from tolerant animals could suppress CML and MLR anti-donor MHC responses from naïve pigs matched to the recipient. No T cell proliferation or cytotoxicity to donor or self antigens in tolerant mixed chimeras following SpTx was observed, suggesting that a peripheral regulatory mechanism had developed that controlled both recipient anti-donor and donor anti-recipient alloreactiv-

ity, as previously demonstrated after HPC Tx (54). Furthermore, SpTx may act as a continuous donor-specific blood transfusion, and this may also be involved in its 'tolerogenic' effect.

In summary, following SpTx, donor and recipient hematopoietic elements interact and interchange over a long time-period, associated with *in vitro* and *in vivo* hyporesponsiveness towards both donor and recipient, and donor-specific suppression. SpTx would therefore seem worthy of further investigation in suitable large animal models as a clinically-applicable approach to the induction of tolerance to allografts, and possibly to xenografts (55).

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Primitive Hematopoietic Cell Populations reside in the Spleen: Studies in the Pig, Baboon, and Human

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Frank JMF Dor, David KC Cooper. Engraftment potential of adult spleen cells in vitro and in vivo.
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ABSTRACT

Objective: We previously observed high levels (>40%) of multilineage hematopoietic cell chimerism following SpTx across full MHC barriers in immunosuppressed miniature swine. We therefore investigated the spleen as a source of hematopoietic progenitor cells (HPCs).

Materials and Methods: Specific cell surface markers were used to identify HPCs in the spleen and bone marrow (BM) of young adult (n=15) and fetal (n=9) miniature swine by flow cytometry. Hoechst dye-effluxing Side Population (SP) cells were analyzed in adult spleen, BM and blood for their expression of c-kit. Functional HPC activity of varying repopulation potential *in vitro* was investigated by the ability of spleens and BM to give rise to colony-forming units (CFUs) and cobblestone area-forming cells (CAFCs) in long-term stromal cultures. Studies were also carried out on baboon and human spleens and BM.

Results: Spleen c-kit$^+$ cells co-expressed more lymphoid markers, but equal myeloid markers, when compared with BM c-kit$^+$ cells. BM and spleen both contained significant percentages of c-kit$^+$ SP cells. Although the frequency of early-forming CFUs in the spleen was only 0.1-1.3% of that in the BM, the frequency of CAFCs developing after 8 weeks in culture was comparable to that of BM. Secondary CFUs in long-term culture-initiating cell assays confirmed the presence of long-term repopulating cells at comparable frequencies in spleen and BM. Similar findings were found with regard to baboon and human spleen cells.

Conclusion: The adult spleen is a relatively rich source of very primitive HPCs, possibly HSCs, and may be of therapeutic value.

INTRODUCTION

Hematopoietic progenitor cells (HPCs) have been isolated from several mammalian sources, including bone marrow (BM), adult blood after cytokine-mobilization of BM cells, umbilical cord blood, neonatal blood, and adult liver (1). The potential therapeutic applications for HPCs are extensive, and include cell therapies for malignancies, autoimmune diseases, neurological disorders, and genetic syndromes (2). Furthermore, in rodents (3), pigs (4,5), dogs (6), nonhuman primates (7), and humans (8), the successful transplantation of donor HPCs results in a state of hematopoietic cell chimerism in the recipient which, in turn, is associated with the induction of tolerance to a donor-specific organ.

Numerous studies in rodent models have demonstrated that successful SpTx can lead to a state of tolerance to a subsequently-transplanted donor-specific organ without the need for exogenous immunosuppression (9). We have recently developed a model of allogeneic SpTx in MHC-defined miniature swine (10), and have demonstrated that the spleen can be used as an effective alternative to BM for providing a state of donor-specific unresponsiveness (11). Suc-

cessful SpTx has been associated with the development of a high level (>40%) of multilineage hematopoietic cell chimerism in the recipient's blood, with evidence for engraftment of donor cells in the recipient BM, thymus and lymph nodes for at least 2-6 months (11), thus indicating the presence of relatively primitive long-term repopulating HPCs in the porcine spleen.

Recently, it has been reported that the injection of mouse spleen cells into non-obese diabetic mice leads to the regeneration of pancreatic islets, believed to be due to the presence of cells in the spleen that rapidly differentiate into islet and ductal epithelial cells within the pancreas (12).

We have investigated naïve (not transplanted) pig spleens by flow cytometry for the presence of side-population (SP) cells and c-kit expression. We also investigated cells that have short-term colony-forming unit (CFU) potential, and longer repopulating progenitor populations, employing an *in vitro* limiting dilution assay to identify cobblestone area-forming cells (CAF-Cs) and long-term culture-initiating cells (LTC-ICs) in pig spleens. The results were compared with studies in pig BM and blood. Preliminary studies have also been carried out on naïve baboon and human spleens.

METHODS

Sources of spleens and BM

Spleens and BM were harvested from 'adult' (3-10 months; *n*=15) and fetal (60-70 days; *n*=9) MHC-defined miniature swine (13). All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996). The Subcommittee on Research Animal Care (SRAC) of the Massachusetts General Hospital approved the research protocols. Baboons (*n*=4, *Papio hamadrayas,* 10-15kg) were from the Mannheimer Foundation, Homestead, FL. Human spleens (*n*=3) and BM (*n*=1) were obtained from deceased male adult organ donors (40-64 years) (through the New England Organ Bank, Newton, MA) with family consent. To our knowledge, these organ donors did not have any form of blood dyscrasia.

Studies on adult and fetal pig spleens and BM

Phenotypic analysis of HPC by flow cytometry

A single cell suspension was made in flow cytometry buffer. After red cell lysis, the concentration was adjusted to $1x10^7$cells/mL. Pig immunoglobulin (50µg/$1x10^6$cells) was added to

avoid nonspecific binding. Biotinylated recombinant porcine stem cell factor (pSCF; BioTransplant Inc., Charlestown, MA) followed by phycoerythrin streptavidin (Pharmingen, San Diego, CA) staining detected c-kit$^+$ cells. FITC-conjugated antibodies (counterstain) were anti-CD1 (76-7-4mouse IgG2a/K) (14), anti-CD2 (MSA4mouse IgG2a) (15), anti-CD3 (898H2-6-15mouse IgG2a/K) (16), anti-CD4 (74-12-4mouse IgG2b/K) (14), anti-CD5 (BB6-9G12mouse IgG1), anti-CD8α (76-2-11mouse IgG2a/K) (17), anti-CD9 (1038H-4-6mouse IgM/K) (18), anti-CD16 (G7mouse IgG1) (19), anti-CD25 (K231.3B2mouse IgG1), anti-CD45RA (STH267mouse IgG1), anti-SWC3 (74-22-15mouse IgG1/K)(20), anti-MHC class I (2-27-3mouse IgG/K)**,** anti-MHC Class II (TH16mouse IgG2a), with biotinylated 12.2.2 and FITC 36.7.5 as negative istotype-matched controls (to IgM/K, IgG2a, respectively). Data were acquired using Becton Dickinson FACScan (San Jose, CA), and analyzed with WinList software (Verity Software House, Topsham, Maine).

Identification of SP cells and enrichment for c-kit in adult porcine BM and spleen

Mononuclear cells from above-mentioned tissues were stained with Hoechst 33342 according to Goodell et al. (21, 22). In brief, cells were suspended at 1x10^6 nucleated cells/mL in Dulbecco's modified Eagle's medium (Gibco), with 2% bovine calf serum (Hyclone), 10 mM HEPES buffer (Gibco), and 5 µg/mL Hoechst 33342 (Sigma) and incubated at 37°C for 120min. Some samples were also stained at 5 x 10^6 per milliliter cell density in Hank's balanced salt solution containing 2% bovine calf serum for 90 minutes at 37°C with 6.5 µg/ml Hoechst dye (Molecular Probes). After the Hoechst staining, cells were pelleted by centrifugation and resuspended at 20-40 x 10^6 cells/mL in cold Hank's balanced salt solution containing 2% bovine calf serum and 10 mM HEPES (HBSS+) for staining with antibodies on ice. Cells were incubated with biotinylated pSCF for 20min, washed in excess with HBSS+, and then incubated with phycoerythrin streptavidin for 20min before a final wash and resuspension in HBSS+ containing 2 µg/mL propidium iodide (Sigma). Flow cytometric analysis was performed on a dual-laser MoFlo apparatus (Cytomation, Inc. Fort Collins, CO). An argon ultraviolet laser was used to excite the Hoechst dye. Fluorescence emission was collected with a 450/20 BP filter (Hoechst blue) and 630/30 BP filter (Hoechst red). A second 488nm laser was used to excite propidium iodide and phycoerythrin; emissions were collected with standard filters.

CFU assay for the detection of HPC from adult spleen, BM, and blood

The CFU assay was set up as previously described for pig BM (23-25). Single cell suspensions were plated at 5 million (spleen) and 50,000 (BM) cells, respectively, in duplicate 35-mm petri dishes (Nunc, Naperville, IL) in 1.5mL of Methocult H4230 (Stem Cell Technologies, Vancouver, Canada), supplemented with 30% FBS, 25ng/mL pSCF, 2ng/mL rpIL-3 (BioTransplant Inc.), 5ng/mL rpGM-CSF; BioTransplant Inc.) and 2U/mL rh-erythropoietin (Amgen, Thousand Oaks, CA). As controls, 50 million blood cells from 2 different pigs were plated. After 10-14 days of incubation (5%CO_2, 37°C), each culture dish was scored using inverted light microscopy and evaluated for CFU-GM, BFU-E, CFU-E, and CFU-GEMM. Only colonies containing >50 cells were counted and classified.

The same techniques were employed with spleen and BM from 9 pig fetuses (50,000 cells plated).

CAFC assay by limiting dilution in adult spleen, BM, and blood

CAFC assays were performed with spleen and BM from the same adult pigs (all aged >8 months) (spleen n=3, BM n=2) and with blood from 2 different adult donors, as described (26), using porcine cytokines (27). A stromal cell line (EUR-FLS-72/1C5A, from Dr Rob Ploemacher, Erasmus MC Rotterdam, The Netherlands), derived from day -12 fetal livers of CBA x C57BL/10 F1 mice, was used. Stromal cells were cultured in 96-well plates coated with 0.3% porcine gelatin in stromal cell line medium, consisting of Iscove's modified Dulbecco's medium with glutamax-1 (Gibco, Grand Island, NY), supplemented with 0.2% gentamicin (10mg/mL), 10^{-4}mol/L 2-mercaptoethanol, 20% fetal bovine serum, and 0.36% hydrocortisone (0.1mg/mL, Sigma, St Louis, MO) at 37°C, 5%CO_2. Spleen and BM cell suspensions to be evaluated for CAFC activity were plated over 9 dilutions (from 200,000-783 cells) and cultured at 37°C, 5%CO_2 for 12 weeks in Myelocult H5100 (Stem Cell Technologies) supplemented with 25ng/mL pSCF, 2ng/mL pIL-3, and hydrocortisone (10^{-6}mol/L), fed weekly. The percentage of wells with at least one phase-dark hematopoietic clone of >4 cells beneath the stromal layer was determined every two weeks.

LTC-IC assay by limiting dilution in adult spleen, BM, and blood

The adherent cells (that had been cultured for 12 weeks in the CAFC assay) were recovered after trypsin-EDTA (Gibco) digestion for 5min. The content of each well, containing adherent and non-adherent cells, was then seeded into 1.5mL CFU media. After 2 weeks, the presence of hematopoietic colonies was scored (wells with >2 CFU considered positive). The frequencies and 95% confidence intervals of CAFC and LTC-IC were computed using the L-calc software, version 1.1 (Stem Cell Technologies).

Studies on adult baboon and human spleens and BM

Phenotypic analysis of HPC by flow cytometry

Applying identical procedures for spleen and BM, we stained baboon and human cell suspensions with biotinylated pSCF, FITC-conjugated anti-human CD9 (M-L13mouse IgG1, BD Biosciences, San Diego, CA), and FITC-CD34 (8G12mouse IgG1, BD Biosciences), with appropriate controls; pSCF-DNA has 80-90% homology with that of other species (28) and crossreacts with human (25) and baboon cells (29).

CFU assays for the detection of HPC

For baboon and human CFU assays, the methylcellulose-based medium contained the rhSCF, rhGM-CSF, rhIL-3, rhIL-6, rhG-CSF, and rhEPO (Methocult H 4435; Stem Cell Technologies).

CAFC and LTC-IC assays

Methods for CAFC and LTC-IC assays were identical to those in pigs, except that Myelocult H5100 (Stem Cell Technologies) with 2ng/mL rhIL-3 (R&D systems, Minneapolis, MN), pSCF (25ng/mL), and hydrocortisone (10^{-6}mol/L) was used for CAFC cultures. For LTC-IC, the human/baboon CFU medium was used.

Statistical methods

Statistical analysis used paired and unpaired t-tests; p values of <0.05 were considered significant.

RESULTS

Studies on adult and fetal pig spleens and BM

Phenotypes of HPCs in adult and fetal spleen and BM by flow cytometry

-C-kit$^+$ cells

In adult spleen and BM, 5.6±3.1% and 11.1±2.7% of the nucleated cells were c-kit$^+$, respectively (P<0.01) (Figure 1A and B). These c-kit$^+$ cells were found predominantly in the monocyte population. c-kit^{high} populations were found in the BM, but were lacking in most spleens. In fetal pigs, the mean frequencies of c-kit$^+$ spleen (9.7±3.2%) and BM (10.6±1.8%) cells were similar (P=0.32) (Figure 1C and D). Also, c-kit^{high} populations were detected in both the fetal spleen and BM.

-CD9$^-$ and CD9$^-$/c-kit$^+$ cells

In adult pigs, the mean percentages of CD9$^-$ cells in spleen (69.6±7.1%) and BM (20.6±5.6%) were significantly different (P<0.001) (Figure 1A and B). In contrast, fetal pigs had a significantly higher CD9$^-$ cell population in BM (28.5±8.9%) than in spleen (17.3±5.6%) (P<0.01) (Figure 1c and d). The mean population of CD9$^-$/c-kit$^+$ cells in adult pigs represented 2.1±1.4% and 4.4±1.4% of spleen and BM cells, respectively (P<0.01) (Figure 1A and B), and in fetal pigs, 1.5±0.9% and 2.2±0.9%, respectively (P=0.01) (Figure 1C and D).

-Markers co-expressed by c-kit$^+$ cells

In adult pigs, spleen c-kit$^+$ cells expressed all of the markers investigated, whereas BM c-kit$^+$ cells mainly co-expressed CD9, CD16 and SWC3 (Figure 2A). (Pig CD9 and SWC3 resemble Mac-1 and Gr-1 expression in mice, and represent markers for the myeloid lineage (30)). There was no statistical difference in the expression of these markers between spleen and BM, but there was for the other markers (P<0.05); spleen c-kit$^+$ cells expressed both lymphocytic and myeloid lineage

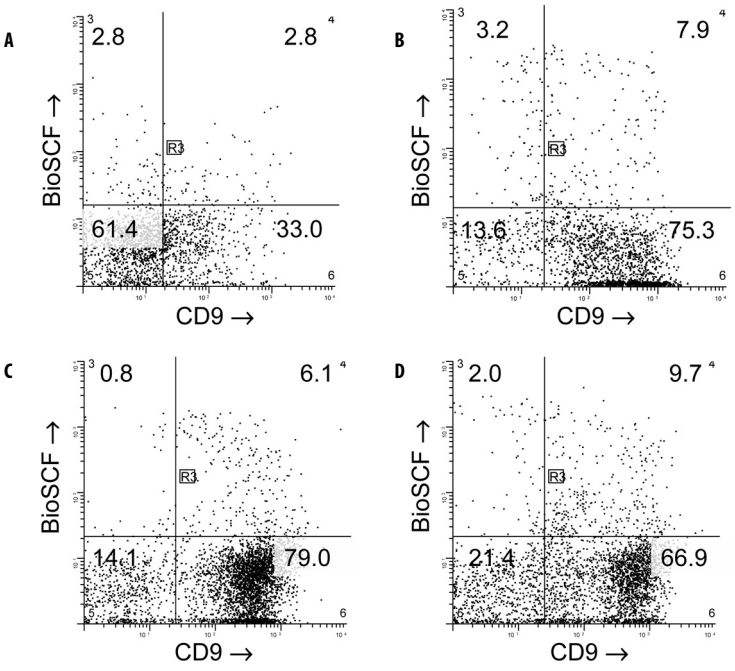


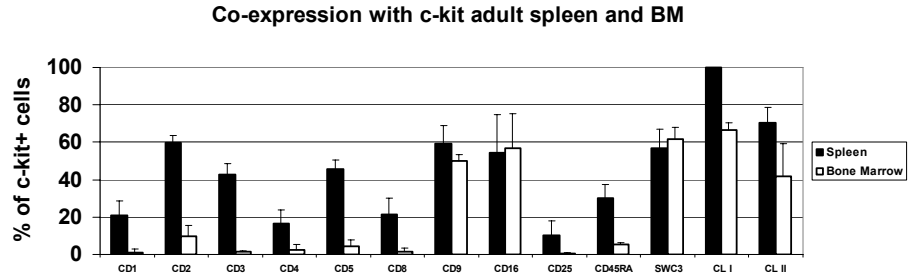
Figure 1. C-kit$^+$ and CD9$^+$ subpopulations of pig spleen and BM.
Differential expression of c-kit (staining with pSCF) and CD9 in adult spleen (A) and BM (B) and in mid-gestational fetal spleen (C) and BM (D). Shown are representative data from 15 adult and 9 fetal pigs.

markers, whereas the BM c-kit$^+$ population was restricted to the myeloid markers. Fetal pig c-kit$^+$ cells from spleen and BM did not differ statistically in their co-expression of other lineage markers (Figure 2B). In general, fetal BM c-kit$^+$ cells demonstrated similar phenotype to adult BM c-kit$^+$ cells, although with rather less co-expression of CD16 and SWC3 (not significant), whereas fetal spleen c-kit$^+$ cells had significantly higher co-expression of CD9 (P=0.001).

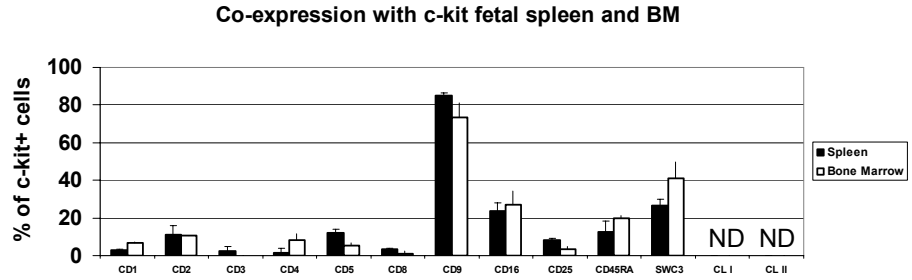
Together, these data indicate that porcine BM c-kit$^+$ cells show similar phenotypic properties to HPC in other species, e.g., mice, dogs, and primates (23, 31-33). Porcine spleen c-kit$^+$ cells, hitherto not described, differ from porcine BM c-kit$^+$ cells in that they co-express more markers than their BM counterparts. Fetal porcine c-kit$^+$ cells are phenotypically similar in spleen and BM, but differ slightly from adult BM c-kit$^+$ cells in that they co-express fewer myeloid markers.

SP cells and co-expression of c-kit

The average percentages of SP cells in BM, spleen, and blood were 0.07%, 0.06%, and 0.06%, respectively. SP cells were enriched for c-kit expression in all tissues. In the spleen, 26% of the SP cells were c-kit$^+$, compared to 46% in BM, and 12% in blood. Figure 2C-F demonstrates the SP in the spleen, and the enrichment for c-kit. Of the total number of mononuclear cells, c-kit$^+$



A



B

Figure 2.

A-B: Phenotyping of c-kit+ cells in pig spleen and BM

Co-expression of various markers by c-kit$^+$ cells in (A) adult spleen and BM and (B) fetal spleen and BM. (A) c-kit$^+$ cells from adult spleen and BM showed similar co-expression of myeloid markers, but c-kit$^+$ cells from adult spleen had greater co-expression of lymphoid markers. (B) Fetal spleen and BM c-kit$^+$ cells showed similar co-expression of all markers tested. ND = not determined.

SP cells were 0.016% in the spleen, 0.032% in BM, and 0.007% in blood. The enrichment for c-kit was even more pronounced in the "tip" SP cells that exhibit the most efficient efflux of the Hoechst-33342 dye (data not shown).

CFUs in spleen and BM

CFUs from all hematopoietic lineages were identified in cultures of both spleen and BM cells as CFU-GM, BFU-E, CFU-E, and CFU-GEMM (Figure 3A-D). The frequency of CFU per million spleen cells plated was 0.1-1.3% of that found in BM from the same pig, with a mean of 0.47% (Table 1A). No significant age-related differences (between 3-10 months) were observed (not shown).

CFUs in peripheral blood of adult pigs

Preliminary experiments had shown that, when blood cells were plated at similar concentrations to spleen cells, CFU growth from blood was too minimal to quantify (Dor 2003, unpublished data). Even when blood was plated at ten times the concentration of spleen cells, CFU frequencies in the blood were only 0-4.5% of those in the spleen (Table 1A).

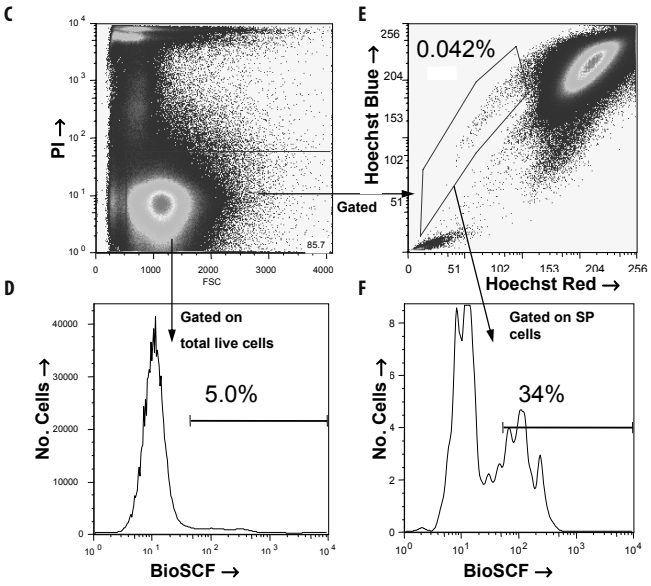


Figure 2.

C-F: SP cells and their expression of c-kit in pig spleen

Flow cytometry data of pig spleen. Dead cells were excluded on the basis of propidium iodide staining, and only live cells were analyzed (C). Nearly 5% of the spleen cells expressed c-kit, as shown above in figure 1a (D). Flow cytometry revealed the Hoechst-33342-staining profile of spleen cells. The selected cell population represents the cells that efflux the fluorescent DNA-binding dye, Hoechst 33342, and identifies a rare, so-called side population (SP) (E). The SP cells in the spleen were highly enriched for c-kit (34% in this figure), compared to the total spleen cell population (F).

CFUs in spleen and BM of fetal pigs

Fetal spleen cells were plated at the same concentration as fetal BM, since the spleen is recognized to be a hematopoietic organ in the pig at the gestational stage of 60-70 days (34). The frequencies of CFUs in the fetal spleen and BM were similar (Table 1B), but the BM contained more CFU-GM than the spleen (P=0.02). A decrease in spleen CFU activity (by 95-99%) and an increase in BM CFU activity (by 148-269%) were seen with the transition from fetal to adult life. Nonetheless, the adult spleen continued to be a source of HPC.

CAFCs and LTC-ICs in adult blood, spleen, and BM

CAFC assays were used to determine the size of the pool of primitive progenitor populations in blood, spleen and BM. Early-appearing CAFCs represent transient repopulating cells equivalent to CFUs, whereas late-appearing CAFCs (day 28-35 in mouse BM and later in human BM) are representative of long-term repopulating stem cells (35). Frequencies of CAFC-week 1-4 in the blood were comparable to those in the pig spleen (Figure 4A), but there was a marked reduction in CAFC-week 6 frequency in the blood, after which CAFC were undetectable. The

Table 1A. CFU frequencies in adult pig spleen, BM and blood

Colony	Spleen	BM	(Spleen as % of BM)	Blood	(Blood as % of spleen)
No. of cells plated	**($5x10^6$)***	**($5x10^4$)***		**($5x10^7$)***	
CFU-GM	170.1+/-109.8	130.8+/-54.8	(1.3)	77.0+/-13.0	(4.5)
CFU-GEMM	5.2+/-5.3	19.5+/-16.2	(0.27)	2.0+/-1.0	(3.8)
BFU-E	5.8 +/-6.7	58.3+/-34.7	(0.1)	1.5+/-2.1	(2.6)
CFU-E	4.2+/-4.5	22.3+/-12.3	(0.19)	0	(0)

* Note differences in number of cells plated

Table 1B. CFU frequencies in fetal pig spleen and BM (at 60-70 days gestational age)

Colony	Spleen	BM	(Spleen as % of BM)
CFU-GM	35.5+/-13.6	79.8+/-24.5	(45)
CFU-GEMM	8.3+/-4.6	7.3+/-2.1	(114)
BFU-E	30.8+/-6.7	27.5+/-14.3	(112)
CFU-E	22.5+/-10.5	15.0+/-7.5	(150)

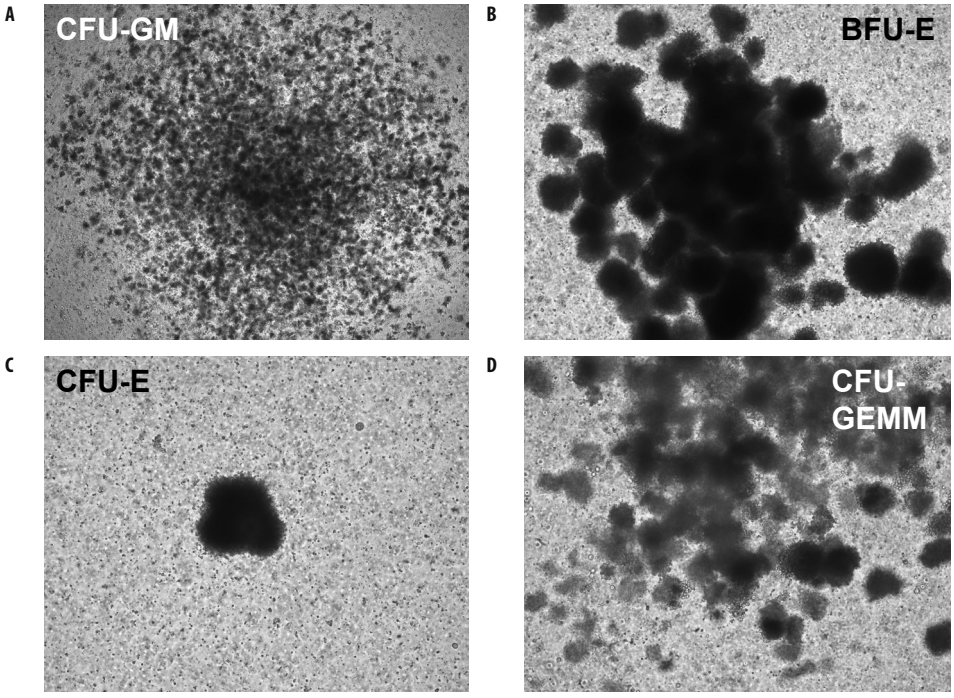


Figure 3. CFUs from pig spleen.

Morphology of colonies in CFUs from adult spleen. Shown are CFU-GM (A, 10x), BFU-E (B, 20x), CFU-E (C, 40x), and CFU-GEMM (D, 20x).

difference in frequency between CAFC-week 4 and CAFC-week 2 was greater in the spleen than in the BM from the same naïve pig (Figure 4B and C). Frequencies of CAFC-week 2-8 in spleen were consistently 10-fold lower than those in BM. However, frequencies of CAFC-week 10 and later in spleen and BM approximated each other, indicating that spleen and BM have the same frequencies of the very primitive subsets of HPC. This was confirmed by the comparable frequencies of LTC-IC at 12 weeks in cultures of both spleen and BM (Figure 4D). The characteristics of CFUs that arose from week-12 CAFC cultures were similar in LTC-IC from spleen and BM, and were restricted to colonies of myeloid morphology. The similarities between the CAFC and LTC-IC endpoints are consistent with the previous report on porcine BM populations separated on the basis of c-kit expression (23).

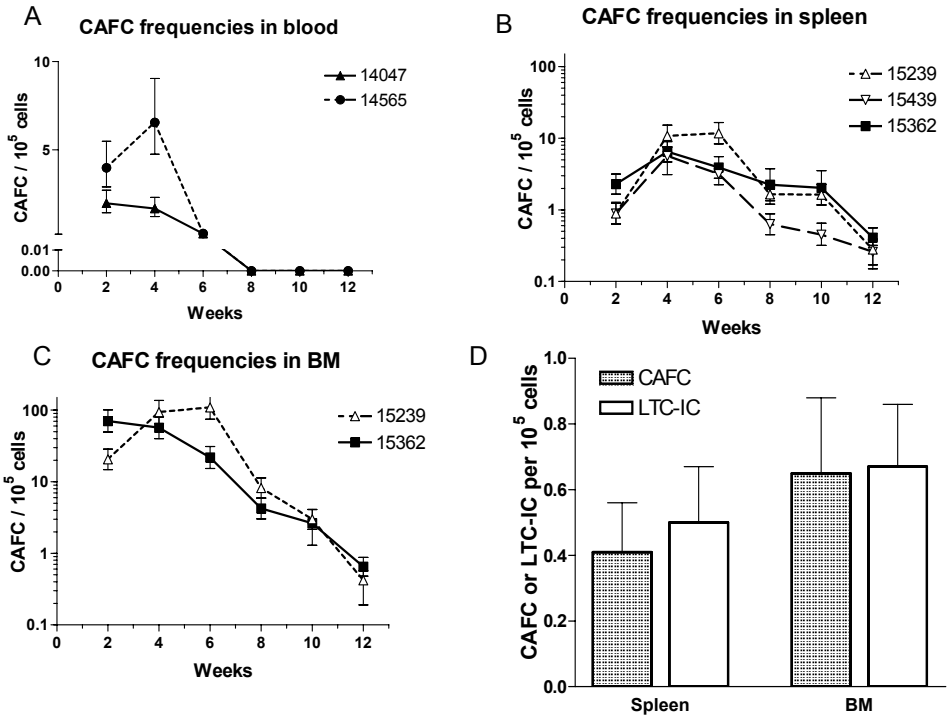


Figure 4. Frequencies of CAFC from adult pig blood, spleen and BM.

Frequencies (per 10^5 cells) of CAFC from adult blood (n=2) (A), spleen (n=3) (B) and BM (n=2) (C). Initially, the frequencies in blood and spleen were comparable, but from week 4 blood CAFC frequencies decreased until undetectable on week 8, whereas spleen CAFC frequencies were present in significant numbers until week 12. During the first 6 weeks of culture, CAFC frequencies in BM were approximately 10-fold higher than in spleen, but thereafter CAFC frequencies were similar in spleen and BM (at approximately 1 per 10^5 cells). Error bars represent 95% CI. (D) Frequencies (per 10^5 cells) of week 12 CAFC and subsequent LTC-IC were comparable in spleen and BM. There were no statistical differences between spleen and BM.

Studies on adult baboon and human spleens and BM

Phenotypes of HPCs by flow cytometry

The expression of HPC markers in baboon and human spleen and BM was studied (Table 2). There were higher percentages of c-kit$^+$, CD34$^+$, and CD9$^-$/c-kit$^+$ cells in BM than in spleen. The percentages were lower than in the pig.

Table 2. Frequency of HPC markers in baboon and human spleen and BM

Cell population	Baboon spleen	Human Spleen	Baboon BM	Human BM
c-kit$^+$	2.5%	1.6%	5.6%	5.1%
CD-34$^+$	1.8%	2.4%	3.4%	3.6%
CD9$^-$	64.4%	72.7%	72.8%	75.6%
CD9$^-$/c-kit$^+$	0.59%	0.62%	1.6%	1.5%

CFUs in the spleen

Baboon and human spleen cells were plated at the same concentration as porcine splenocytes (5x10^{6}cells/3mL CFU media). In comparison with the pig spleen CFU, some differences were observed in the frequencies of baboon spleen CFU. The baboon spleen had fewer cells that gave rise to "white and mixed" colonies, i.e., CFU-GM, CFU-GEMM, whereas the number of "red" colonies, i.e., BFU-E, CFU-E, tended to be higher (Table 3). This may reflect differences in spleen function between the two species. The human and baboon spleen had comparable CFU frequencies (Table 3).

Table 3. CFU frequencies in adult baboon and human spleens in comparison with adult pig spleen

Colony	Pig Spleen	Baboon Spleen	(% of pig spleen)	Human Spleen	(% of pig spleen)
No. of cells plated	**(5x10^6)**	**(5x10^6)**		**(5x10^6)**	
CFU-GM	170.1+/-109.8	29.7+/-2.1	(17)	9.0+/-2.5	(5)
CFU-GEMM	5.2+/-5.3	1.3+/-2.3	(25)	2.5+/-2.7	(48)
BFU-E	5.8+/-6.7	11.0+/-13.1	(190)	37.0+/-12.1	(638)
CFU-E	4.2+/-4.5	4.0+/-3.5	(95)	15.5+/-14.8	(369)

CAFCs and LTC-ICs in the spleen and BM

CAFC-week 2 frequencies in spleen and BM were similar (approximately 5 per 10^5 cells). However, CAFC-week 4-8 showed decreasing frequencies in both spleen and BM, with only ~0.1 per 10^5 cells from week 8. In contrast, frequencies of CAFC-week 6-10 in the spleen were slightly higher than in CAFC-week 2-4 (i.e., ~1 per 10^{5}cells), and were 10-fold higher than in the BM (Figure 5A). LTC-IC from the 12-week CAFC cultures also demonstrated a similar pat-

tern in frequencies (overlapping 95% confidence intervals) of cells that were able to give rise to colonies (Figure 5B).

Figure 6 shows the morphology of "cobblestones" from an adult human spleen after 12 weeks in culture.

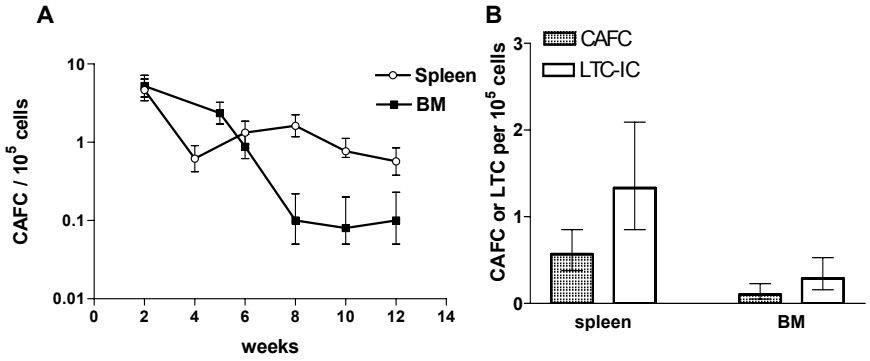


Figure 5. CAFC and LTC-IC in human spleen and BM.

Frequencies (per 10^5 cells) of CAFC from adult human spleen and BM (A). During the first six weeks of culture, the frequencies in BM were higher than in spleen, but thereafter, frequencies in spleen remained constant, while the BM-CAFC frequency diminished rapidly.

Frequencies (per 10^5 cells) of week 12 CAFC and subsequent LTC-IC were comparable in each tissue (B). There were no statistical differences between spleen and BM.

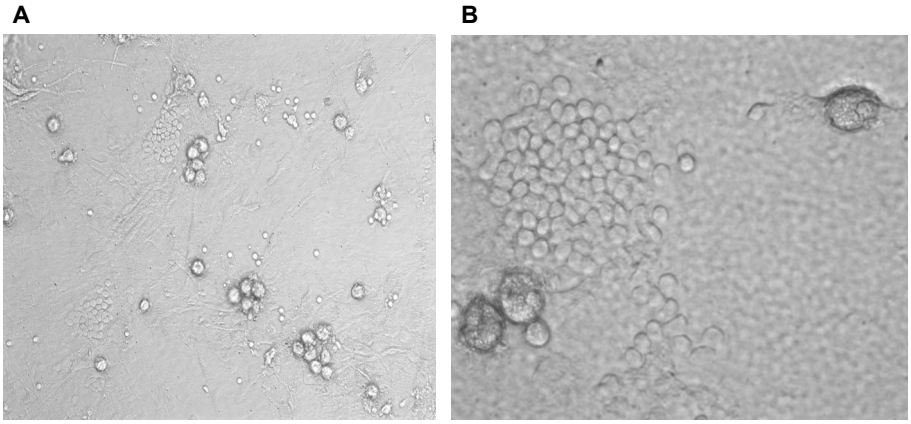


Figure 6. Cobblestone morphology.

(A&B) Morphology of "cobblestones" from an adult human spleen after 12 weeks in culture (A, 40x; B, 200x).

DISCUSSION

Hematopoiesis in humans is sustained by a population of pluripotent stem cells that are capable of self-renewal and differentiation into all lineages of functional hematopoietic cells. During early embryonic development, these stem cells reside in the yolk sac and the aorta-gonad-mesonephros region, and later migrate to the liver, spleen and BM (36,37). Adult hematopoiesis essentially occurs in the BM, and pluripotent stem cells are rarely detected in the circulation. In humans and other higher mammals, the spleen plays an important role in blood formation during much of the period of fetal development, but this function rapidly decreases after birth, and the spleen is thought not to participate in hematopoiesis in healthy individuals (38).

In contrast, in rodents the spleen continues to be an accessory hematopoietic organ during adult life, although its role may largely be limited to erythropoiesis and megakaryocytopoiesis (39-41). As early as 1949, Jacobsen (42) clearly demonstrated the ability of hematopoietic cells from the shielded spleen to reconstitute lethally-irradiated mice. Recently, Wolber et al. demonstrated that the spleen from adult mice contained low numbers of primitive HPCs capable of long-term reconstitution of hematopoiesis in irradiated hosts (43).

In higher mammals, when hematopoiesis in the BM is failing from e.g. dysplasia or myelofibrosis, the spleen can regain its fetal role in exhibiting extramedullary hematopoiesis (44). It is unclear whether the spleen regains its hematopoietic function by stimulation of precursor cells that are already present in the spleen or by the *de novo* migration of circulating HPCs to the spleen (38,45).

Our own studies indicated that allogeneic SpTx in pigs induced a state of mixed hematopoietic chimerism in the blood and lymphoid tissues of the host, including the thymus, and that donor cell engraftment was established in the recipient BM (11). We therefore wished to evaluate whether the naïve porcine spleen contains sufficient hematopoietic activity to account for engraftment in recipients of spleen allografts. Previously, HPCs have been characterized in BM and fetal liver of miniature swine (18,24), but not in the spleen.

For flow cytometry, the choice of markers specific for HPC in the pig is limited. Porcine-specific CD34 markers, for example, have not yet been developed and antibodies recognizing CD34 in other species do not cross-react with pig cells (46). C-kit is the receptor for SCF, and the use of biotin-labeled recombinant pSCF has been reported to contain long-term-repopulating hematopoietic stem cell (HSC) activity as determined in both the *in vitro* CAFC assay and *in vivo* engraftment in NOD/SCID mice (23).

CD9 is associated with cellular activation, and quiescent HPC possibly increase CD9 expression upon activation. CD9 expression has been shown to be absent on primitive or early porcine HPC (47). Lack of expression of CD9 on HPC may be associated with their retention in the tissues of their origin (i.e., BM, spleen) (18). In long-term CAFC assays, which are thought to test for more primitive cell populations than CFU assays, $CD9^{negative/low}$ activity was enriched 10-fold relative to $CD9^{high}$ cells (18).

In the present study, no age-related differences in phenotype of c-kit^+ spleen and BM cells were found in the adult population (>3 months old), but there were notable phenotypic differences between the adult tissues and mid-gestational fetal tissues. In fetal pigs, c-kit^+ cells in both spleen and BM co-express a variety of other markers equally, whereas in adult pigs the spleen c-kit^+ cell population differs considerably from that in the BM in co-expression of other markers. Expression of c-kit^+/$CD9^-$ was used as an indicator of pig HPC to compare spleen and BM cells; significantly higher numbers of these cells were present in the BM than in spleen. The functional relevance of these differences on flow cytometry remains unclear.

It has previously been shown that murine HSCs can be highly enriched with the use of the fluorescent DNA-binding dye Hoechst 33342 whereby the SP cells can be distinguished by their ability to efficiently efflux the dye (21,22). Hoechst dye efflux has also been used to purify primitive stem cells from different organisms, tissues, and developmental states (21,48-50). Thus, in contrast to many cell-surface markers, Hoechst efflux appears to be a physical property that correlates with a general state of "stemness" and could potentially identify the HSC with the most potent self-renewal activity.

In pigs, SP cells have previously been demonstrated in BM (18, 21). We demonstrate for the first time that the adult pig spleen also contains this population. Parmar et al. recently demonstrated that SP cells of cytokine-mobilized peripheral blood (and not unmodified blood) contain HSC activity (51) and that these cells were enriched for c-kit. In our study, spleen SP cells were enriched for c-kit (26%), which is more than 2-fold higher than in blood, although lower than in BM. A qualitative hierarchy within the SP population may also exist for porcine spleen and BM as the expression of c-kit was more pronounced in the "tip" SP fraction, the region that is already known to contain the highest frequency of primitive stem cells in the mouse bone marrow (21,51-54).

The pig spleen is relatively larger (on average 8g/kg body weight) than the human or baboon spleen (on average 2g/kg body weight), which might reflect different functions. However, the present study would suggest that primate spleens appear to harbor HPCs in similar numbers to pig spleens. There were slight differences in frequencies of CFU-GM (pig>primate), CFU-GEMM (pig>primate), and BFU-E (primate>pig). The numbers of baboon and human spleens that have become available to us to date have been small, and further studies would be beneficial.

Total nucleated cell numbers of adult porcine spleen and BM are comparable ($2.2x10^{10}$ vs. $2.0 x10^{10}$, respectively). These cell numbers were obtained by harvesting all accessible BM and whole spleen from adult pigs. Therefore, the relative frequencies allow a 1:1 comparison when estimating total progenitor cell numbers in spleen and BM. Unfortunately, we do not have data on total cell numbers in fetal porcine or adult human tissue.

There are very few reports relating to CFU growth from the human spleen. Freedman et al reported that human spleen cells gave rise to multiple hematopoietic colonies only when there was evidence for extramedullary hematopoiesis, generally in patients with a malignant or other hematological disease (38); however, no spleens from healthy subjects were examined. Meytes (55) documented a significant decrease in BFU-E from the blood after splenectomy; tissue from the excised spleen contained significant numbers of BFU-E. In deceased organ donors, CFU frequencies from human spleen cells (that had been maintained in liquid nitrogen) were 10-20 fold higher than in the present study (56).

Our data clearly demonstrate that the spleen from adult pigs contains a significant number of early HPCs in the form of CAFCs and LTC-ICs. The frequency of more primitive CAFC (>8 weeks of culture) closely approximated that in the BM. While CAFC-week 6 have been reported to correlate with the long-term repopulating ability of hematopoietic cells in NOD/SCID mice and in human BM transplants (57,58), extension of such cultures for longer periods, such as in our study, may be necessary to identify the most primitive HSC subset (59). In the present study, the murine stromal cell line, EUR-FLS-72/1C5A, proved to be important in allowing maintenance of the cultures to 12 weeks without the problems of stromal cell detachment that are commonly encountered within the first 6 weeks for many other stromal cell lines.

Correlative data between phenotype and function, especially c-kit vs. CAFC/LTC-IC or repopulation in NOD/SCID mice, already exists. LeGuern et al. (23) reported that purified pig c-kit$^+$ BM cells were substantially enriched for both CFUs and CAFCs *in vitro* and their transplantation led to improved long-term porcine hematopoiesis *in vivo* in mice.

In summary, porcine spleens harbor very early HPCs that can give rise to hematopoietic colonies of different lineages *in vitro*. It remains unclear whether the HPCs found in the spleen actually actively contribute to hematopoiesis in a normal naïve pig or are generally dormant and become hematopoietically active when there is an extra demand on blood formation that cannot be met by the BM alone. The fact that the frequency of CAFC from spleen and BM is comparable only in the week 8-12 cultures supports the theory that the spleen harbors HSCs that are generally quiescent. Irradiation or other myeloablative therapy to the host may provide a stimulus for HPC in a transplanted spleen to migrate from the graft to the recipient BM, as suggested by our own studies (11). The data obtained in the present study correlate

well with our *in vivo* observations that SpTx can lead to multilineage mixed hematopoietic cell chimerism in the blood, and engraftment in recipient BM (11).

Although our observations on primate spleens are preliminary, they suggest that human adult spleens are equally a source of early HPCs. The adult spleen may therefore be a valuable source of early HPCs, or even HSC, that may prove of clinical use.

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Lack of Cardiac Differentiation in c-kit-enriched Porcine Bone Marrow and Spleen Hematopoietic Cell Cultures using 5-azacytidine

7

appendix

Mario L Ramirez, Isabel M McMorrow, Todd M Sanderson, Courtney J Lancos, Yau-Lin Tseng, David KC Cooper, Frank JMF Dor. Lack of cardiac differentiation in c-kit-enriched porcine bone marrow and spleen hematopoietic cell cultures using 5-azacytidine.
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ABSTRACT

The adult spleen is a source of early hematopoietic stem cells (HSC). We therefore studied whether culturing spleen or bone marrow (BM) HSC in medium containing 5-azacytidine could induce a cardiac phenotype. C-kit-enrichment and -depletion of adult pig spleen and BM mononuclear cells was obtained by magnetic bead separation using biotinylated pig stem cell factor (SCF; c-kit ligand). Cells were incubated with 5-azacytidine for 24 h, and refreshed with 5-azacytidine-free medium every 48 h. Western Blot was used to detect cardiac troponin and myosin heavy chains.

Although 5-azacytidine treatment led to the formation of ball-like cell clusters in both c-kit enriched populations, these clusters showed no rhythmic contractions (beating), as observed by others. Furthermore, neither cardiac troponin nor myosin was detected in cells derived from either source. Our methodology and treatment with 5-azacytidine did not induce cardiac gene expression in porcine HSC derived from either pig spleen or BM.

INTRODUCTION

Although the presence of hematopoietic stem cells (HSC) is well characterized in the bone marrow (BM), we have recently shown that the spleens of pigs, baboons, and humans are also sources of early HSC (1). Although CD34 is often used as a marker of the earliest HSC, it is also known that, in the sequence of events associated with hematopoietic differentiation, porcine cells expressing c-kit (c-kit$^+$) contain pluripotent HSC as well (2). Through the use of the cobblestone area-forming cell (CAFC) assay, one assay that defines the presence of early hematopoietic stem cells (3,4), we documented an equal frequency of these earliest stem cells in BM and spleen (1). The question was then raised whether these c-kit$^+$ stem cells might reflect an early pluripotent state that could be directed away from hematopoietic fate.

Recent work at understanding the repair of infarcted myocardial tissue and the ability of fetal myocardial cells to potentially repair cardiac tissue has generated interest in this field as a potential therapeutic option (5). At the same time, ethical ramifications and technical problems of fetal tissue acquisition have generated great interest in adult stem cell populations and the ability of these cells to dedifferentiate and be directed toward other fates (6-10). Along these lines, the ability of 5-azacytidine, a cytosine analog, to induce altered gene expression and *in vitro* cellular rhythmic contractions, associated with a cardiac phenotype, has been well documented (11-14). Fukuda has demonstrated that regenerative cardiomyocytes can be generated in the rat from BM stromal cells (MSC) using 5-azacytidine (14), and Tomita et al. (15) have demonstrated that porcine heart function improved following the infusion of 5-azacytidine-treated MSC into infarcted myocardium.

In view of the work of Rangappa et al. (16), which demonstrated the ability of adipose-derived rat mesenchymal stem cells to differentiate towards cardiac fate, a similar approach was applied to the c-kit -expressing HSC from the spleen and BM. The spleen, like the BM, is a hematopoietic tissue of mesodermal origin and, as such, may serve as a repository for stem cells that retain their plasticity. Recent work by Kodama et al. (17), which showed that injected mouse splenocytes contribute to the regeneration of functional pancreatic islet cells, suggests that the transdifferentiation of splenic cells to an alternate phenotype may, in fact, be possible.

Based on the shared embryonic origins of the splenic and BM HSC, and further supported by their similar CAFC frequencies at 12 weeks of culture (1), we attempted transformation of c-kit expressing HSC from spleen and BM cells toward cardiac fate by an approach that has been reported to be successful by others working with mesenchymal stem cells.

MATERIALS AND METHODS

Animals

Four naïve pigs were obtained from the Massachusetts General Hospital herd of partially-inbred MHC-defined miniature swine (aged 3-5 months), whose characteristics have been described in detail previously (18). All procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* produced by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996).

Primary culture of porcine c-kit$^+$ cells

Spleen and sternal BM samples were obtained in a sterile fashion from adult pigs immediately following the administration of an overdose of pentobarbital to induce euthanasia. The samples were washed in Hanks Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY), and minced in a dish of RPMI 1640 culture medium (Mediatech, Herndon, VA). The resulting suspension was passed through a 40-micron filter (BD Biosciences, San Jose, CA), diluted 1:1 with HBSS, and mononuclear cells isolated using a gradient of lymphocyte separation medium (ICN Biomedicals, Aurora, OH). The buffy coats were harvested and residual erythrocytes lysed using ACK lysing buffer (Cambrex Bio Science, Walkersville, MD). The cells were resuspended in culture medium consisting of RPMI 1640, 15% fetal bovine serum, penicillin (1U/mL), streptomycin (1mg/mL), gentamicin (0.05mg/mL), amphotericin B (0.34µg/mL) (all from

Gibco), Hepes buffer (10mM, Mediatech), and glutamine (2 mM, Gibco). The cells were incubated with 1:40 porcine biotinylated recombinant porcine stem cell factor (pSCF; c-kit ligand) (BioTransplant, Charlestown, MA) (2), and then with magnetic cell separation (MACS) anti-biotin magnetic microbeads (MiltenyiBiotec, Auburn, CA), and separated over MACS LS columns (MiltenyiBiotec) with the negative fraction being retained for use in the c-kit-depleted (c-kit$^-$) cultures. The c-kit$^+$ cells were then eluted for use as the HSC. 1x10^6 cells were removed from each population for FACS analysis to evaluate purification of the different populations. Both the c-kit$^+$ and c-kit$^-$ cell populations for the spleen and BM were counted, and separately seeded onto 25-cm^2 flasks at a concentration of 1x10^6 cells/mL in the same culture medium as above. At 24 h, the cells were trypsinized (0.25% trypsin with EDTA, Mediatech) and resuspended in the above culture medium.

Treatment with 5-Azacytidine

Half of the cells from both c-kit$^+$ and c-kit$^-$ cultures were transferred from the culture flasks into 6-well plates in culture medium (as described above) with the addition of 5-azacytidine (Sigma-Aldrich, St. Louis, MO) at a concentration of 9μM. This was the same concentration used in previous experiments in which a cardiac phenotype was observed. The other half was cultured in the same manner in the absence of 5-azacytidine. After 24 h incubation with 5-azacytidine, 40% of the medium was removed from each well, and replaced with fresh culture medium without 5-azacytidine. Medium (40%) was again removed and replaced after 36h and 48h in order to titrate down the concentration of 5-azaycytidine as quickly as possible. Culture medium was thereafter changed every 48 h. The cells were observed under an inverted light microscope at several time points over 5 weeks.

Phenotypic assessment of cell cultures by flow cytometry

Cell suspensions for flow cytometry from the BM and spleen were resuspended in flow cytometry buffer, consisting of HBSS, 0.1% BSA, and 0.4% sodium azide (diluted 1:1:1). Pig immunoglobulin (5 μg/10^6 cells) was added to prevent nonspecific binding. Cells were labeled with biotinylated recombinant pSCF; biotinylated 12.2.2 (IgM) and fluorescein isothiocyanate (FITC) 36.7.5 (IgG2a) were used as negative isotype-matched controls.

Western Blot and visualization

After 5 weeks in culture, cells were removed from the bottom of the plates using 0.25% trypsin with EDTA and lysed at a concentration of 100μL/10^6 cells using a solubilization buffer, consisting of 100mM NaCl, 1.0% SDS, 50mM TEA (pH 7.4), 1mM DTT, 1mM PMSF, and Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts 1:1000 (Sigma-Aldrich). The

resulting lysates were loaded under reducing conditions onto SDS-PAGE gels (7.5% and 12.5% acrylamide for myosin and troponin staining, respectively). The proteins were then transferred to nitrocellulose membrane (Hybond C, Amersham Biosciences, Piscataway, NJ) and stained with Ponceau Red to assess protein transfer. The nitrocellulose was incubated in blocking buffer (PBS with 10% fetal bovine serum and 0.1% Tween) overnight, washed, and incubated for 1 h with either mouse anti-myosin heavy chain (with known pig reactivity) (0.2µg/mL) (Biodesign International, Saco**,** ME) or anti-troponin I (known to have pig reactivity) (0.2µg/mL) (Novus Biologicals, Littleton, CO) monoclonal antibodies diluted in the same. The membranes were washed in blocking buffer and incubated with HRP-conjugated anti-mouse IgG (1:25,000) (Southern Biotechnology Associates, Birmingham, AL). The SuperSignal West Pico chemiluminscent substrate kit (Pierce Biotechnology, Rockford, IL) was used to develop the blots.

RESULTS

c-Kit purity by MACS cell separation

The isolation of c-kit$^+$ populations from the BM was substantially more efficient than that from the spleen tissue (Table 1). Following cell separation, in the BM, there was a significant difference in mean c-kit expression between the populations: 66.01%±14.67% c-kit$^+$ cells in the enriched population, and 3.31%±1.76% in the depleted population (p=0.01). In the spleen, the separation came close to, but did not achieve, statistical significance; enriched cultures contained a mean of 19.98%±7.07% c-kit$^+$ cells, and depleted samples contained 3.44%±1.06% c-kit$^+$ cells (p=0.05). When BM and spleen were compared, there was no statistical difference between the respective c-kit-enriched populations (p=0.07) or the respective c-kit-depleted populations (p=0.93).

Growth and morphology of cultures prior to 5-azacytidine treatment

Following separation, there was a gross difference in the overall morphology of the cells from the two tissues. BM c-kit$^+$ isolates had cells that were larger and well rounded compared to splenic c-kit$^+$ isolates, where the cells were much smaller and had more variation in shape (Figure 1). In the c-kit$^-$ cultures, the BM cells were somewhat smaller than in the corresponding c-kit$^+$ BM cells, but still remained well rounded. Morphologically, splenic c-kit$^-$ cultures were not as distinct from splenic c-kit$^+$ cells as in the BM. Both splenic populations were of the same relative size, and featured variation in cell morphology that included both smaller, well-rounded cells, and other more spindle-shaped cells.

Table 1. Enrichment of c-kit hematopoietic stem cells in porcine bone marrow and spleen

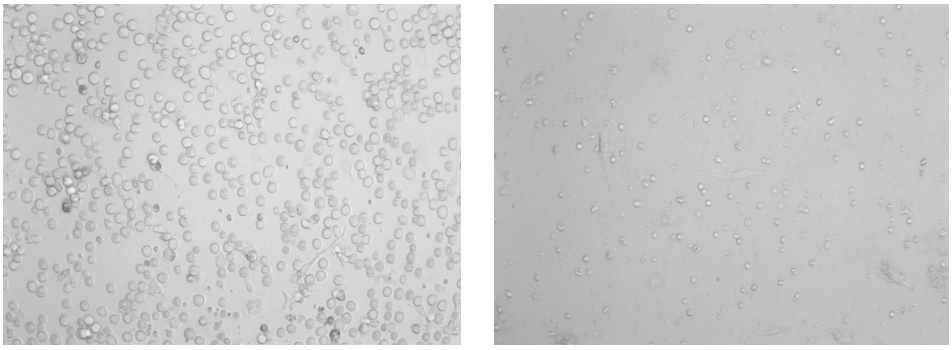
Tissue	Trial	%c-kit unseparated[1]	%c-kit enriched[2]	%c-kit depleted[3]
Bone Marrow	1*	20.38	81.84	5.34
	2	7.36	52.88	2.34
	3	8.06	63.31	2.28
Mean		11.93	66.01	3.32
Standard Deviation		7.32	14.67	1.75
Spleen	1	4.99	12.1	2.77
	2*	4.49	25.79	2.89
	3	8.79	22.04	4.67
Mean		6.09	19.98	3.44
Standard Deviation		2.35	7.07	1.06

[1] % c-kit unseparated = % c-kit$^+$ cells prior to separation over column

[2] % c-kit enriched = % c-kit$^+$ cells in enriched cultures

[3] % c-kit-depleted = % c-kit$^+$ cells in depleted cultures

* indicates those samples that showed multinucleate ball masses



A **B**

Figure 1. BM and spleen c-kit-enriched cultures.
One day after separation over a magnetic column, BM c-kit-enriched cultures show larger and more rounded cells (A) compared to spleen c-kit-enriched cultures (B).

Effects of 5-azacytidine treatment

Bone marrow

Regardless of exposure to 5-azacytidine, there were substantial morphological variations between the samples (Table 2). In only one of the three 5-azacytidine-exposed, c-kit -enriched BM samples were multinucleated fusion bodies visualized, that resembled those observed by Taylor and Jones (13) or Rangappa et al. (16). In this single culture, at one week there were several separate foci of fibroblast or triangle-like morphology. This growth continued through

Table 2. Relationship between exposure to 5-azacytidine and morphology

Source of Cells	c-kit	5-azacytidine	Morphology of Cells (3 samples in each group)
Bone Marrow	enriched	+	1 with fusion bodies, 2 with fibroblastic activity but no fusion bodies
	depleted	+	3 with fibroblastic activity but no fusion bodies
	enriched	-	3 with comparatively decreased fibroblastic activity; no fusion bodies
	depleted	-	3 with comparatively decreased fibroblastic activity; no fusion bodies
Spleen	enriched	+	1 with fusion bodies 1 with fibroblastic activity and no fusion bodies 1 stagnant with no growth
	depleted	+	3 stagnant with no growth
	enriched	-	3 stagnant with no growth
	depleted	-	with strong fibroblastic-like growth, but no fusion bodies 2 stagnant with no growth

week 2, with the joining of separate fibroblastic foci and the presence of multinucleated masses by day 23 (Figure 2A). The masses continued to grow in size until day 29, when they assumed a constant size without further change in morphology. These masses ranged from 1 to 9 per well, and were only present in foci with high fibroblastic activity. At no point over the 5 weeks of culture was either rhythmic or nonrhythmic beating observed in any of the structures.

The other two 5-azacytidine-exposed, c-kit-enriched BM samples showed similar fibroblastic activity through week 2, but there was no subsequent evidence of multinucleated masses. A growth pattern without multinucleated masses was consistently observed in each of the c-kit-depleted cultures exposed to 5-azacytidine in addition to both groups of cultures that were not exposed to 5-azacytidine at any time (Figure 2B). Growth in cultures not exposed to 5-azacytidine was relatively decreased, with some cultures showing foci of growth that did not join with neighboring colonies.

Spleen

In the spleen, there was considerably greater variation (Table 2). Multinucleated cell masses similar to those seen in the BM were visualized in only one culture that was enriched for c-kit and exposed to 5-azacytidine. The observed changes followed a similar time course to that in the corresponding BM culture (Figure 2C). Of the other two c-kit -enriched samples exposed to 5-azacytidine, however, fibroblastic activity was present in only one. The other culture remained static with cells fixed to the bottom of the well, but with no visible sign of cellular extensions (Figure 2D). This same growth was observed in the cultures that were exposed to 5-azacytidine and depleted of c-kit, as well as in each of the cultures that were neither

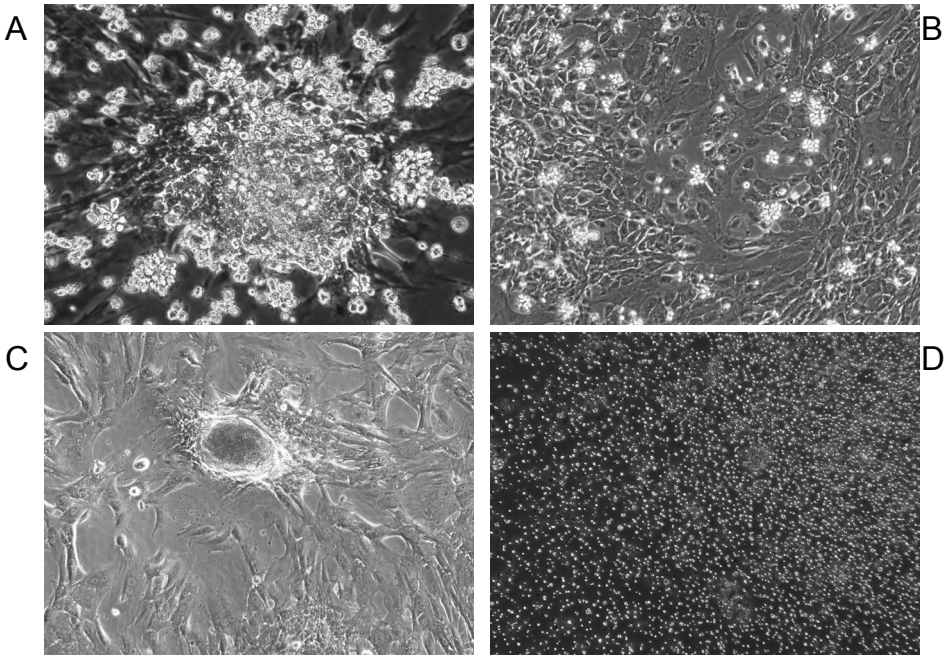


Figure 2. BM and spleen c-kit-enriched populations showed various morphologies at day 21 following exposure to 5-azacytidine.
(A) c-Kit enriched BM culture, exposed to 5-azacytidine, showing a multinucleate fusion body
(B) c-Kit enriched BM culture, exposed to 5-azacytidine, showing fibroblastic growth, but no fusion bodies
(C) c-Kit enriched spleen culture, exposed to 5-azacytidine, showing a multinucleate fusion body similar to that in (A)
(D) c-Kit enriched spleen culture, exposed to 5-azacytidine, showing stagnation, with no growth or fibroblastic activity

exposed nor enriched. In the c-kit-depleted populations that were not exposed to 5-azacytidine, strong fibroblastic activity was present in only one sample, which displayed the same clustered cell masses as in the c-kit -enriched population.

Western Blot analysis

Analysis by SDS-PAGE did not detect the presence of either cardiac troponin I or cardiac myosin heavy chain in any of the cultures generated, regardless of exposure to 5-azacytidine. In those single BM and spleen cultures that showed multinucleated cell masses, expression of these proteins was again negative. Control samples of freshly processed porcine cardiac tissue were used as positive controls for the myosin heavy chain and troponin antibodies; fresh splenic tissue was used as a negative control in each gel (Figure 3). Extended exposures of each blot were done in order to detect low-level expression of either myosin or troponin, but no signals were observed in any of the samples (data not shown).

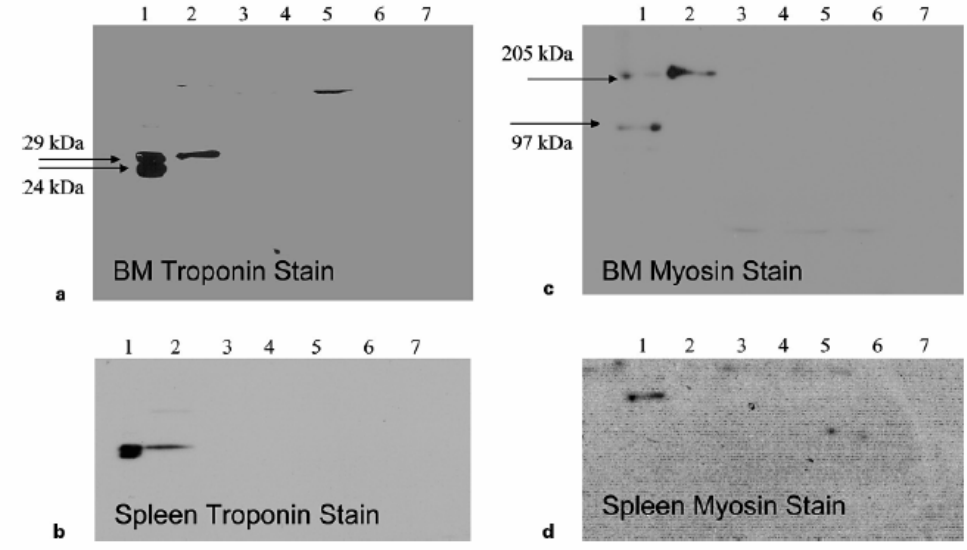


Figure 3. Absence of expression of troponin I or myosin in BM or spleen cultures on Western Blot.
BM and spleen stained for troponin (a , b); BM and spleen stained for myosin (c , d).
1 = Molecular marker; 2 = cardiomyocytes (control); 3 = BM c-kit enriched + 5-azacytidine, 4 = BM c-kit depleted + 5-azacytidine; 5 = spleen c-kit enriched, no 5-azacytidine; 6 = spleen c-kit depleted, no 5-azacytidine; 7 = homogenized spleen (control). Lanes 2 and 7 are positive and negative control, respectively.

DISCUSSION

The ability of 5-azacytidine to induce novel gene expression has been documented by several different groups over the last few decades (11-16). Although Constantinides et al. (11), were the first to describe the appearance of multinucleated striated muscle cells in non-myeloblast precursors following 5-azacytidine treatment, it was Makino et al. (19) who first described the appearance of cardiomyocytes from immortalized BM stromal cells in 1999. Because it was necessary for cell lines to be immortalized for this conversion, any clinical utility of this methodology remained questionable. However, soon after this, Tomita et al. (20) reported that MSC in a primary culture were also capable of achieving this phenotype, and Rangappa et al. (16) have recently reported that mesenchymal stem cells derived from fatty tissue in rats assume a rhythmic beat consistent with cardiac phenotype following exposure to 5-azacytidine. However, there is some conflict in the literature, as Liu et al. (21) have reported an inability to reproduce these results in rat primary BM stromal cell cultures. Their work suggested that, unless the cell line was immortalized, a cardiac phenotype was not achievable. The present study was an attempt to add further experience by assessing the utility of 5-azacytidine to influence the fate of porcine pluripotent HSC.

Based on our previous work, it was known that a small fraction of c-kit$^+$ cells that were also CD9$^-$, and therefore non-mobilized, were present in the spleen (1). The work of Le Guern et al. (2), which showed that c-kit$^+$ was a marker for HSC, and that this cell population displays early engraftment capacity, lead us to believe that these cells might be very early stem cells present in the spleen. Indeed, CAFC assays indicated that the earliest stem cells in the spleen were present at an equal frequency to those in the BM, raising the question of whether this population, or another splenic cell population, may have the same potential as the HSC and MSC described above. It was a central premise to our study whether enrichment for c-kit would improve the conversion of HSC to cardiac phenotype, if this were at all achievable.

Although CD34 is a widely-used marker for HSC in rodents and humans, there is not yet a commercially-available anti-CD34 antibody for pigs. However, c-kit$^+$ expression is known to have a strong correlation with stem cell activity, and can therefore reliably be used as a stem cell marker in pigs (1,2). By using both BM and spleen as sources of HSC, it has been possible to compare the results, not only between these two sources, but also between splenic HSC and the MSC described by others (16,19-21). If such an increase in efficacy had been seen, there would have been some argument for including c-kit as a possible marker for increased plasticity and potential conversion to cardiomyocytes. If c-kit expression were unrelated to phenotype conversion, there would have been similar frequency of cardiac conversion regardless of the enrichment status, provided that the cells were still exposed to 5-azacytidine. If this were the case, it was hypothesized that separation over a magnetic column would not affect the results. However, it should be noted, that we were unable to achieve the same efficacy in separation in the spleen as in the BM, despite the same methodology. This may result from the fact that, although spleen cells express c-kit, they do not express c-kit^{high} as do the HSC of the BM (1). This distinction may be one cause for the difference in purity of the cells obtained from the MACS columns. Although the effect of this variation remains uncertain, we believed that it was more important to maintain an identical, standardized separation protocol for both tissues. Thus, we included any cells expressing c-kit, whether c-kit^{high} or c-kit^{low}, in the c-kit+ group, and only those cells failing to express any c-kit at all in the c-kit- group.

Our results were consistent with the observations of Liu et al (21). Although we observed a similar fibroblastic growth pattern to Rangappa et al. (16), our cultured cells not only failed to beat, they also failed to express cardiac-specific proteins at any level. The gross appearance of the multinucleate structures in one BM and one spleen from separate animals was most consistent with the formation of chondrocyte structures first described by Taylor and Jones (13). As noted by Chacko et al. (22) and Benya et al. (23), however, the chondrocyte phenotype in culture is quite unstable and degrades with subculture. As a result, it was impossible to better analyze the composition of these structures. We do not exclude the possibility that the multinucleate masses we observed may have been foci of cell types other than cardiac

cells. In view of our interest in applying the successful approach of other scientists working with 5-azacytidine and cardiac transformation, our experiments focused only on determining the presence of cardiac-specific troponin and myosin. However, the infrequent and seemingly random appearance of the multinucleate structures in our work attests to the argument by Liu et al. (21) that genetic reprogramming by 5-azacytidine may be nonspecific. Because it is a cytosine analogue, and appears to work by altering gene methylation (24), it is more feasible to hypothesize a sequence of random gene activation than specific cardiac gene potentiation.

The basis for the discrepancy between our results and those of some others (11-13,14-16) is difficult to assess. Since we did not carry out a 'control' experiment in an attempt to confirm that, following exposure to 5-azacytidine, porcine MSC can differentiate into cardiomyocytes, or compare porcine HSC with their murine counterparts, the negative result we obtained could be associated with technical discrepancies or failures. For example, the particular culture conditions we employed may not have been appropriate for porcine cells. Furthermore, our lack of better 'control' experiments is explained by the considerably low cell yield associated with the purification and separation procedures; this afforded us only enough cells to carry out the 'experimental' segment of our protocol. Increasing the number of experiments would not have addressed this problem, as the issue was one of low cell yield following separation and not necessarily variation between the samples of each tissue examined. A final possibility is that, although we were not able to confirm troponin and myosin expression in these cells at the 5-week point, other cardiac proteins, such as Nk2.5 or alpha-actinin, may have been present at other time points during the experiment. As the scope of our study was limited by the commercial availability of porcine markers for these proteins, we were not able to invesitgate for their presence. As this was an initial study into the "plasticity" of splenic HSC, we restricted our Western blot analysis to those cardiac proteins described by others and did not attempt detection of proteins that may be expressed by bone, cartilage, or endothelial cells. These limitations can only be resolved by further investigations, possibly on a larger scale.

The goal of our work was not to definitively answer the question of HSC plasticity in a porcine model, but rather to stimulate new thinking with regard to an alternative source of adult stem cells for transdifferentiation studies, namely the adult spleen. One other important difference that may account for variability in results is the donor species. Although Tomita et al. (15) assessed porcine myocardial function following infarction and the subsequent infusion of 5-azacytidine-treated MSC, the majority of *in vitro* studies have been in rodent species. Pig BM and splenic cells may not have the same pluripotency or transforming capabilities as those in lower mammals. It is also possible that, in the pig and higher mammals, culture by the method we utilized may drive the cells into a state of senescence, and therefore less susceptible to the effects of 5-azacytidine. Additionally, if 5-azacytidine requires a certain state of cellular interaction, enrichment or depletion of c-kit+ stem cells may be detrimental to the

intercellular contact or cytokine-mediated microenvironment that may be necessary. The enrichment of c-kit+ cells by MACS may have resulted in the removal or destruction of critical cellular components through specific or non-specific binding to the column; however, the lack of correlation between fibroblastic growth states and individual c-kit concentrations suggests another mechanisms may be at work. Finally, only one concentration of 5-azacytidine (9μM) was tested, based upon previous reports that this concentration was the most effective at inducing cardiac phenotype (16). As our cells were plated at a higher density than that previously reported (16), the concentration of 5-azacytidine may not have been sufficient. While the absence of any clear cardiomyocyte phenotype is consistent with the failure to detect either myosin or troponin by Western Blot, it is also possible that this technique may not have been sensitive enough to distinguish expression of these proteins by a small subpopulation of cells.

Our study does not preclude the ability of BM and splenic cells to be transformed towards cardiac phenotype. It does, however, suggest that our methodology for the enrichment or depletion of c-kit+ stem cells does not efficiently affect this process.

CONCLUSIONS

Treatment with 5-azacytidine of cultures derived from the porcine BM and spleen led to an unpredictable pattern of growth. Regardless of exposure to 5-azacytidine, fibroblastic activity was present to varying extent among separate populations of c-kit$^+$ and c-kit$^-$ populations. Although there were some ball-like formations in both BM and spleen c-kit$^+$ populations that were similar to those observed by others, these cell clusters showed no sign of *in vitro* beating. Furthermore, neither cardiac troponin nor myosin heavy chain were detected by Western blot in cells derived from either source. These results suggest that, using the protocol we employed, MACS of c-kit+ stem cells and treatment with 5-azacytidine is not an efficient means of inducing cardiac gene expression in porcine HSC derived from either BM or spleen.

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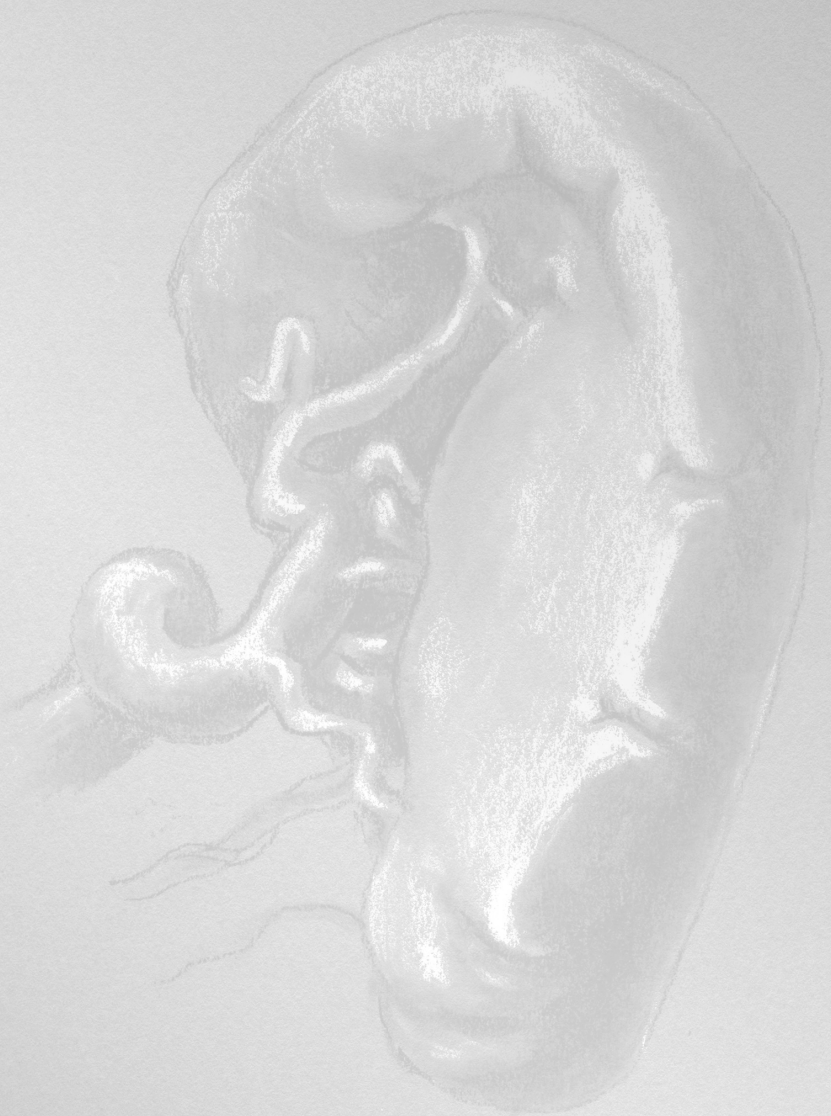
Pig Spleen Transplantation induces Transient Hematopoietic Cell Chimerism in Baboons



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Frank JMF Dor*, Yau-Lin Tseng*, Kenji Kuwaki, Dicken SC Ko, David KC Cooper. Pig spleen transplantation induces transient hematopoietic cell chimerism in baboons.
Xenotransplantation 2004;11:298-300.
*authors contributed equally

In several rodent models spleen allotransplantation has been shown to be associated with donor-specific tolerance to allo-antigens (1). To study the tolerogenic capacity of the spleen in a systematic manner in large animals with known MHC barriers, we have developed an allogeneic spleen transplantation (SpTx) model in MHC-defined miniature swine (2). One of the goals of investigating SpTx as a potential for tolerance induction was its extrapolation to xenoTx.

In our allogeneic SpTx experiments, we have observed that all recipients develop hematopoietic cell chimerism in the blood. Across full MHC barriers, when recipients were treated with 100cGy of whole body irradiation, 700cGy thymic irradiation, and a 45-day course of cyclosporine, multilineage chimerism was present in the blood and lymphoid tissues, and in some cases resulted in engraftment of donor cells in recipient bone marrow (BM). This was associated with *in vitro* donor-specific hyporesponsiveness in mixed leukocyte reaction (MLR) and cell-mediated lympholysis (CML) assays. Long-term survivors demonstrated evidence for donor-specific regulatory T cell activity, since peripheral blood mononuclear cells from these pigs contained a cell population that was able to suppress anti-donor responses of cells from naïve recipient-MHC-matched pigs in suppressor-CML and -MLR assays (Chapter 6).

We have performed xenogeneic pig SpTx in two baboons, in the first of which we established that the surgical technique used in our pig alloTx model was also feasible in the pig-to-baboon model. The second baboon (B221) also received donor-specific BM Tx 4 hours later. We here report our observations in B221 and compare them with those in a baboon (B219) that received BM Tx only (with no SpTx).

Both baboons were conditioned with a fairly complex regimen based on the pig-to-baboon BM Tx regimen reported previously (3,4). Both donor pigs were miniature swine bred by nuclear transfer/embryo transfer with greatly reduced expression of α1,3-galactosyltransferase RNA, associated with reduced levels of Galα1-3Gal (Gal) when compared with wild-type swine; some Gal was detectable by flow cytometry on hematopoietic cells. No measures were taken to deplete natural anti-Gal antibodies, but complement depletion was performed using cobra venom factor, which was administered for the first 21 days.

SpTx was carried out in B221 in the abdomen using the previously-described technique (2). BM was harvested from the donor pigs and prepared for infusion into the baboon as previously described (5). BM cells (1.33 and 0.8 x 10^9/kg, respectively, in B221 and B219) were infused intravenously without untoward effect (in B221 four hours after SpTx). Blood was drawn at intervals to determine the extent of hematopoietic cell chimerism by flow cytometry.

The maximum percentage of pig cells (using a 'pan-pig' antibody) in the blood of B221 was 16.8% (2 hours after SpTx), but only 1.9 % in B219 (on day 2) (Figure 1), and the duration of

pig cell macrochimerism was 12 days in B221 compared to only 5 days in B219. In B221, the absolute number of pig cells was 689/μL after SpTx and 876/μL after BM Tx (Figure 2) (2 hours after SpTx and BM Tx, respectively); in contrast, in B219 the peak absolute number of pig cells 2 hours after the BM Tx was 85/μL (Figure 2). In both cases, loss of chimerism was most likely related to phagocytosis of pig cells by host macrophages (3,6).

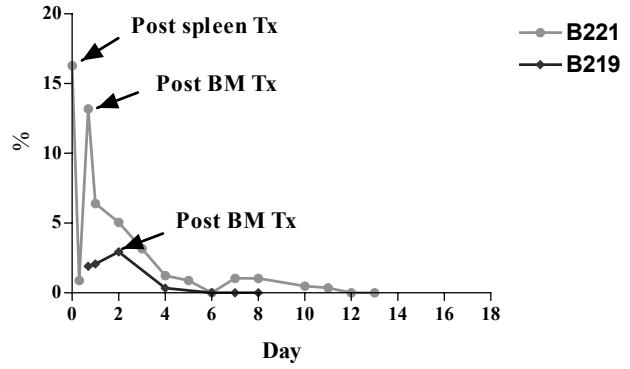

Figure 1.

Percentage and duration of pig cell macrochimerism (detected by flow cytometry) in B221 that underwent pig SpTx followed 4 hours later by donor-specific BM Tx, and in B219, that underwent pig BM Tx only.

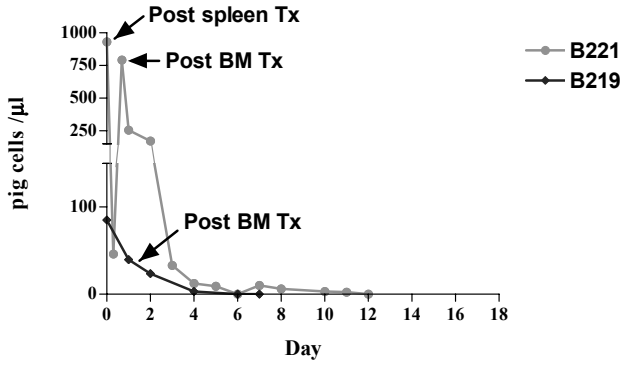

Figure 2.

Absolute number of pig cells detected in the recipient's blood following pig spleen + BM Tx (B221) or BM Tx alone (B219).

In B221, two hours after SpTx, multilineage chimerism was present (Figure 3A), but two hours later (before BM Tx) no pig cells were detectable in the blood. Two hours after BM Tx these percentages were similar (Figure 3B). The phenotypes of the cells released by the spleen graft were different from those detected after BM Tx; after SpTx, more pig lymphoid cells in the T

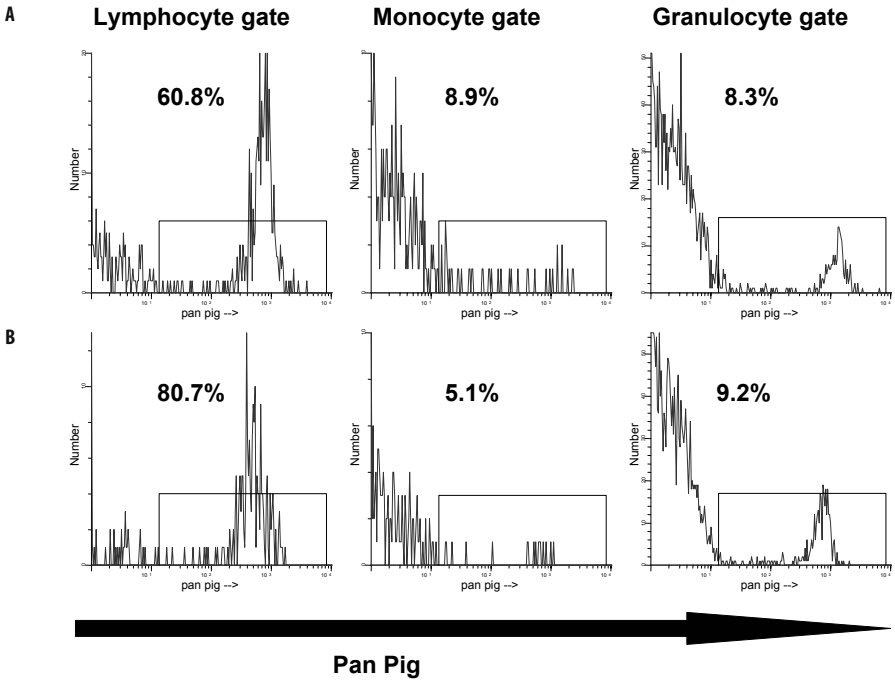

Figure 3.

Multilineage pig cell chimerism detected by flow cytometry in the blood of B221 two hours after initial SpTx (A) and after subsequent BM Tx (B).

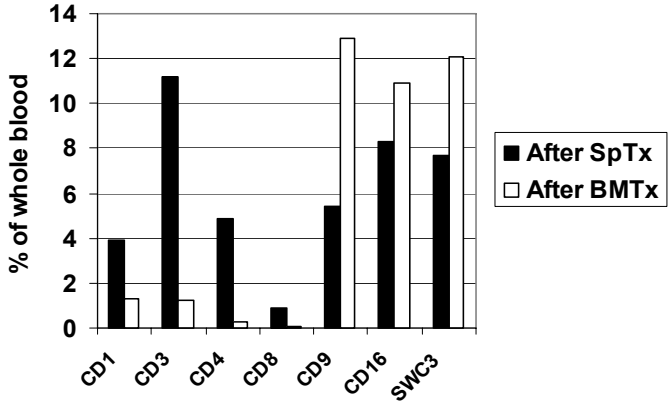

Figure 4.

Phenotypes of pig cells after SpTx (black bars) and BMTx (white bars). Following SpTx, more pig lymphoid cells in the T cell lineage (expressing either $CD1^+$, $CD3^+$, $CD4^+$, or $CD8^+$) could be detected. After BMTx, there was a higher number of myeloid ($CD16^+$, $SWC3^+$) cells. SWC3 is a marker for the porcine myeloid lineage.

cell lineage (expressing either $CD1^+$, $CD3^+$, $CD4^+$, or $CD8^+$) could be detected than after BM Tx, when there was a higher number of myeloid ($CD16^+$, $SWC3^+$) cells (Figure 4). (Pig SWC3 expression resembles that of Mac-1 and Gr-1 in mice, and represents a marker for the myeloid lineage (7)).

Biopsy of the spleen graft was performed on day 18, when it was found to be rejected (8), which was most likely due to anti-Gal antibody-mediated injury (since the histologic picture resembled antibody-mediated rejection but no anti-nonGal antibody could be detected in the blood).

To our knowledge, this is the first report of SpTx in a discordant pig-to-baboon xenoTx model. As in allogeneic SpTx, multilineage hematopoietic cell chimerism could be detected early after reperfusion of the spleen graft. The level of chimerism was comparable to that seen after the subsequent BM Tx. The level and duration of chimerism were greater when the spleen was transplanted in addition to the BM. The cells released from the spleen were different in phenotype from those after BM Tx, which correlated well with the phenotypes of hematopoietic stem cells identified in naïve spleens and BM (9). A relatively large population of $CD3^+$ T cells was detected after SpTx, but no features of graft-versus-host disease were observed during follow-up, an observation that correlates well with that of Eguchi et al. in the pig-to-mouse model (10).

The adult pig spleen is a relatively rich source of hematopoietic progenitor cells (9). In an allogeneic setting, the transplanted spleen releases progenitor cells into the recipient's blood, from which they reach the lymphoid tissues (lymph nodes, thymus) and engraft in the BM.
Based on previous studies in the pig-to-baboon model using either cytokine-mobilized peripheral blood progenitor cell Tx from Gal-positive miniature swine (3) or BM from α1,3-galactosyltransferase gene-knockout miniature swine (4), we conclude that there is a need for a proper microenvironment for pig cell engraftment in the baboon. SpTx from the BM donor potentially provides an additional source of stem cells but also hematopoietic stroma that may facilitate seeding of pig BM cells and support hematopoiesis. Further studies of combined spleen and BM Tx, using α1,3-galactosyltransferase gene-knockout pigs, would seem worthwhile.

ACKNOWLEDGEMENTS

We thank Henk-Jan Schuurman PhD and David H. Sachs MD for helpful comments on the presentation of this experience.

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Post-Transplant Lymphoproliferative Disease after Allogeneic Transplantation of the Spleen in Miniature Swine

9

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ABSTRACT

Background: Post-transplant lymphoproliferative disease (PTLD) is an important complication of immunosuppression after transplantation (Tx). We have performed spleen transplantation (SpTx) in miniature swine across full major histocompatibility complex barriers in order to study the tolerogenic effect of the spleen. We describe the development of PTLD after allogeneic SpTx.

Methods: Recipient pigs were immunosuppressed with whole body irradiation (100cGy), thymic irradiation (700cGy), and cyclosporine given intravenously daily to maintain a trough level of 400-800ng/mL for 45 days. Native splenectomy and SpTx were performed on day 0. Two control pigs received the immunosuppressive regimen and underwent native splenectomy, but did not undergo SpTx.

Results: After SpTx, 2 of 7 pigs developed PTLD (29%) on days 30 and 32, respectively. One pig developed a malignant proliferation of host-type B-lymphocytes, whereas the other had proliferation of donor-type B-lymphocytes. The 2 pigs that developed PTLD had significantly greater T cell depletion on the day of SpTx with higher trough levels of cyclosporine in the first week after transplantation than similarly treated pigs that did not develop PTLD and control animals. Early changes associated with PTLD included increased PLHV-1 viral loads in blood and tissues, increased numbers of leukocytes, B-(CD3-CD16-) cells, and total serum IgM. These changes occurred prior to the development of clinical features of PTLD.

Conclusions: PTLD can occur after allogeneic SpTx in swine. This model may provide a useful system for studies of the pathogenesis of PTLD.

INTRODUCTION

One of the major complications in patients with organ transplants receiving immunosuppressive therapy is an increased risk of malignant disease, particularly lymphoid tumors. Post-transplant lymphoproliferative disease (PTLD) comprises a heterogeneous group of lymphoid proliferations, usually of B-cell origin, that are associated with ineffective T cell function and exogenous immunosuppression. A strong correlation has been reported between B-cell neoplasms developing in immunosuppressed patients and the presence of the B-lymphotropic gammaherpesvirus, Epstein-Barr virus (1).

In miniature swine, PTLD has been described most commonly after allogeneic hematopoietic cell transplantation (Tx) (2), but has also been reported after liver Tx (3) and kidney Tx (Huang CA, unpublished data). Because of the clinical importance of PTLD, our laboratory has characterized this complication in more detail in the pig model (2,4). As in humans, a lymphotropic gammaherpesvirus, porcine lymphotropic herpesvirus-1 (PLHV-1), has been found

to play a major role in the pathogenesis of PTLD (2,4,5). Risk factors for PTLD identified after hematopoietic cell transplantation include T-cell depletion, the degree of histocompatibility mismatch, and the intensity of PLHV-1 infection (4).
SpTx has been performed in miniature swine to examine the ability of the spleen to induce a state of tolerance (6, 7). We describe for the first time the occurrence of PTLD in miniature swine after SpTx across a full major histocompatibility complex (MHC) barrier.

MATERIALS AND METHODS

Animals

Transplant donors and recipients were selected from our herd of partially-inbred, MHC-defined miniature swine (8). Donors (n=7, 3.4±1.2 months of age, weighing 22.7±9.6 kg), and recipients (n=7, 3.0±0.9 months, weighing 18.9±8.8 kg) were fully MHC-mismatched. To be able to study hematopoietic cell chimerism, donor and recipient pairs were also mismatched for the pig allelic antigen (PAA), a non-MHC cell-surface marker of no known function (9), donors being PAA+ and recipients PAA-.

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

Spleen transplantation

The technique of heterotopic SpTx in miniature swine has been described in detail previously (7).

Recipient pretransplant conditioning and immunosuppressive treatment

The recipients received a low dose of whole body irradiation (WBI; 100 cGy on day -2), which is non-myeloablative (Chapter 6), and thymic irradiation (700 cGy on day -1) from a cobalt irradiator (10). Cyclosporine (CyA; Sandimmune, generously provided by Novartis Pharmaceutical Corporation, East Hanover, NJ) was administered intravenously (iv) as a single daily infusion at a dose of 10-30 mg/kg (adjusted to maintain a whole blood trough level of 400-800 ng/mL) for 45 days, beginning on the day of SpTx (day 0), through an indwelling catheter in the neck. CyA levels were determined by a fluorescence polarization immunoassay (Abbott Laboratories, Dallas, TX), which measured the parent compound, but not metabolites.

Control experiments

As controls, two pigs (#15312, #15330) underwent the identical immunosuppressive regimen, but no spleen was transplanted.

Supportive therapy and monitoring

Supportive therapy consisted of daily prophylactic enrofloxacin and dalteparin (6). Blood cell count, serum chemistry, and CyA levels were determined daily. Monitoring for graft-versus-host-disease included daily inspection of the skin and stools, and daily determination of liver enzymes. In case of a skin rash, punch biopsies were taken from affected and non-affected skin for histological examination. Serum lactate dehydrogenase (LDH) was followed routinely as a potential marker of spleen graft rejection.

Flow cytometry

Flow cytometry was performed twice weekly to monitor the level of hematopoietic cell chimerism. T cell depletion was assessed by flow cytometry before the immunosuppressive regimen was initiated on day-3 and on the day of the SpTx (day 0), and the number of circulating CD3+ T cells in the recipient's blood was subsequently determined at frequent intervals. In one case (#15446), B-cell proliferation in the blood was monitored, and flow cytometry was used to determine the phenotype of the cells involved in the PTLD. Monoclonal antibodies used included: Anti-CD1 (76-7-4, mouse IgG2aK) (11), anti-CD3 (898H2-6-15, mouse IgG2aK) (12), anti-CD4 (74-12-4, mouse IgG2bK), anti-CD5 (9G12 (BB6-9G12), mouse IgG1) (13), anti-CD8α (76-2-11, mouse IgG2aK), anti-CD9 (1038H-4-6, mouse IgM/K), anti-CD16 (G7, mouse IgG1), anti-PAA (1038H-10-9, mouse IgMK) (9), anti-Igμ (5C9, IgG1/K), and anti-Igκ (K139.3E1, mouse IgG2a). Staining of blood and tissue cell suspensions was achieved as previously described (14). Data were analyzed using WinList mode analysis software (Verity Software House, Topsham, ME).

Enzyme-linked immunosorbent assay (ELISA) to assess level of total IgM in spleen transplantation recipients

Ninety-six-well flat-bottomed Nunc maxisorp ELISA plates (Nalge-Nunc International, Rochester, NY) were coated with 5 μg/mL of purified porcine IgM (PPIgM) (Rockland, Gillbertsville, PA) in carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. Plates were washed three times with PBS containing 0.1% Tween 20 (Sigma-Aldrich). Non-specific binding sites were subsequently blocked with 1% bovine serum albumin (BSA; Fisher Scientific, Hampton, NH) in PBS for one hour at room temperature. Serially-diluted porcine IgM

standards (100-0.14µg/mL) were included on each plate. The test sera were serially diluted such that at least two points from each could be extrapolated to the linear part of the standard curve. Both the standards and the serum samples were tested in triplicate. To initiate the competition, the IgM standards and the diluted sera were incubated for one hour at room temperature with HRP-conjugated goat anti-porcine IgM (Serotec Oxford, UK) in an uncoated ELISA plate that had been blocked as previously described. Both the standards and the serum samples were tested in triplicate. The competition reaction was then transferred to the blocked, porcine IgM-coated plate and incubated for 60 min at room temperature. The plates were developed using O-phenylenediamine (Sigma-Aldrich) at 0.9mg/mL in phosphate-citrate buffer with urea hydrogen peroxide (Sigma-Aldrich) for 20 min at room temperature. The reaction was stopped with the addition of 2N sulfuric acid (Fisher Scientitfic). Plates were read on a microplate reader (Molecular Devices, Menlo, Calif.) at 490 nm.

Polymerase chain reaction (PCR) to identify porcine lymphotropic herpesvirus-1 (PLHV-1)

DNA extraction

Blood samples were taken before SpTx and weekly thereafter. Peripheral blood mononuclear cells were isolated from blood by density gradient sedimentation using histopaque-1077 (Sigma-Aldrich, St. Louis, MO). Tissue samples were snap frozen at the time of biopsy or necropsy. DNA was isolated using the Purgene DNA Isolation Kit (Purgene, Minneapolis, MN). Tissue DNA was quantified by the Hoechst dye fluorescence assay on a DNA fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Quantitative real-time PCR

Target DNA sequences were quantified by real-time PCR using the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA), as described previously (15). In brief, sequence-specific primers and probes were generated for each gene target using Primer Express software (Perkin-Elmer). PCR conditions were identical for all assays. Each PCR reaction mix consisted of target DNA, 900 nM primers, 200 nM probe, TaqMan Universal PCR 2x MasterMix (Applied Biosystems, Branchburg, NJ) containing the passive reference dye ROX (6-carboxy-x-rhodamine) in a 50µL final reaction volume loaded into a 96-well plate. The PCR conditions were as follows: an initial cycle at 50°C for 2 min, 95°C for 10 min, 50 cycles of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. Under these conditions, target DNA was detected with a linear dynamic range from 10^0 to 10^6 copies (data not shown). The threshold cycle or C_t values of PCR amplification of the standards were used to generate a standard curve for quantification of target DNA. Samples from which reproducible amplification signals were detected at levels below the lowest standard quantification limit are referred to as +/- or "below 6 copies", with 6 being the lower limit of quantitative detection.

Measurement of porcine lymphotropic herpesvirus type-1 (PLHV-1) DNA
Primers and probe for PLHV-1 were derived from the PLHV-1 polymerase gene and were as follows: PLHV-1 sense: 5-AAG GTG ACA TGC AAT GCT GTG-3; antisense: 5-TGC AAT CTT GAG ACA GGG CA-3; probe: 5′ 6FAM-TGG GTT CAC TGG TGT TGC ATC TGG TAT G-TAMRA 3′ (16-18). Reverse primer and probe were specific for PLHV-1 by blast analysis, while the forward primer was able to detect PLHV-1 and 2. Sequencing of the product amplified by the conventional primers confirmed the specificity for PLHV-1. No cross-reactivity was detectable with porcine cytomegalovirus in *in vivo* tissues samples and in DNA preparations derived from *in vitro* cytomegalovirus-infected cell cultures, both with known high copy numbers of cytomegalovirus. The quantitative detection limit for this assay was 6 copies per reaction. Results were expressed as copy number of PLHV-1 per 10^4 pig MHC class I molecules or 5 x 10^3 cell equivalents.

Measurement of species-specific porcine DNA
Pig MHC class I gene (pig MHCC1) primers and probe were developed as internal controls for porcine cellular DNA (15, 19). Primers and probe were derived from the pig MHCC1 gene and were as follows: MHCC1 sense: 5′-GCC CTG GGC TTC TAC CCT AA-3′; antisense: 5′-TCT CAG GGT GAG TGG CTC CT-3′; probe: 5′ 6FAM-CCA GGA CCA GAG CCA GGA CAT GGA GCT CGT-TAMRA 3′. Quantitative sensitivity was 3 copies per reaction. In the peripheral blood mononuclear cell samples, the number of cells was quantified as one cell per two copies of MHCC1. PLHV-1 copy numbers were calculated per 5 x 10^3 mononuclear cells. In tissues, MHCC1 served as internal control for the input total DNA amount.

Microscopic examination of tissues

Tissue samples were fixed in 10% formalin and subsequently embedded in paraffin, or frozen and stored at -80°C. Five-micron histologic sections were cut from paraffin blocks, stained with hematoxylin and eosin and studied by light microscopy.

RESULTS

Hematopoietic cell chimerism

All recipients of SpTx developed multilineage mixed hematopoietic cell chimerism after reperfusion of the spleen graft, whereas the control pigs had only very low and transient chimerism, mostly restricted to CD3+ T cells, resulting from blood transfusions during the splenectomy procedure. Details of hematopoietic cell chimerism following SpTx have been reported in Chapter 6. There was no significant difference in initial levels of chimerism in pigs without and with PTLD.

Incidence of PTLD

Of the 7 SpTx recipients treated with the same immunosuppressive regimen, 2 (29%) pigs developed PTLD; in one (#15399), the PTLD developed from native (recipient) B-cells and, in the other (#15446), from donor B-cells. PTLD was not observed in the two control pigs that received the identical immunosuppressive regimen without SpTx.

Clinical course of pigs with PTLD

Laboratory abnormalities associated with PTLD were detected before clinical features of disease became apparent in two pigs (#15399 and #15446) that developed PTLD (days 30 and 32), (Table 1). An increased viral load of PLHV-1 in the blood was detected by 14 days after transplantation, followed by B-cell proliferation (by flow cytometry day 28) in the blood and on routine LN biopsy. The pig with recipient-type PTLD (#15399) demonstrated a decline in lymphoid chimerism, while the pig with donor-type B-cell proliferation (#15446) had increased lymphoid chimerism. These changes in lymphoid chimerism preceded increases in WBC, which began 29-31 days after SpTx (Figure 1A). There were no signs of infection and surveillance blood cultures remained negative.

Table 1. Time-course of events in the development of PTLD in two pigs with spleen allografts

Day	Pig #15399	Pig #15446
7		
14	PLHV-1 upregulation in blood	PLHV-1 upregulation in blood
21		
27	Increase in total serum IgM	B-cell proliferation in blood
28	PLHV-1 upregulation in LN	B-cell proliferation in LN
	atypical lymphoid proliferation LN	
29	Increase in WBC count	PLHV-1 upregulation in LN
30	**Clinical features detected; CyA stopped**	Increase in WBC count
32	Palpable LN	**Clinical features detected; CyA stopped**
		Increase in total serum IgM
34		Palpable LN
35	Euthanized for respiratory difficulty	
37		Atypical lymphoid proliferation LN
		Euthanized for respiratory difficulty

Increased levels of serum total IgM were detected before or accompanying clinical signs of PTLD. The increase in total serum IgM levels in both animals coincided with B-cell expansion. In #15399, the maximum IgM level (57 mg/mL) was more than 17-fold higher than the upper

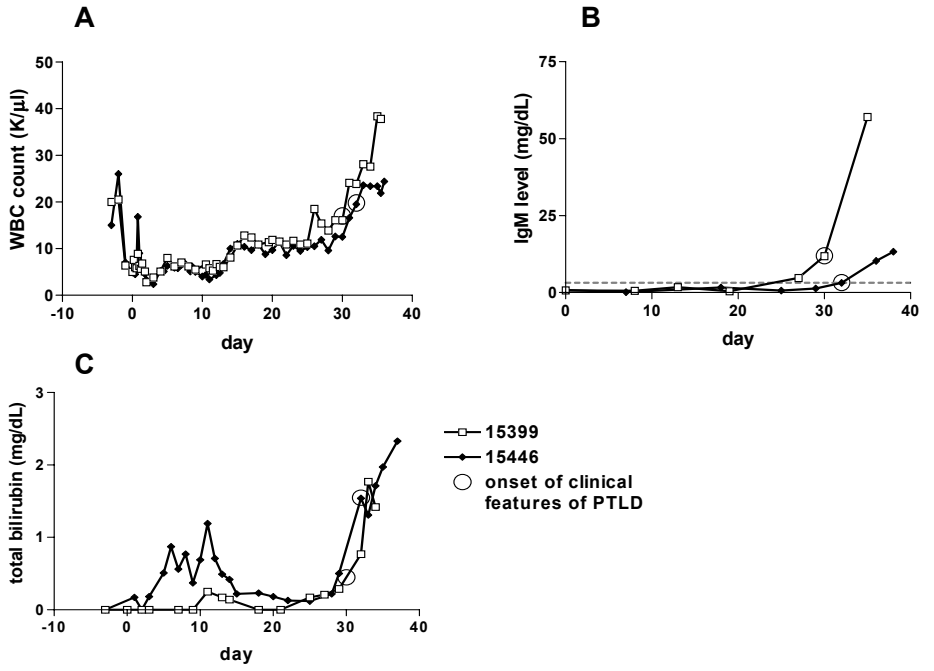


Figure 1.

In this figure, the open boxes indicate pig #15399, the closed diamonds pig #15446. The circle represents the day that clinical features became apparent. (A) White blood cell counts of the two pigs with PTLD. Both pigs had significant increases in leukocytes that began just prior to the recognition of clinical features of PTLD. (B) Serum total IgM levels in the 2 pigs that developed PTLD. The upper limit of normal in pigs is 3.2 mg/mL, represented by the dashed line. The increase in serum IgM preceded clinical features in pig #15399 by 3 days. (C) Serum total bilirubin levels in the two pigs with PTLD. Both pigs demonstrated intrahepatic cholestasis due to liver involvement of PTLD, which was confirmed by histological examination (Figure 5). In #15446, the increase in bilirubin began before clinical features were obvious.

limit of the normal level (0.2-3.2 mg/mL, Bajwa JA et al, unpublished data), and in #15446, IgM was 13.3 mg/mL, more than 4-fold higher than the upper normal limit (Figure 1B).

Clinical features of systemic illness included loss of appetite, lethargy, and weight loss in both affected animals. Within two days, enlarged lymph nodes could be easily palpated, those in the pre-femoral region being most accessible and the easiest to examine. Although CyA treatment was discontinued when suspicion of PTLD arose, in both cases the disease progressed rapidly to respiratory failure associated with obstructive enlargement of the tonsils and compression of the trachea by enlarged lymph nodes in the neck and mediastinum, necessitating euthanasia of both animals.

Both pigs demonstrated significant rises in total bilirubin and lactate dehydrogenase (LDH) in the serum, concomitant with the onset of clinical disease. The LDH levels at the time of clinical

onset were 1469 U/L and 1137 U/L (2.9 and 2.3-fold higher than normal level). Total bilirubin levels were 0.5 and 1.54 mg/dL at the time of clinical appearance of PTLD rising to 1.42 and 2.33 mg/dL by the day of sacrifice (figure 1C).

At necropsy, both animals had massive enlargement of the lymph nodes, including the tonsils (with advanced necrosis in one case) and the lymph nodes of the abdomen (Figure 2A), mediastinum, neck, and groin. The spleen grafts (Figure 2B) were also involved in the lymphoid proliferation, particularly in the pig with the recipient-type PTLD (#15399) that also had two distinct solid hepatic tumors, each with a diameter of 1cm.

T cell depletion

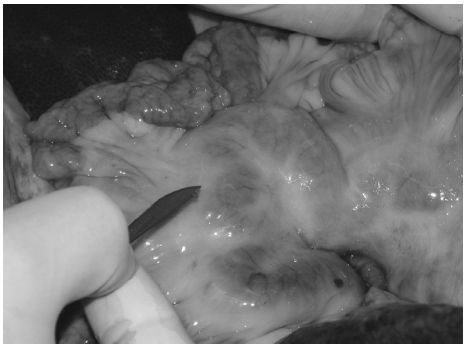
Although T cells were not depleted specifically, following whole body and thymic irradiation, all 9 pigs showed a significant decrease in the absolute number of T cells. The two pigs that went on to develop PTLD had significantly greater T cell depletion (90.6%) than unaffected animals (70.6%) ($p=0.019$) (data not shown). Recovery of T-cells was similar in both affected and unaffected animals with the absolute T cell counts increasing over 14 days to >1500/mm^3 (data not shown).

Cyclosporine dosage and levels

Despite adjusting of the CyA dosage on a daily basis, the two pigs that developed PTLD had significantly higher CyA levels in the first week after SpTx ($p=0.001$) (Figure 2C). CyA levels also increased, despite a reduction in dosage or cessation of therapy, when the clinical features of PTLD developed**.** This resulted in overall higher CyA trough levels in the pigs with PTLD over the course of the experiment ($p<0.001$) (Figure 2D). Renal function remained normal in both pigs.

Flow cytometry

In the animal that developed donor-type PTLD (#15446), B-cell proliferation in the blood began on day 27 before any clinical features of PTLD were obvious (day 32) (Figure 2E). Figure 3 shows flow cytometry from lymphoid tissues taken on day 28 (day of spleen and lymph node (LN) biopsies) and day 37 (day of euthanasia) in #15446. On day 28, 40% of LN cells were donor B-cells (CD3-CD16-PAA+), but recipient CD3-CD16-PAA- B-cells were still present (32 %) (Figure 3A). By the day of euthanasia (day 37), donor B-cells made up almost 75% of the total cellular LN content. In the BM (Figure 3B), engraftment of donor (PAA+) cells was observed on day 28, with approximately 2% being donor B-cells. However, by day 37, almost 54% of BM cells were of donor origin, of which 66% were B-cells (of note, in a normal PAA+ pig, only 50-



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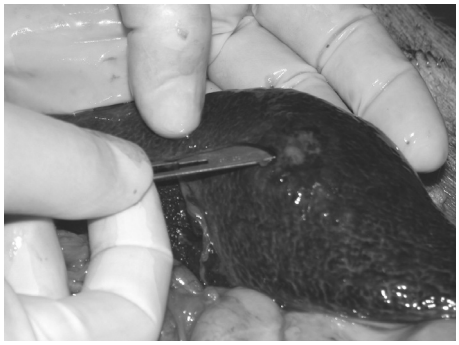

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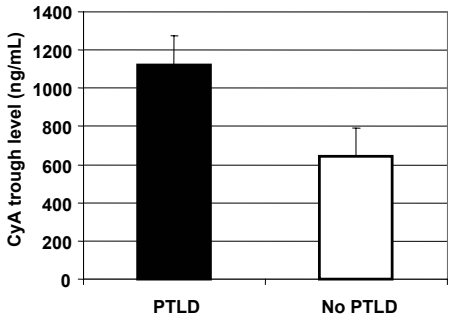

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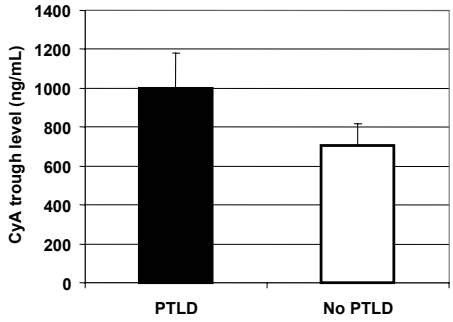

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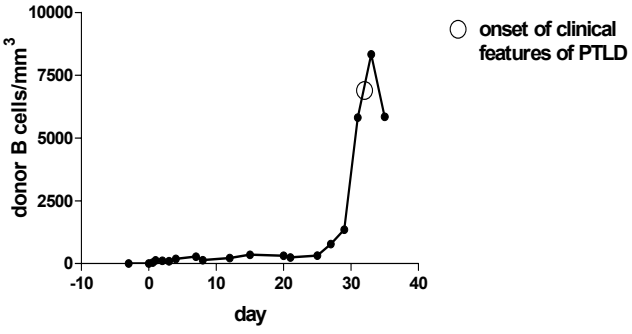

E

Figure 2.

(A) Macroscopic appearance of mesenteric lymph nodes in pig #15399 at necropsy (day 35). (B) Spleen graft of pig #15399 on day 35 (necropsy) with a distinct lymphomatous nodule. (C) Mean CyA trough levels were significantly higher in the first week after spleen Tx in the 2 pigs that developed PTLD compared with the 7 pigs that did not ($p=0.001$), even though CyA dosages were adjusted on the basis of the daily CyA trough levels. (D) Similarly, pigs that developed PTLD had significantly higher CyA trough levels during the entire course of therapy than the pigs that did not develop PTLD ($p<0.001$). This resulted partly from the fact that CyA trough levels increased despite reduction in dosage or even complete withdrawal when PTLD became clinically apparent. (E) Absolute numbers of donor B-cells (CD3-CD16-PAA+) in the blood of the recipient that developed donor-type PTLD (#15446). The expansion of the donor B-cell pool in the recipient blood was clear before clinical features of PTLD developed. (PAA+ is a cell surface marker of no known function, expressed on all donor cells).

60% of BM cells express PAA (9)). The spleen graft (of PAA+ origin) had a high B-cell content on day 28 (almost 50%), and a PAA+ B-cell content of 63% on day 37 (Figure 3C).

Phenotyping of PTLD cells

Previous studies confirmed the CD3-CD16- peripheral blood cells to be B-cells based on surface and cytoplasmic Igμ and Igκ staining (2).

In the current series, CD3-CD16- PTLD cells in the blood did not express any of the porcine B-cell markers CD1, Igμ and Igκ. However, in LN and bone marrow samples, low levels of expression of Igμ chains, but not Igκ and CD1 could be seen in the clonally-expanded B-cell pool (not shown).

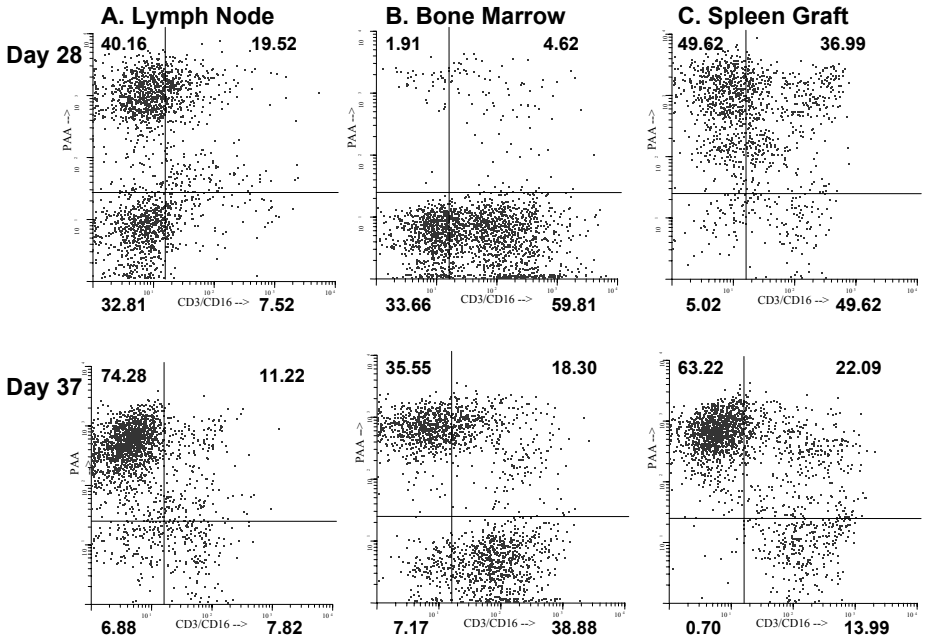

Figure 3. Flow cytometry of lymphoid tissues on day 28 and day 37 (necropsy) from pig #15446 that developed donor-type PTLD.

(A) In the LN (top), a distinct population of donor B-cells (CD3-CD16-PAA+) could be seen on day 29, making up 40% of the total cells. At necropsy (day 37), this percentage was increased to almost 75% (bottom).

(B) The bone marrow biopsy on day 29 (top) revealed the presence of some donor cells (~6.5%), but only approximately 2% donor B-cells, which was not uncommon after SpTx. However, on the day of necropsy (day 37) (bottom), there was an unusually large number of donor B-cells, making up 35% of the total.

(C) The spleen graft (which was from a PAA+ animal) contained a large number of donor B-cells on day 29 (top). This number increased and made up almost 2/3 of the total spleen cells at the time of necropsy (day 37) (bottom).

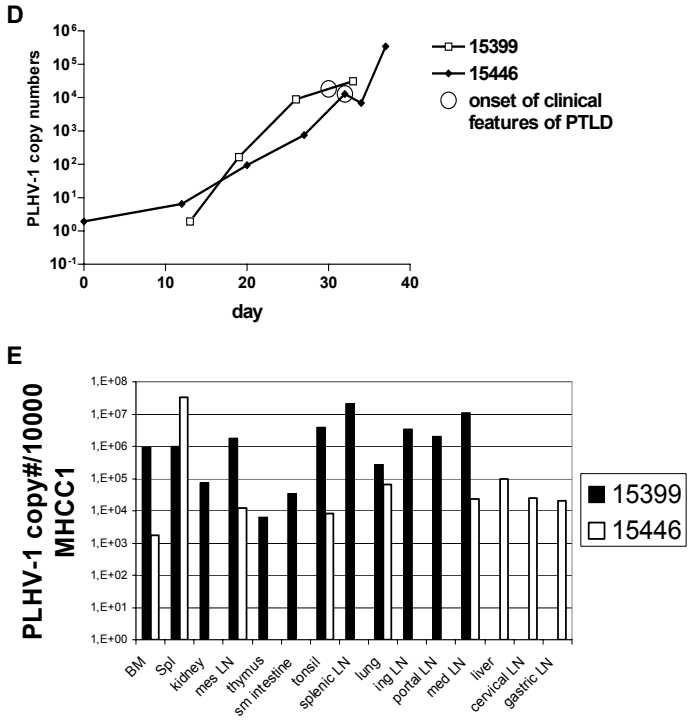

Figure 3.

(D) Copy numbers of PLHV-1 in recipient blood after SpTx in two pigs with PTLD. Approximately two weeks before the onset of clinical features of PTLD, both animals demonstrated significant copy numbers of PLHV-1.

(E) Tissues taken at necropsy revealed very high PLHV-1 copy numbers per 10,000 MHC-Class I copy numbers.

PLHV-1 activation in blood and tissues

Both pigs with PTLD had a significant increase in copy numbers of viral DNA in the blood, beginning 2 weeks after SpTx, 2 weeks before the onset of clinical signs of PTLD (Figure 3D). In addition, biopsies from LN from both animals also revealed high copy numbers of PLHV-1 DNA (not shown). All tissues obtained at necropsy revealed high PLHV-1 DNA content (Figure 3E). In the pig with the donor-type PTLD (#15446), the spleen graft had high copy numbers of PLHV-1. The control pigs and the 5 other SpTx recipients did not develop an increase in PLHV-1 viral load in the blood or in tissue samples taken at biopsy and necropsy.

Histopathology

In pig #15399, histological examination of protocol biopsies of mesenteric LN and spleen graft revealed features of PTLD on day 28 (two days before clinical features were detected). In the

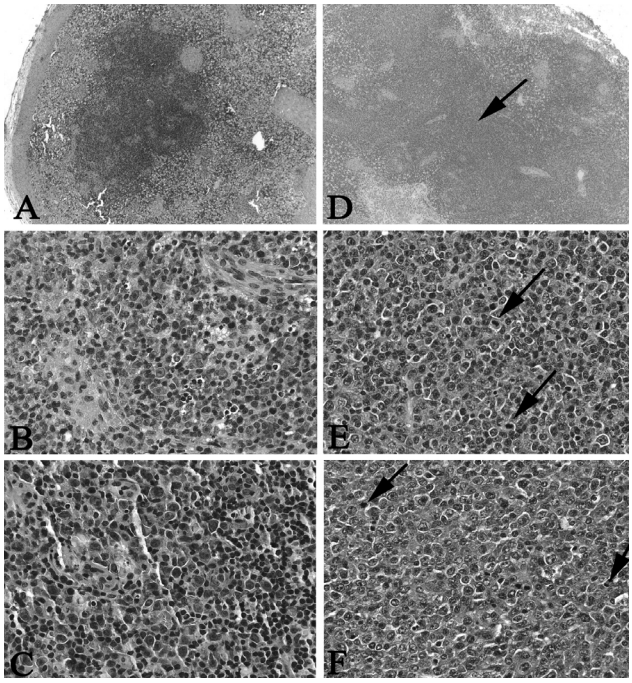

Figure 4. Histologic changes in the morphology of spleen and LN in pig #15399 on post-operative days 29 (A-C) and 35 (D-F).
A moderate increase in size of a Malpighian corpuscle in the spleen (A), H&E x50, was associated with mild cellular atypia (B), H&E, x400. A mesenteric LN showed mild cellular atypia (C), H&E, x400. The spleen later showed a marked increase in size of a Malpighian corpuscle (D), H&E, x50 (arrow), associated with marked cellular atypia and mitotic activity, (E), H&E x400 (arrows point to mitoses). A LN showed marked cellular atypia and mitotic activity (arrows point to mitoses), (F), H&E x400.

spleen, white pulp expansion (Figure 4A) with some cellular atypia was observed, although few mitotic figures were seen (Figure 4B). The red pulp had an abundance of lymphocytes. The mesenteric LN taken on the same day contained atypical lymphocytes and plasma cells (Figure 4C). Tingible body macrophages marked a high degree of cell turnover. At necropsy on day 35, the above findings were more pronounced in both tissues; in the spleen graft, the white pulp expansion was massive (Figure 4D), with sheets of atypical cells (Figure 4E). Many mitoses were noted. Few normal lymphocytes were found. In the LN (Figure 4F), the atypia and mitotic activity were pronounced as well. The liver, which contained macroscopically distinct tumors, had evidence of PTLD histologically (Figure 5A). The tonsil, that was macroscopically affected, demonstrated sheets of atypical cells and necrosis (not shown). The bone marrow was clearly involved (Figure 5B), and malignant cells filled most of the space of the hematopoietic microenvironment.

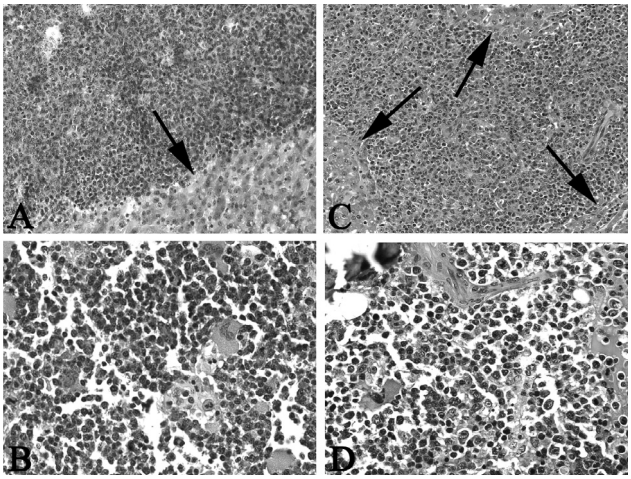

Figure 5. Tumor involved extra-splenic and extra-nodal tissues in both animals on post-operative day 35.
In pig #15399, tumor infiltrated both liver (A), H&E, x100 (arrow points to discreet margin of tumor and normal liver), and bone marrow (B), H&E x100. Similarly, in pig #15446, liver (C), H&E x200 (arrow points to discreet margin of tumor and normal liver), and bone marrow (D) showed tumor infiltration.

In pig #15446, protocol biopsies on day 28 (4 days before clinical onset of PTLD), no diagnostic abnormality of either spleen graft or LN could be observed. Necropsy on day 37 revealed identical histopathological features of PTLD in LN (with necrosis), spleen graft, tonsils (not shown) as well as in liver and BM (Figure 5C&D) as in #15399. In neither pig did the lungs demonstrate any atypical cells.

DISCUSSION

To our knowledge, this is the first report that describes PTLD after SpTx. PTLD has been described in miniature swine after hematopoietic cell Tx (2), in 3 cases after liver Tx (3), and there has been a single case after kidney Tx (Huang CA, unpublished data). PTLD or lymphoma has developed in only one solid organ (kidney) transplant recipient in the several hundred miniature swine receiving kidney, heart or lung allografts at our center. The incidence of PTLD in miniature swine undergoing hematopoietic cell Tx has been reported to be as high as 28% (4), with a greater incidence (42%) in animals receiving a full two-haplotype MHC-mismatched transplant. In pigs with PTLD that did not receive WBI, the incidence of donor-type was 7.7 % and host-type 92.3 %. In current studies with BMTx using a preparative regimen including 100 cGy WBI 1 out of 8 pigs developed PTLD of donor-type (Cina RA, personal communication). In six pigs receiving 300 cGy WBI, none developed PTLD. Cho et al. defined risk factors in the development of PTLD in a series of 47 miniature swine (4); a greater initial T cell depletion, wide

MHC-disparity between recipient and donor, a greater intensity of immunosuppression, and the presence of PLHV-1 infection contributed to the development of PTLD.

In the present small series, PTLD developed in 2 of 7 pigs (29%) that underwent two-haplotype MHC-mismatched SpTx. Myelosuppressive host conditioning, including WBI, results in a reduction of the host B-cell pool, which may contribute to a reduction in the prevalence of host-type PTLD. However, following SpTx, one of the two pigs developed a host-type PTLD. Host-type PTLD is more often seen in human organ transplant recipients, probably because the lymphoid system remains of host-type, whereas that of recipients of hematopoietic cell Tx is generally converted to donor-type, as shown in previous studies at our center when whole body irradiation was used in the preparative regimen (2). In mixed chimeras without myeloablation, however, host- or donor-type PTLD may be seen, with a predominance of host-type PTLD, presumably because recipient B-cell precursors are more prevalent at the time of origin of the disease than are the corresponding donor elements. Following SpTx, the recipients developed a high level of mixed hematopoietic cell chimerism (Chapter 6), and therefore PTLD would appear to be able to arise from donor or recipient cells.

The clinical picture of PTLD in our two cases resembled that reported previously (2,4). Loss of appetite, lethargy, and weight loss were predominant features. Within days, lymphadenopathy was evident. Even though CyA therapy was discontinued as soon as suspicion of PTLD arose, the disease progressed rapidly to respiratory failure, necessitating euthanasia.

An important observation was that an increase in PLHV-1 DNA copy numbers in the blood was apparent well *before* clinical features of PTLD became apparent, as described by Cho et al. (4); no such increase was seen in pigs that did not develop PTLD, although copy numbers of PLHV-1 were present at low numbers in the blood. In addition, in pigs with PTLD, a high copy number of PLHV-1 was present in a LN, which remained macroscopically and histologically normal, 2-3 days before clinical features developed. Flow cytometry of this node revealed B-cell expansion; shortly thereafter, B-cell proliferation was detected in the blood. This may be of clinical diagnostic relevance, since the detection of viral replication in the blood would appear to be the first indication of the disease. Clinically, intensive and frequent monitoring for Epstein-Barr virus may therefore reveal early PTLD that may be reversible.

Another new finding was the disproportionate rise in serum IgM levels in the pigs with PTLD. To our knowledge, this is the first time that this phenomenon has been described in relation to PTLD. Other changes that occurred before clinical signs appeared were an increase in white blood cell count, a relatively sudden increase or decrease in lymphoid chimerism, B-cell proliferation, and histological evidence of atypical lymphoid proliferation in a LN on protocol biopsy. A marked increase in CyA level (without a change in dosage) accompanied the devel-

opment of clinical features of PTLD; a recipient's lack of ability to metabolize CyA rapidly may be a factor in the development of PTLD.

In clinical cases of PTLD, a reduction or cessation of immunosuppressive therapy has been suggested as a means of reversing PTLD (20, 21). However, when the serum lactate dehydrogenase has risen >2.5 times above the upper limit of normal, or when organ dysfunction is present, or when the disease has spread to multiple visceral sites, a response to a reduction in therapy has been unlikely (22). Successful cessation or reduction of immunosuppression has led to recovery of only 3 of 9 pigs affected with PTLD in our laboratory (2). In our two affected animals, the serum lactate dehydrogenase at the time of clinical onset was 1448 U/L and 1137 U/L (2.9 and 2.3-fold higher than normal level), respectively. Lymphadenopathy was present throughout the body, and organ dysfunction developed rapidly. Based on these risk factors for unresponsiveness, there was no reasonable likelihood of preventing death in these 2 animals.

Although the number of pigs in our study was small, our observations correlate with those of Cho et al (4) in that a greater extent of T cell depletion in the recipient appeared to be a factor that contributed to disease development. Clinically, PTLD is more common in patients treated with T cell-depleting regimens, such as those including anti-thymocyte globulin and OKT3 (23-27). Increased T cell depletion in association with the presence of PLHV-1 would appear to be combined risk factors for disease development. Irradiation (WBI and thymic) is a known activator of latent herpes viral infection (28) and may further impair T cell function and immune surveillance (4). Furthermore, low-dose WBI (100 cGy and less) may cause mutations in B-cells that ultimately develop into malignancies**.**

In summary, this study provides evidence for the occurrence of PTLD after Tx of allogeneic spleens in miniature swine receiving a relatively modest immunosuppressive regimen. SpTx offers a promising strategy to induce Tx tolerance (6). However, the risk of over-immunosuppression in the induction phase of tolerance clearly needs to be emphasized. This model may provide a useful system for studies of the pathogenesis of PTLD.

ACKNOWLEDGEMENTS

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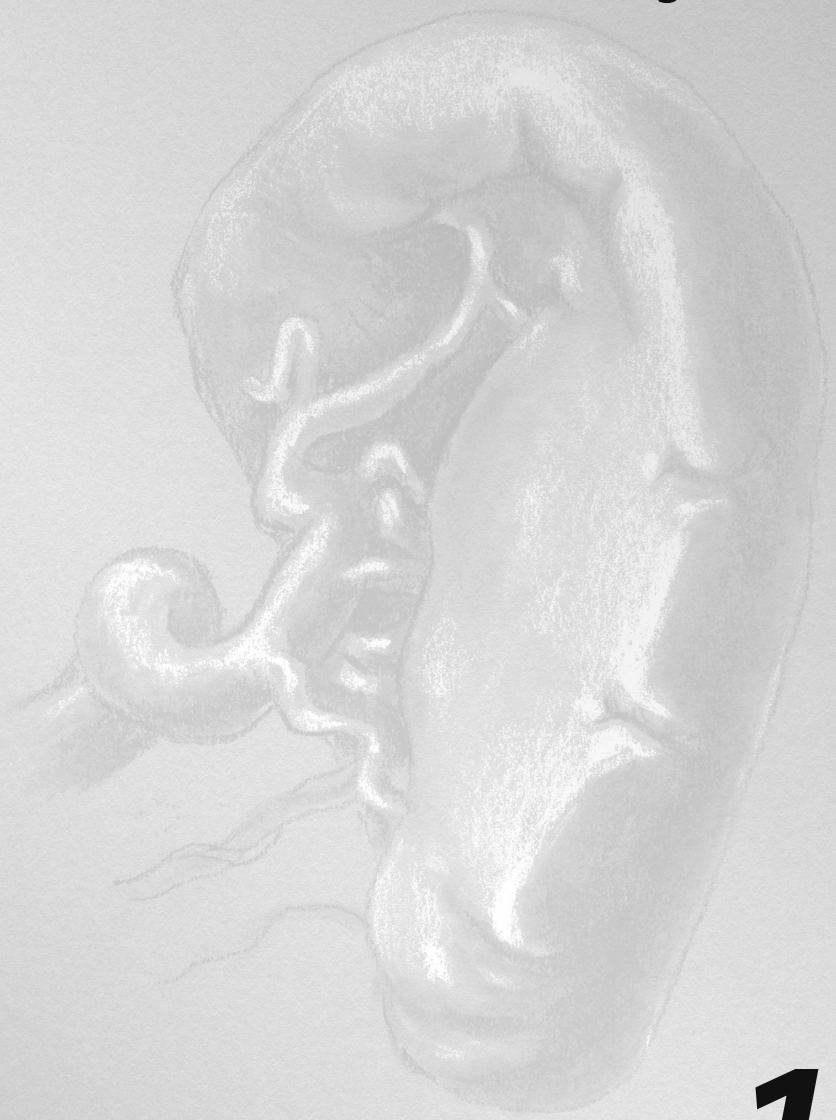
Summary and General Discussion: Transplantation of the Spleen: an Approach to the Induction of Immunological Tolerance



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Frank JMF Dor, Bernd Gollackner, Jan NM IJzermans, David KC Cooper. Transplantation of the spleen: an approach to the induction of immunological tolerance.
Submitted

MINI-ABSTRACT

This paper summarizes experience with spleen transplantation in a pre-clinical large animal model with an emphasis on the potential of the spleen to induce immunological tolerance.

ABSTRACT

Objective: To summarize experience with allogeneic spleen transplantation (SpTx) in swine.
Summary Background Data: Successful SpTx in rodent models has been demonstrated to induce a state of donor-specific tolerance. The relevance of this phenomenon remains to be demonstrated in large animal models and in humans.
Methods: SpTx has been carried out in miniature swine across known MHC antigen barriers. Tolerance to the spleen could be obtained by a nonmyeloablative conditioning regimen and cyclosporine, allowing *in vitro* investigation of the mechanism of tolerance induction and *in vivo* investigation of whether tolerance to a second donor-specific organ had been achieved. Naïve pig spleens were studied as a source of hematopoietic stem cells.
Results: Survival of the spleen was associated with prolonged periods (months) of hematopoietic cell chimerism and with the induction of *in vitro* donor-specific cellular unresponsiveness, with evidence for suppression by regulatory cells. No or minimal features of graft-versus-host disease were observed. In two cases (MHC class I- or full-mismatched), the subsequent transplantation of a kidney (MHC-matched to the spleen donor) without exogenous immunosuppression resulted in prolonged kidney survival (months), but eventual failure despite viability of the spleen and *in vitro* donor-specific unreponsiveness; evidence for donor-specific tolerance was therefore inconclusive. As a source of hematopoietic stem cells, immunologically-naïve adult pig spleens were found to be equivalent to bone marrow.
Conclusions: Allogeneic SpTx may be a means of achieving donor-specific tolerance. The naïve adult spleen is a relatively rich source of hematopoietic stem cells.

INTRODUCTION

The spleen, although not fully understood, has a number of functions as a secondary lymphoid organ, a blood filter, storage for platelets and red blood cells, and as a hematopoietic organ (1-5).

In general, absence of the spleen may result in a higher risk of certain bacterial infections, and also by mycobacteria, viruses, and parasites, now generally termed "overwhelming post-splenectomy infections" (6-8). Furthermore, decreased levels of IgM (9-11), increased platelet

counts (4), and the presence of abnormal red blood cells in the blood (7, 12) are phenomena typically seen after splenectomy (**Chapter 1**).

There are numerous reports in the literature indicating that successful spleen transplantation (SpTx) in some rodent models can lead to the development of donor-specific immunological tolerance (reviewed in 13). Tolerance allows for long-term survival of the spleen in the absence of exogenous immunosuppression, and also the successful transplantation of other organs from the spleen donor without the need for further therapy.

In large animals and humans, however, although SpTx has been carried out on a number of occasions, there is no conclusive evidence that it is capable of inducing tolerance either to itself or to other donor-specific organs (13). However, there is evidence that a state of hematopoietic cell chimerism exists after SpTx, at least for some weeks (14-16). Furthermore, graft-versus-host disease has been documented after SpTx, and in one clinical case proved fatal (15), indicating that the transplanted spleen can initiate an immune response against the host (**Chapter 3**).

The absence of conclusive evidence for tolerance induction in large animal models is probably related to the fact that in none of the studies carried out was knowledge available of the major histocompatibility complex (MHC) of either donor or recipient known, and the immunosuppressive regimens administered were not aimed at the induction of tolerance. Furthermore, most of these studies were performed several decades ago when only limited immunosuppressive agents and laboratory techniques for the assessment of tolerance could be used.

The major aim of our studies was to examine whether the spleen can induce tolerance in a large animal model across known MHC barriers (further aims are described in **Chapter 2**). The MHC-defined MGH miniature swine have been used for almost 30 years in transplantation research and were considered a suitable model in which to study the spleen's capacity to induce tolerance.

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996).

SURGICAL TECHNIQUE OF SPLEEN TRANSPLANTATION IN MINIATURE SWINE

The surgical technique has been described previously (17 and **Chapter 4**) and involved taking a long donor vascular pedicle of splenic artery and celiac axis to its origin from the aorta and of splenic vein to its junction with the portal vein. Carrel patches of aorta and portal vein were taken with the donor spleen to facilitate implantation in the host. The recipient procedure involved end-to-side anastomoses of the donor aortic cuff to the recipient abdominal aorta and of the donor portal venous cuff to the recipient inferior vena cava. A number of potential surgical complications have been experienced, particularly kinking, twisting or compression of the vascular pedicle, leading to either splenic vein thrombosis (with resulting engorgement or even rupture of the spleen) or splenic artery thrombosis (leading to ischemic necrosis).

In all of the studies performed in MHC-defined miniature swine (18), the donor has been positive for the pig allelic antigen (PAA) and the recipient negative for this antigen; the pig allelic antigen is a marker of unknown function, but allows detection of donor hematopoietic cells in the host (19). Whenever SpTx was performed, a native splenectomy was carried out. In view of the considerable volume of blood required to reperfuse the pig spleen (which is approximately four times the size of the human spleen in relation to body weight, i.e., 8g/kg in comparison to 2g/kg) a transfusion of donor red blood cells was given immediately prior to reperfusion. In control experiments, native splenectomy was performed and a donor red blood cell transfusion was given, but SpTx was not carried out.

EXPERIENCE IN MHC-MATCHED, MINOR ANTIGEN-MISMATCHED SPLEEN TRANSPLANTATION IN MINIATURE SWINE (CHAPTER 4)

Four such transplants were performed to establish the surgical technique and to study donor spleen survival in donor-recipient combinations with minor antigen differences only. When no immunosuppressive therapy was given to the host, rejection of the spleen developed within approximately one month. The major histopathological features of the rejected spleen were multifocal hemorrhage, fibrinoid necrosis and vessel wall necrosis. Recipient cells ($CD3^+$/$CD8^+$ and $CD16^+$ cells) rapidly invaded the graft, and loss of function of the spleen was associated with the presence of Howell-Jolly bodies in the blood **(Chapter 5)**.

When a 12-day course of cyclosporine (CyA) was administered to the host (days 0-11) with or without augmentation of immunosuppression by pretransplant thymic irradiation (700cGy on day -1), the spleen was not rejected, and the histological appearance of the spleen remained normal for >200 days, despite the fact that there was a slow replacement of donor cells in the spleen by recipient cells (Figure 1A-C).

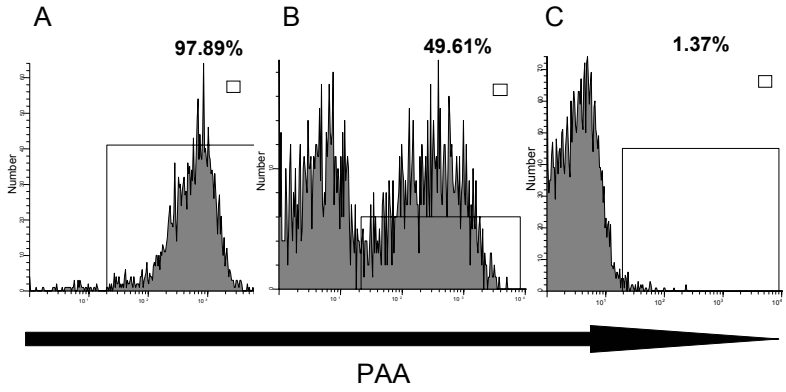

Figure 1. Flow cytometry data of a spleen graft at different time points. All spleen grafts were derived from PAA^+ donors and transplanted into PAA^- recipients.
(A) Pre-Tx, 98% of the spleen cells expressed PAA.
(B) On day 60 after SpTx, the spleen graft consisted of approximately 50% PAA^+ cells (donor-type). This indicates that 50% of the cells in the spleen graft were of recipient-type at this stage.
(C) By 150 days after SpTx, PAA^+ cells (donor-type) in the spleen had largely been replaced by PAA^- cells (recipient-type). This indicates a near-complete (99%) repopulation of the spleen graft by recipient cells.

In all cases, mixed hematopoietic cell chimerism was detected in the host, i.e., PAA-positive donor cells were found circulating in the blood. Chimerism was lost rapidly when rejection developed, but was maintained for approximately two months when rejection did not develop. In addition, in one non-rejector, chimerism in the thymus was observed at multiple time points. The period of chimerism in the blood correlated to some extent with the replacement of PAA-positive cells in the transplanted spleen by host cells.

This small experience established the surgical technique and demonstrated that tolerance could be induced to the spleen, and was associated with transient chimerism in blood and thymus.

EXPERIENCE IN MHC CLASS I-MISMATCHED SPLEEN TRANSPLANTATION IN MINIATURE SWINE (CHAPTER 6)

One transplant was performed, the recipient receiving pretransplant thymic irradiation (700 cGy on day -1) and a 12-day course of CyA. Again, multilineage chimerism was detected in the blood for approximately three months, and replacement of donor cells by recipient cells took place in the spleen graft during this period.

Cell-mediated lympholysis (CML) assays, that reflect cytotoxic T lymphocyte anti-donor reactivity across the Class I barrier, demonstrated that the host-versus-donor response was lost during the first month after SpTx, and there was a virtual absence of response for the next 10 months during the remainder of the experiment. This indicated that successful SpTx had induced a state of donor-specific hyporesponsiveness, since the response of the host to third-party cells remained vigorous. Further *in vitro* studies indicated that a state of endogenous active suppression had developed; when recipient cells were mixed with donor-type cells from a naïve donor, the host cells suppressed the immune response of naïve recipient-matched cells in suppressor-CML assays, indicating the presence of regulatory cells in the spleen recipient (20).

Interestingly, even during the period of hematopoietic cell chimerism, no donor-versus-host response was detected in CML assays. This correlated well with the clinical absence of graft-versus-host-disease (GVHD).

In the recipient, flow cytometry did not indicate donor cells in the host bone marrow (BM) or thymus, and there was no evidence by polymerase chain reaction (PCR) on colony-forming unit (CFU) assays for donor hematopoietic cells that had engrafted in the BM. Our conclusion therefore was that the hyporesponsiveness that had developed was due to the induction of peripheral, rather than central, tolerance.

Four and a half months after SpTx, a donor-matched kidney was transplanted into the host without any exogenous immunosuppressive therapy. This kidney was followed for a further 7.5 months (approximately one year after SpTx) at which time spleen histology remained pristine and kidney histology showed some interstitial fibrosis, but no features of acute cellular rejection. However, during this period, the serum creatinine had risen to 2.5mg/dL during a period of hydronephrosis due to ureteral scarring that had been corrected surgically. [In the absence of exogenous immunosuppression, a MHC Class I-mismatched kidney transplant survives in a naïve miniature swine for approximately 14 days (21), and a MHC Class I-mismatched heart transplant for 8 days (22).] Altogether, *in vivo* donor-specific tolerance correlated well with the *in vitro* tests that indicated donor-specific hyporesponsiveness.

EXPERIENCE WITH FULL MHC-MISMATCHED SPLEEN TRANSPLANTATION IN MINIATURE SWINE (CHAPTER 6)

Fourteen such studies have been performed **(details in Chapter 6)**. The regimen of a 12-day course of CyA and pretransplant thymic irradiation was found to be inadequate to induce

a state of tolerance to the transplanted spleen in this model (12). Long-term survival of the spleen graft was only obtained when the regimen consisted of pretransplant thymic irradiation (day -1), a 45-day course of CyA (rather than a 12-day course), and the addition of whole body irradiation (100 cGy on day -2). The combined dose of whole body and thymic irradiation was insufficient to cause myeloablation, as evidenced by normal white blood cell counts throughout the post-SpTx period and only marginally reduced platelet counts, which recovered within three weeks.

When this full regimen was applied, rejection of the spleen developed in only one of 9 experiments; the cause for failure in this one case was uncertain. When the full regimen was not applied, but the host was either untreated or received only elements of this regimen, 4 of 5 grafts rejected (within 15 days after cessation of CyA) and the remaining animal died of infection on day 23 (while still on CyA) before rejection had occurred. In pigs that had undergone successful SpTx, there were recipient deaths from post-transplantation lymphoproliferative disease (associated with abnormally high levels of CyA) (23 and **Chapter 9**) and infection (due to an outbreak of circovirus in the pig facility).

The full regimen was associated with the prolonged presence of donor cells in the histologically normal spleen, which were slowly replaced by recipient cells over a period of 100-150 days. Spleen graft survival was also associated with the development of multilineage chimerism in the blood, particularly of T cells, with lesser chimerism of B cells, monocytes and granulocytes. In several recipients, donor T cells outnumbered host T cells (Figure 2A), yet no features of GVHD (e.g., skin rash, diarrhea, hepatic dysfunction) were seen. Multilineage chimerism was slowly lost, particularly after cessation of CyA therapy, but could persist for >6 months. In three pigs, a rapid increase in granulocyte and monocyte chimerism was seen during the fourth week to levels >50%, although lymphocyte chimerism was maintained at <20%.

Chimerism (generally of 3-15%) was also detected in the thymus in 6 of the 9 recipient pigs from one month after SpTx, with evidence for donor thymopoiesis. In one recipient it even rose to a maximum of approximately 30% by day 57 (Figure 2B). In the same 6 pigs, donor cells represented up to 25% of the BM cells. In this respect, it should be noted that, in PAA-positive pigs, only 50-60% of BM cells carry this marker (19), and therefore the actual number of donor cells in the BM following SpTx may be considerably more than the 25% measured by flow cytometry. True engraftment was confirmed by PCR and subsequent Southern Blot analysis on DNA derived from colony-forming units from recipient BM, which indicated donor hematopoiesis. Our observations were that engraftment in the BM was more likely to occur when T cell depletion had been particularly good.

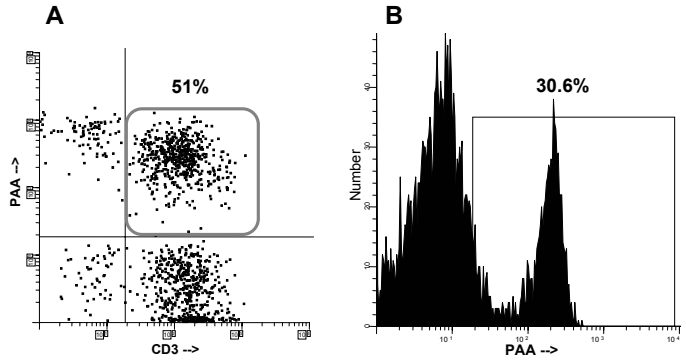

Figure 2.

(A) Flow cytometry data of peripheral blood of a SpTx recipient during CyA therapy. Donor T cells ($CD3^+PAA^+$) are circled. Donor T cells frequently made up approximately 50% of the T cell pool (in the absence of GVHD).

(B) Flow cytometry data of thymus in a SpTx recipient, at 57 days after SpTx. Donor-derived cells made up approximately 31% of the T cells in the thymus at this point.

As with the Class I-mismatched experiment, anti-donor reactivity on CML was lost within the first few weeks following successful SpTx, and the host remained unresponsive to donor cells thereafter, whereas it retained full reactivity against third-party cells. Mixed leukocyte reaction (MLR) assays (MHC Class II-restricted T cell responses) determined that recipients were hyporesponsive to donor-specific or donor-matched cells. Suppressor CML and MLR assays clearly documented active suppression in the host, which was again donor-specific, indicative of the presence of regulatory cells.

In fully MHC-mismatched donor-recipient combinations, anti-donor antibodies were found only in those recipients that did not receive the full immunosuppressive protocol and rejected the spleen graft.

A donor-matched kidney transplant was performed on day 60 (by which time no CyA could be detected in the blood) in one spleen recipient that demonstrated *in vitro* hyporesponsiveness to the donor, without any immunosuppressive drug therapy. This graft functioned for >4 months, although there was a steady rise in serum creatinine, which was 8.1mg/dL at the time the animal was euthanized. The kidney graft showed fibrotic changes with some vasculopathy and glomerulopathy, and a cellular infiltrate (similar to that seen on previous biopsies), but no endothelialitis. The exact cause of graft failure remains uncertain.

The histology of the spleen graft revealed no rejection. Nevertheless, although *in vitro* evidence for tolerance existed and the spleen graft was not rejected, a state of *in vivo* tolerance could not be confirmed. [Graft survival for a period of >4 months is in marked contrast to

kidney graft survival across a full MHC barrier in the absence of immunosuppression in a naïve pig, which is rarely more than 10-20 days (24).]

For comparison, two pigs received the full immunosuppressive regimen, including native splenectomy and red blood cell transfusion, but without SpTx. Although temporary chimerism (due to white cell contamination of the red cell transfusate) was seen in these two control animals, it was at a very low level (maximum 5.9%) and was lost within 11 days (Figure 3A). There was no evidence of the development of *in vitro* hyporesponsiveness on either MLR or CML assays (data not shown). In one case, a subsequent kidney transplant from a pig MHC-matched to the red blood cell donor (performed on day 60) was rejected in an accelerated manner with evidence for both cellular and humoral rejection within four days (Figure 3B). After the kidney transplant had been excised, hyperresponsiveness to donor cells was seen on both CML and MLR assays. It therefore appeared that the regimen alone was insufficient to develop hyporesponsiveness.

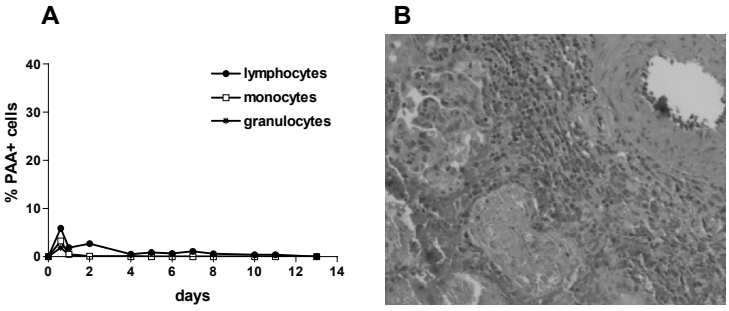

Figure 3.

(A) Peripheral blood chimerism of a control pig that received the full immunosuppressive regimen and a blood transfusion, but no SpTx. By day 11, no chimerism is present.

(B) Histology of the kidney graft (matched to the blood donor, fully MHC-mismatched with the recipient) in a control pig. By day 4 after KTx, severe rejection can be seen (H&E 200x). Interstitial hemorrhage, severe cellular infiltration, and endothelialitis point to both cellular and humoral rejection.

INVESTIGATION OF NAÏVE PIG SPLEENS FOR HEMATOPOIETIC PROGENITOR CELLS (CHAPTER 7)

In view of the clear development of multilineage hematopoietic cell chimerism following SpTx, with subsequent engraftment in recipient BM, we investigated the naïve spleen as a source of hematopoietic progenitor cells (HPC), and compared this source with the BM from the same pig (**Chapter 7**).

The fetal spleen, even in higher mammals, is a major contributor to hematopoiesis (5). However, under normal circumstances, this function of the spleen is lost before birth. In the adult pig, nonhuman primate or human, the spleen can become an hematopoietic organ (extramedullary hematopoiesis), but only when there is diminished or absent BM hematopoietic activity, for example in BM aplasia or myelofibrosis (25, 26).

In rodents, however, the spleen continues to be a major source of HPC. Indeed, the first demonstration of successful restoration of the hematopoietic system was carried out by shielding the spleen from lethal whole body irradiation by Jacobsen in 1949 (27); this seminal study demonstrated that the spleen was a source of HPC that, through engraftment in the aplastic BM, could restore full hematopoiesis to the rodent.

C-kit is an essential marker of long-term-repopulating HPC with early engraftment capacity (28). The fetal pig spleen and BM are rich sources of c-kit^{+} cells. By adult life, however, the pig spleen is a source of far fewer c-kit^{+} cells than the BM, particularly of cells that are c-kit^{high} on flow cytometry (Figure 4).

In mice and humans, it has been demonstrated that side population (SP) cells are highly enriched in hematopoietic stem cells (HSC) (29-31). We demonstrated for the first time that in the adult pig spleen, SP cells are enriched for c-kit, as well as in BM (32). It may therefore be concluded that HSC in the adult spleen reside in the c-kit^{+} SP compartment.

Culture of adult pig spleen cells demonstrated them to be a source of HPC of all forms of CFU. However, the frequency of CFU from the adult spleen was approximately only 0.5% of that from the adult BM. The fact that the CFU activity of HPC in the spleen was not solely due to the presence of blood in the spleen was confirmed by the observation that CFU from pig blood were only 0 - 4.5% of those in the spleen.

The ability of either spleen or BM to produce CFU indicates the presence of HPC, but these are believed to be at a relatively late stage of differentiation (33). The cobblestone area-forming cell (CAFC) assay indicates the presence of hematopoietic stem cells (HSC) (33,34). When the capacity of the spleen to develop CAFC was compared with that of the BM, the frequency of these cells was initially approximately 10-fold higher in the BM. However, after 8 weeks of culture, CAFC frequencies in spleen and BM were equal, indicating that HSC (that provide the ability for long-term cell culture) were equal in number in the spleen and BM. When spleen CAFCs were re-plated in the so-called long-term culture-initiating cell assay, colonies were found at much the same frequency, indicating that these HSC had the ability to form colonies after prolonged culture (32).

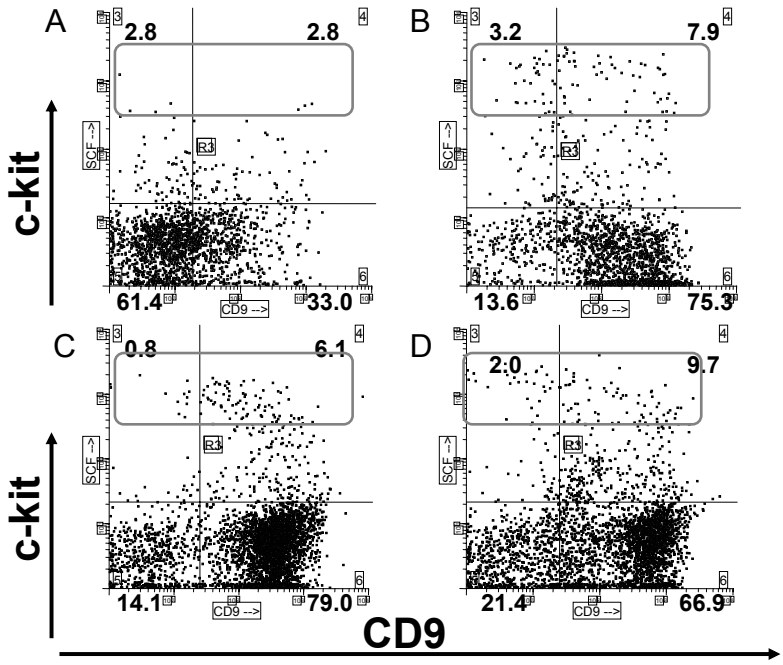

Figure 4. Flow cytometry data of spleen and adult bone marrow (BM) (A+B, respectively), and fetal spleen and BM (C+D, respectively). Stem cell factor (SCF) is a marker for c-kit$^+$ cells, expressed on hematopoietic progenitor cells (HPC). CD9 is a marker for the myeloid lineage, CD9$^-$ cells have been shown to be more primitive cells. Cells that are both CD9$^-$ and c-kit$^+$ are primitive HPC. Each quadrant within figure A,B,C and D represents a specific sub-type (e.g. CD9-/c-kit$^+$), from which the percentage of the total amount of cells is given in each corner.
In adult spleen and BM (A+B, respectively), c-kit$^+$ cells made up 5.6% and 11.1%, respectively. The spleen (A) lacked c-kit^{high} populations (within the rectangles).
In fetal spleen and BM (C+D, respectively), c-kit$^+$ cells made up 6.9% and 11.7%, respectively, and both BM and spleen contained c-kit^{high} populations (within the rectangles).

INVESTIGATION OF NAÏVE BABOON AND HUMAN SPLEENS FOR HEMATOPOIETIC PROGENITOR CELLS (CHAPTER 7)

As the pig spleen is approximately four times the size of the spleen in the baboon or human in relation to body weight, it is possible that this ability to produce HPC was specific for the pig spleen and not for the baboon or human spleen. We therefore carried out a limited number of studies on naïve human and baboon spleens, which indicated that they also contain HPC that could produce CFU of all lineages. CAFC were also present in the human spleen at the same or even greater frequency than in the BM of the same donor. Long-term culture-initiating cells could also be demonstrated from human spleen CAFC (32).

We conclude that the adult spleen is a source of HPC (32), which correlates well with the observations made in our SpTx studies. Since the spleen in mammals is not an active participant in adult hematopoiesis, it is not surprising that relatively low frequencies of HPC were found in comparison with the BM, but it is surprising that more undifferentiated earlier HSC are found at comparable frequencies in spleen and BM. There is evidence from studies of others (35-38) that the adult spleen may be a source of other types of stem cells in addition to HSC.

DISCUSSION

After SpTx, there is a dynamic exchange of cells between the host and the donor tissues. Donor cells can be found in the host blood circulation, BM and thymus, as well as other lymphoid organs (e.g., lymph nodes), whereas recipient cells slowly repopulate the donor spleen (12,20).

This hematopoietic cell chimerism is associated with clear *in vitro* evidence of donor-specific unresponsiveness, that might be due to the development of regulatory cells that developed in both tolerant MHC-class I-mismatched and fully MHC-mismatched SpTx recipients. We have addressed the question whether the unresponsiveness observed after SpTx was truly donor-specific and not due to a "bystander" effect, in which regulatory T cells, activated by donor-antigen, suppress immune responses in an antigen-nonspecific manner. In different *in vitro* analyses with cells from unresponsive SpTx recipients, there was no evidence for "bystander suppression". Therefore, it seems unlikely that bystander suppression played a role in our *in vivo* experiments.

In the fully MHC-mismatched SpTx recipients, there was evidence for both central and peripheral tolerance mechanisms. However, macrochimerism slowly decreased in all recipients, even when engraftment in the BM had occurred. Evidence for regulatory mechanisms persisted in all animals.

Recipients of long-term surviving spleen grafts receiving the full immunosuppressive regimen did not develop anti-donor antibodies. Although we did not investigate this phenomenon specifically, it suggests the possibility that SpTx also induces antibody-mediated unresponsiveness.

The spleen is known to be a source of immature and other subtypes of dendritic cells (39-41) that would be released into the circulation and may be important in mediating tolerance (42,43). A distinct subpopulation of immature dendritic cells has been identified in the spleen with limited T cell stimulation capacity (44). By releasing cells into the recipient circulation

over a number of weeks, SpTx may also have the same effect as a prolonged donor-specific blood transfusion, and may induce hyporesponsiveness through a similar mechanism. However, the mechanisms by which donor-specific blood transfusion (DSBT) promotes organ allograft acceptance are still largely unclear (45), although mainly peripheral mechanisms have been described. It has been demonstrated in a number of rodent models that, early after DSBT, alloantigen-specific regulatory T cells are present in secondary lymphoid organs, where they gradually expand, and can be found in the graft as early as 2 weeks after Tx (46). In large animal models, the beneficial effect of DSBT has also been shown (47-49), although there were no full MHC-mismatches in these studies. Other studies showed no beneficial effect of DSBT (50,51). The disadvantages of DSBT include immunization against alloantigens in 50% of the cases, which could jeopardize a subsequent organ transplant (49), transfusion-associated GVHD (52), a rare but often lethal complication (occurring in 90% of the reported cases), infections (53), and malignancies (49). Potential advantages of SpTx over DSBT are (i) both central and peripheral mechanisms are involved in tolerance induction through SpTx, (ii) donor cells are released continuously in the recipient's circulation after SpTx rather than in one or two "bolus" transfusions (which could prevent GVHD and facilitate donor-recipient immune interaction), (iii) SpTx provides a stroma for APC-T cell interaction, and (iv) SpTx seems to be more effective in preventing an anti-donor antibody response than DSBT (20,50,51).

Relatively high levels of chimerism were documented after successful SpTx, particularly of T cells, and notably *in the absence of any features of GVHD*. Why GVHD was not seen in these experiments, whereas it is not uncommon after MHC-mismatched BM Tx in large animals (54,55) (and in humans) remains uncertain, but there are several differences that may account for this. First, the spleen releases donor cells into the host circulation over a matter of weeks or months rather than as a single infusion as occurs with BM Tx. Second, the differences in phenotype between spleen and BM HPC may be of importance in this respect. Third, regulatory T cells are known to control graft-versus-host reactions (56); the regulatory T cells induced by SpTx may be more effective in controlling GVHD compared to these induced by BMTx.

SpTx may also be compared to intestinal Tx, because of the large load of lymphoid tissue that is transplanted. Chimerism has been described after intestinal Tx, though this is usually limited to microchimerism, in contrast to SpTx. The level of chimerism positively correlates with graft survival (57). However, microchimerism is transient, unless a concomitant BMTx is performed, leading to macrochimerism and engraftment of donor BM cells (58). In contrast to SpTx, GVHD was noted in 4.7-6.5% of intestinal Tx recipients (59). An important complication of intestinal Tx, besides infection and GVHD, is PTLD (60), the incidence of which has been reported to be higher after intestinal Tx than after any other organ transplants (61). There are no reports that intestinal Tx induces donor-specific tolerance. It does not seem realistic to believe that intestinal Tx will be used clinically for the purpose of inducing Tx tolerance.

Interestingly, in a recent report on multivisceral Tx, it was indicated that multivisceral grafts seem to facilitate survival of organs, suggesting that this procedure offers a degree of immunologic advantage (62). The authors concluded that this advantage could at least partly be attributed to the inclusion of the spleen in the graft (62).

Another organ that has the potential to induce tolerance is the thymus, the major site of self-nonself discrimination in the immune system. Two different immunologic events would allow a vascularized thymic graft to induce tolerance. The first mechanism involves thymic stromal cells, and possibly dendritic cells, in the vascularized thymic graft that may induce central tolerance after Tx by achieving deletion or anergy of T cells reactive to the donor haplotype. The second potential mechanism involves thymic emigrants from the vascularized thymic graft, which could be responsible for the induction of tolerance by means of a peripheral mechanism of regulation that leads to the silencing of alloreactive cells (63). Disadvantages of thymus Tx are its applicability only in the pediatric and adolescent population (since the thymus involutes with adolescence) (64), the need for host thymectomy to successfully induce tolerance (65), and the technically challenging nature of the surgical procedure (64,66).

Based on the studies described in this dissertation, allogeneic SpTx deserves further investigation as a potential means of achieving donor-specific immunological tolerance. Since the spleen is commonly discarded (except for tissue-typing purposes) from deceased human organ donors, it is readily available for Tx along with any other organ, such as the kidney or heart. If indeed it is proved that the spleen can induce a state of tolerance to the kidney or heart, allowing the patient to be weaned from all exogenous immunosuppressive drug therapy (with its concomitant side effects and complications), this would be a significant advance in organ Tx. The potential of SpTx in the induction of tolerance following xenoTx has also been considered (67) (**Chapter 8**).

Our limited studies on the presence of HPC and HSC in the spleen indicate that the human spleen, like its pig counterpart, is an equal source of these cells. This suggests that SpTx in humans might be associated with the same outcome as in pigs.

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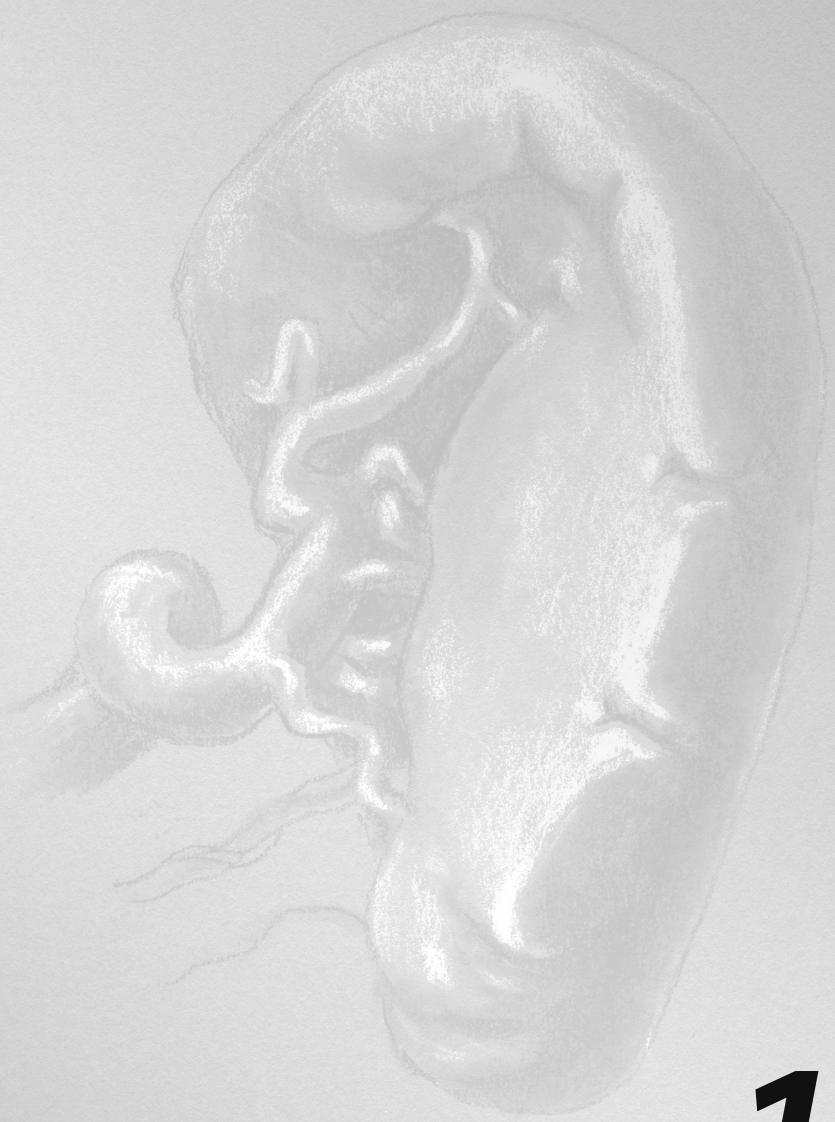
Summary in Dutch



11

ONDERZOEK GERELATEERD AAN DE INDUCTIE VAN IMMUNOLOGISCHE TOLERANTIE DOOR MIDDEL VAN MILTTRANSPLANTATIE IN MINIATUURVARKENS

Het ultieme doel in klinische transplantatie is het bereiken van donor-specifieke immunologische tolerantie ten opzichte van het getransplanteerde orgaan zonder dat continue immunosuppressieve therapie noodzakelijk is. Namelijk, getransplanteerde organen zouden dan langdurig kunnen overleven zonder dat de patiënt de risico's en complicaties van farmacologische immunosuppressieve therapie ondervindt. Het transplanteren van de milt wordt in dit proefschrift onderzocht als methode om immunologische tolerantie te induceren.

In **hoofdstuk 1** van dit proefschrift wordt een uitgebreide introductie gegeven over de milt. Vanuit de medische geschiedenis wordt met name duidelijk dat de functie(s) van de milt langdurig onbekend is (zijn) geweest, en het orgaan met mystiek werd omhuld. Er wordt een overzicht gegeven van de huidige inzichten betreffende de embryologie, anatomie, histologie, en fysiologie van de milt, waarbij specifieke aspecten van de varkensmilt worden uitgelicht. Daarna volgt een aantal paragrafen over transplantatie-immunologie, waarin met name aandacht is voor het begrip "transplantatie-tolerantie". Ter afsluiting van de introductie wordt de lezer geïnformeerd over het miniatuurvarkensmodel, met name in relatie tot transplantatiestudies.

Hoofdstuk 2 verwoordt puntsgewijs de doelstellingen van het onderzoek:

1. het ontwikkelen van een pre-klinisch groot proefdiermodel voor milttransplantatie.
2. het bepalen van histologische kenmerken van afstoting (rejectie) van milttransplantaten in het miniatuurvarken.
3. door middel van milttransplantaties in het miniatuurvarken in verschillende donor-ontvangercombinaties (Major Histocompatibility Complex (MHC)- gematched, MHC-klasse I mismatch, volledige MHC mismatch) zal worden gepoogd om langdurig gemengd hematopoietisch chimerisme te induceren, en worden onderzocht of milttransplantatie leidt tot donor-specifieke non-responsiviteit en langdurige overleving van het milttransplantaat. Tevens zal worden onderzocht welk immunosuppressieve regime het meest succesvol is om immunologische tolerantie te bewerkstelligen.
4. het onderzoeken of succesvolle MHC-gemismatchte milttransplantatie (met of zonder inductie van gemengd hematopoietisch chimerisme en/of cellulaire non-responsiviteit) tolerogeen is, zodat vervolgens een niertransplantaat dat MHC-gematcht is aan de donor kan overleven zonder dat de ontvanger immunosuppressiva krijgt. Tevens zal worden onderzocht welk mechanisme aan deze tolerantie of non-responsiviteit ten grondslag ligt.
5. het onderzoeken van de milt als bron van hematopoietische voorlopercellen of stamcellen.

6. het onderzoeken of milttransplantatie ook een rol kan spelen bij tolerantie-inductie in xenotransplantatie (transplantatie tussen verschillende soorten), in een varken-naar-baviaan transplantatiemodel.
7. het identificeren en beschrijven van mogelijke nadelen van milttransplantatie en/of het immunosuppressieve regime.

Hoofdstuk 3 is een review van bestaande literatuur met als hoofdvraag: kan de milt tolerantie induceren? In diverse knaagdiermodellen is overtuigend bewijs gevonden voor een tolerogeen effect door een getransplanteerde milt onder verschillende omstandigheden. In meerdere studies wordt beschreven dat er een balans kan worden verkregen tussen host-versus-graft (ontvanger-versus-transplantaat) en graft-versus-host (transplantaat-versus-ontvanger) reacties na milttransplantatie, hetgeen leidt tot lange-termijns overleving van zowel de getransplanteerde milt als een tweede donor-specifiek orgaan. De inductie van tolerantie zou gepaard gaan met het ontwikkelen van regulatoire T cellen. Echter, in grote proefdieren en mensen is geen definitief bewijs verkregen dat milttransplantatie tolerantie kan induceren. De meeste studies beschrijven dat de getransplanteerde milt, zoals ieder ander orgaantransplantaat, wordt afgestoten. Echter, deze studies zijn alle sterk verouderd. Tevens is in geen van de studies gebruik gemaakt van een model waarvan de transplantatie-antigenen, het MHC, bij donor en ontvanger bekend zijn. Ook is in geen van de beschreven onderzoeken gebruik gemaakt van immunosuppressieve strategieën die gericht zijn op het induceren van tolerantie, waarover de laatste jaren veel kennis is vergaard. Enkele studies die klinische milttransplantatie beschrijven (van mens naar mens) demonstreren dat na milttransplantatie hematopoietisch chimerisme kan optreden, en een sterke graft-versus-host reactie teweeg kan brengen, die zelfs tot dodelijke graft-versus-host-disease kan leiden. Dit toont aan dat de milt in ieder geval de potentie heeft om de gebruikelijke afstotingsreactie van een ontvanger van een orgaantransplantaat tegen te werken.

De voordelen van immunologische tolerantie bij ontvangers van een orgaantransplantaat, zeker in vergelijking met chronische farmacologische immunosuppressie, zijn aanzienlijk. Derhalve lijkt het zinvol om, voordat eventuele klinische studies worden geïnitieerd, milttransplantatie te bestuderen in een groot proefdiermodel, zoals het Massachusetts General Hospital MHC-gedefinieerde miniatuurvarken, waarmee een aanzienlijke ervaring is opgebouwd in transplantatie-onderzoek.

In **hoofdstuk 4** wordt de chirurgische techniek van milttransplantatie in het varken beschreven. Aanvankelijk werd een techniek gebruikt, waarbij de donormilt werd geëxcideerd en de suprapancreatische arteria lienalis end-to-side werd geanastomoseerd op de infrarenale abdominale aorta van de ontvanger en de miltvene op de infrarenale vena cava inferior. Echter, de diameter van de miltvaten in de donorvarkens was klein (<3 mm), waardoor er stenotische

en/of trombotische complicaties optraden ter hoogte van de anastomose. Daarom ontwikkelden we een techniek waarbij in de donor de miltarterie en truncus coeliacus werden vrijgeprepareerd tot aan de aorta teneinde een Carrel patch van de aorta te kunnen uitnemen. De miltvene werd tot aan de confluens met de v. mesenterica superior vrijgeprepareerd zodat een cuff van de vena porta kon worden geïncludeerd. De donormilt werd geëxcideerd, gewogen en vervolgens op ijs geplaatst. De milt werd via de arterie gespoeld met preservatievloeistof totdat de veneuze efflux helder was. Bij alle ontvangers werd een splenectomie verricht, waarna de donormilt werd geïmplanteerd door middel van end-to-side anastomose tussen de cuff van donoraorta en abdominale aorta van de ontvanger. De vena porta cuff werd end-to side op de vena cava inferior geanastomoseerd. Reperfusie van het milttransplantaat resulteerde in een hematocriet-daling van 25%. Derhalve werd een transfusie van donor-erythrocyten gegeven. Het milttransplantaat werd aan de oppervlakte van de darmen gepositioneerd ter voorkoming van "kinking" van de vaatsteel.

De introductie van deze nieuwe chirurgische techniek leidde tot 4 succesvolle MHC-gematchte milttransplantaties, waarvan in dit hoofdstuk de resultaten worden beschreven. Twee van de vier ontvangers kregen geen immunosuppressie. Rejectie van het milttransplantaat trad op na 28-29 dagen. De histologische kenmerken daarvan worden in **hoofdstuk 5** beschreven. De overige twee ontvangers kregen 12 dagen cyclosporine A (en één tevens 700cGy thymusbestraling op dag-1); afstoting van de milt werd niet waargenomen. Alle ontvangers van een milttransplantaat vertoonden gemengd hematopoietisch chimerisme gedurende circa drie weken. Degenen die waren behandeld met cyclosporine A hadden significant langere periodes van chimerisme, tot twee maanden. Tevens was er sprake van thymus-chimerisme in de ontvanger die thymusbestraling had ondergaan en 12 dagen cyclosporine A had gekregen.
We concludeerden dat milttransplantatie in het varken technisch mogelijk is, en dat lange-termijnsoverleving van MHC-gematche milttransplantaten wordt gefaciliteerd door een kortdurende cyclosporine A behandeling, die tevens leidt tot langdurig gemengd hematopoietisch chimerisme. Additionele thymusbestraling faciliteert chimerisme van de thymus.

In **hoofdstuk 5** rapporteren we de histopathologie van milttransplantaat-rejectie in het eerder beschreven miniatuurvarkensmodel, na een overzicht te hebben gegeven van eerdere studies waarin afstoting van milttransplantaten wordt beschreven.
Van 17 tot dan toe verrichte milttransplantaties in ons varkensmodel trad afstoting op in 2 milttransplantaten in onbehandelde MHC-gematchte (minor antigen-gemismatcht) ontvangers (Groep 1) en in 5 transplantaten in volledig MHC-gemismatchte ontvangers (Groep 2), van wie 1 ontvanger onbehandeld was en 4 immunosuppressieve therapie hadden ondergaan. Na biopsie en/of necropsie werd weefsel van het transplantaat met diverse kleuringen bewerkt, gekleurd met immunohistochemische markers, en werden er TUNEL assays uitgevoerd.

In beide groepen konden overeenkomende kenmerken van transplantaatafstoting worden waargenomen, maar deze traden op verschillende momenten in de tijd op. Necrose trad al op dag 8 op in groep 2, en pas op dag 28 in groep 1, variërend van focale fibrinoïde necrose van de vaatwand van arteriolen en sinusoïden tot aan diffuse necrose, meestal geassocieerd met bloeding. Andere kenmerken van rejectie zijn onder anderen: expansie van de witte pulpa door atypische cellen, verminderde aankleuring van basaalmembranen en reticulaire vezels. Een verdubbeling van de TUNEL index ging vooraf aan histologisch waarneembare afstoting. Tevens kunnen binnen een week na afstoting van het milttransplantaat (mits natieve splenectomie is uitgevoerd) Howell-Jolly bodies worden waargenomen in een bloeduitstrijkje.
Op basis van deze studie hebben we histologische richtlijnen opgesteld om acute rejectie van allogene milttransplantaten (waarvan donor en ontvanger MHC bekend is) te kunnen diagnosticeren, en wellicht te kunnen voorspellen (zie tabel hieronder).

Graad	Rejectie	Histologische Kenmerken
0	geen	Normale architectuur van de milt
1	mild	Focale of multifocale fibrinoïde necrose van wanden van sinusoïden en/of arteriolen, met of zonder bloeding
2	matig	Expansie van witte pulpa door grote, atypische, en mitotisch-actieve lymphoïde cellen
3A	matig-ernstig	Focale coagulatieve necrose
3B	ernstig	Diffuse coagulatieve necrose

De meeste milttransplantaten die langdurig overleefden vertoonden histologisch het beeld van atrofie van de witte pulpa gedurende de eerste 5 maanden. De (overigens normale) rode pulpa werd gedeeltelijk vervangen door gevacuoliseerde histiocyten en gladde spiercellen.

In **hoofdstuk 6** wordt de inductie van gemengd hematopoietisch chimerisme en donor-specifieke non-responsiviteit na milttransplantatie beschreven. Bij 13 varkens werd een splenectomie (dag 0) uitgevoerd; allen kregen een transfusie van donor erythrocyten, waarna direct een milttransplantatie werd uitgevoerd met een MHC-klasse I mismatch (n=1) of volledige MHC-mismatch (n=10); twee varkens dienden als controle en kregen geen milttransplantaat. Alle varkens werden intensief gemonitord voor chimerisme en anti-donor immuunresponses. Zes van de 11 milttransplantaten gingen verloren door rejectie (n=5) of trombose van de miltvene. Vijf transplantaten overleefden langdurig. Alle 11 ontvangers van een donormilt ontwikkelden gemengd hematopoietisch chimerisme, dat spoedig verloren ging bij transplantaatfalen. De 2 controledieren vertoonden kortdurend (<11 dagen) laaggradig chimerisme in het bloed. Varkens met functionerende milttransplantaten hadden multilineair chimerisme in bloed, thymus en beenmerg gedurende tenminste 2-6 maanden (met bewijs voor engraftment in beenmerg), zonder graft-versus-host-disease. Deze ontvangers ontwikkelden *in vitro* donor-specifieke non-responsiviteit en suppressie.

In 2 ontvangers van een succesvol milttransplantaat werd van een donor MHC-gematcht varken een nier getransplanteerd zonder enige vorm van exogene immunosuppressie. Tevens werd in 2 controledieren, die geen milttransplantatie hadden ondergaan, maar wel het gehele immunosuppressieve regime, inclusief splenectomie en bloedtransfusie, een niertransplantatie uitgevoerd van een varken MHC-gematcht aan de bloeddonor. Ook deze dieren kregen geen verdere immunusuppressie. In 2 tolerante varkens overleefden de donor-gematchte niertransplantaten >4 en >7 maanden zonder exogene immunosuppressie. In 2 controledieren werden deze niertransplantaten afgestoten na 4 respectievelijk 15 dagen. Wij concluderen dat succesvolle milttransplantatie kan resulteren in engraftment van hematopoietische cellen van de donor en *in vitro* donor-specifieke non-responsiviteit, zodat vervolgens niertransplantaten gematcht aan de oorspronkelijke donor kunnen overleven zonder dat de ontvanger opnieuw immunosuppressiva krijgt.

Hoofdstuk 7

Aangezien we eerder, zoals in **hoofdstuk 6** beschreven, langdurig gemengd hematopoietisch chimerisme (>40%) observeerden na volledig MHC gemismatchte milttransplantatie, werd de milt onderzocht als een bron van hematopoietische voorlopercellen (progenitors). Met behulp van specifieke celoppervlakte-markers werden hematopoietische progenitors in de milt en beenmerg van jong-volwassen (n=15) en foetale (n=9) miniatuurvarkens geïdentificeerd met behulp van flowcytometrie. De zogenaamde "Side Population Cells", waaronder zich mogelijk stamcellen bevinden, scheiden een speciale fluorescerende stof (Hoechst33342) uit, waardoor ze een verlaagde fluorescentie hebben, werden geanalyseerd in volwassen milt, beenmerg, en bloed. Met name werd bekeken in welke mate zij de stamcelmarker c-kit tot expressie brachten. De c-kit$^+$ cellen in de milt hadden meer co-expressie van lymfoïde markers dan c-kit$^+$ beenmergcellen, maar een gelijke co-expressie van myeloïde markers. Zowel de "Side Population Cells" in het beenmerg als in de milt bevatten een significante hoeveelheid c-kit$^+$ cellen.

Functionele hematopoietische progenitor activiteit van de milt en het beenmerg werd *in vitro* onderzocht door middel van de "colony forming unit (CFU)" assays en "cobblestone area-forming cell (CAFC)" assays in lange-termijnkweken.

Hoewel de frequentie van vroege CFUs in de milt slechts 0.1-1.3% was van de frequentie in het beenmerg, was de frequentie van CAFCs die zich na 8 weken in kweek vormden vergelijkbaar met de frequentie in het beenmerg. Secundaire CFUs in zogenaamde "long-term culture-initiating cell assays" bevestigden de aanwezigheid van cellen met lange-termijn repopulatie-capaciteit, in vergelijkbare frequenties in milt en beenmerg. Tevens werden studies uitgevoerd met miltweefsel en beenmerg van bavianen en mensen, waarin vergelijkbare resultaten werden geboekt als in het varken. Concluderend is deze studie de eerste die aantoont dat de milt een relatief rijke bron van zeer primitieve hematopoietische voorlopercellen is, mogelijk stamcellen, die van therapeutische waarde zouden kunnen zijn in de toekomst.

Hoofdstuk 7 appendix

Aangezien we al eerder in **hoofdstuk 7** hebben beschreven dat de volwassen milt een bron is van primitieve hematopoietische voorlopercellen (mogelijk stamcellen), initieerden we een studie waarin we onderzochten of hematopoietische voorlopercellen uit milt- en beenmerg door middel van behandeling met 5-azacytidine (een cytosine-analoog) konden differentiëren in cardiomyocyten, zoals eerder door anderen beschreven is voor beenmergcellen. Daartoe werden van volwassen varkens mononucleaire milt-en beenmergcellen verrijkt met c-kit danwel gedepleteerd voor c-kit door "magnetic bead separation" met varkens-stamcelfactor (=c-kit ligand). Hierna werden de cellen geïncubeerd met 5-azacytidine gedurende 24 uur, waarna het kweekmedium elke 48 uur werd ververst met 5-azacytidine-vrij medium. De morfologie van de cellen werd microscopisch gedurende 5 weken regelmatig beoordeeld. Met behulp van de Western Blot techniek werd de expressie van cardiaal troponine en myosine gedetecteerd.

Hoewel 5-azacytidine behandeling leidde tot morfologische veranderingen in beide c-kit-verrijkte populaties, namelijk het ontstaan van balvormige celclusters, werden er geen ritmische contracties (zoals het kloppen van het hart) waargenomen, in tegenstelling tot eerdere bevindingen van anderen. Verder werd noch troponine, noch myosine gedetecteerd in milt-of beenmergcelkweken.

Concluderend leidde onze behandeling van hematopoietische voorlopercellen vanuit milt of beenmerg met 5-azacytidine niet tot de inductie van hartspecifieke genexpressie.

Hoofdstuk 8 beschrijft de resultaten van de eerste xenogene milttransplantaties in een varken-naar-baviaan model. Eén van de doelstellingen bij het aanvangen van de studies naar de potentie van de milt om tolerantie te induceren was het onderzoeken van de mogelijke extrapolatie van de techniek naar xenotransplantatie. Xenogene varkensmilttransplantatie werd uitgevoerd in twee bavianen, waarvan we de eerste transplantatie met name hebben gebruikt om de chirurgische techniek zoals in **hoofdstuk 4** beschreven te valideren in het varken-naar-baviaan model. De tweede baviaan (B221) kreeg 4 uur na de varkensmilttransplantatie ook een beenmergtransplantatie van dezelfde donor. We rapporteren in dit hoofdstuk onze observaties in deze baviaan en vergelijken deze met een baviaan die alleen een beenmergtransplantatie van een varken had ondergaan (B219).

Beide bavianen werden geconditioneerd met een vrij complex immunosuppressief regime gebaseerd op het varken-naar-baviaan-beenmergtransplantatiemodel. De beide brondiervarkens waren miniatuurvarkens met sterk verlaagde expressie van het xeno-antigen, Galα1-3Gal (Gal). Er werden geen maatregelen getroffen om natuurlijk-voorkomde anti-Gal antistoffen te depleteren, wel werd het complement gedepleteerd met behulp van cobra venom factor.

We onderzochten het optreden van gemengd hematopoietisch chimerisme na xenogene milt- en beenmergtransplantatie. In B221 was er 2 uur na de milttransplantatie multilineair chimerisme in het bloed (16.8%), echter 2 uur erna (voor beenmergtransplantatie) waren er

geen varkenscellen meer detecteerbaar. Twee uur na de beenmergtransplantatie was het percentage chimerisme vergelijkbaar, hoewel de phenotypes van de varkenscellen in het bloed van de baviaan na milttransplantatie verschilden van die na beenmergtransplantatie. Het maximale percentage chimerisme in B219 was slechts 1.9% (op dag 2). De duur van het macrochimerisme in B221 was 12 dagen, in B219 5 dagen. Een biopt van het milttransplantaat op dag 18 vertoonde het beeld van rejectie, waarschijnlijk anti-Gal antistof-gemedieerd.
Concluderend kon, net als na allogene milttransplantatie, hematopoietisch chimerisme worden gedetecteerd na xenogene milttransplantatie, in vergelijkbare percentages als na de hierop volgende beenmergtransplantatie. In vergelijking met een baviaan die alleen een beenmergtransplantatie kreeg, waren er meer en landuriger varkenscellen in de circulatie van de baviaan met milt- en beenmergtransplantatie. Er trad geen graft-versus-host-disease op. Mede gebaseerd op eerdere studies naar varken-naar-baviaan beenmergtransplantatie concluderen we dat er een geschikte micro-omgeving nodig is voor varkenscellen om te kunnen engraften in een baviaan. De milt van de beenmergdonor biedt een extra bron van hematopoietische cellen, maar ook een stroma, noodzakelijk voor hematopoiese.

Hoofdstuk 9

Post-transplant lymphoproliferative disease (PTLD) is een belangrijke complicatie van immunosuppressie na transplantatie. PTLD omvat een heterogene groep van lymfoïde proliferaties, meestal van B-cel origine, die geassocieerd zijn met ineffectieve T cel functie en exogene immunosuppressie. Er is een sterke correlatie gerapporteerd tussen B cel neoplasma's in patiënten die immunosuppressiva ontvangen en de aanwezigheid van het B-lymfotroop gammaherpersvirus, Epstein-Barr virus. In het miniatuurvarken is PTLD beschreven na allogene beenmergtransplantatie, maar ook na lever- en niertransplantatie. Vanwege de klinische relevantie van PTLD is het fenomeen uitvoerig in het varken bestudeerd. Zoals in mensen, speelt een lymfotroop gammaherpesvirus, het "porcine lymphotropic herpesvirus-1" (PLHV-1) een belangrijke rol in de pathogenese van PTLD. Eerder gedefinieerde risicofactoren voor PTLD na beenmergtransplantatie in het varken zijn: T cel-depletie, de mate van MHC mismatch, en de intensiteit van PLHV-1 infectie. In dit hoofdstuk beschrijven we het optreden van PTLD in 2 van de 7 (29%) ontvangers van een milttransplantaat (op dag 30 en 32) die hetzelfde immunosuppressieve regime hadden ondergaan, namelijk totale lichaamsbestraling (100 cGy op dag -2), thymusbestraling (700 cGy op dag -1), en cyclosporine intraveneus gedurende 45 dagen. Twee controledieren kregen dezelfde immunosuppressie, maar geen milttransplantatie.
In één geval betrof de PTLD een maligne proliferatie van ontvanger-type B-lymfocyten, in het andere geval een proliferatie van donor-type B-lymfocyten. In de twee varkens met PTLD bleek er een significant betere T cel-depletie te bestaan op de dag van de milttransplantatie (na totale lichaamsbestraling en thymusbestraling), en hogere cyclosporine waarden (trough levels) in het bloed in de eerste week na transplantatie dan gelijkbehandelde varkens die geen PTLD ontwikkelden en controles. Vroegtijdige veranderingen geassocieerd met PTLD

waren: toegenomen hoeveelheden PLHV-1 in bloed en weefsels, leukocytose, toename van aantal B cellen en verhoogd serum IgM. Deze veranderingen gingen vooraf aan de klinische verschijnselen van PTLD. Het staken van immunosuppressie leidde niet tot verbetering.
Concluderend is dit de eerste studie die het optreden van PTLD na allogene milttransplantatie beschrijft in miniatuurvarkens die relatief milde immunosuppressie kregen. Hoewel milttransplantatie een veelbelovende strategie is om transplantatietolerantie te induceren, benadrukken we de risico's van over-immunosuppressie in de inductiefase van tolerantie. Dit model lijkt tevens geschikt om de pathogenese van PTLD verder te bestuderen.

Samenvattend geven de studies beschreven in dit proefschrift aanleiding tot verder onderzoek naar milttransplantatie als methode om donor-specifieke immunologische tolerantie te bewerkstelligen. Aangezien de milt van postmortale orgaandonoren vaak niet wordt gebruikt (afgezien van weefseltyperingsonderzoek), zou deze beschikbaar zijn voor transplantatie in combinatie met een ander orgaan, zoals een nier of hart. Als inderdaad bewezen kan worden dat de milt tolerantie induceert ten opzichte van een nier- of harttransplantaat, zodat bij patiënten alle immunosuppressiva gestaakt kunnen worden, zou dat een significante vooruitgang zijn in orgaantransplantatie. Ook heeft de milt een potentiële rol bij tolerantie-inductie in xenotransplantatie en als bron van hematopoietische voorlopercellen.
Graag verwijs ik naar **Future directions** voor voorstellen betreffende verder onderzoek.

FUTURE DIRECTIONS

1. Confirm the feasibility of spleen transplantation and the induction of immunological tolerance to concomitantly-transplanted donor-matched kidney grafts.

2. Demonstrate immunological tolerance through spleen transplantation towards donor-matched organs (other than kidneys), either by concomitant or by subsequent transplantation, in a large animal model.

3. Investigate the mechanism of tolerance induction by spleen transplantation in a rodent model, e.g. the role of the thymus or the need for venous drainage of the spleen in the portal circulation.

4. Refine the immunosuppressive protocols in order to make them clinically acceptable, e.g. by replacing irradiation with a pharmaceutical agent.

5. Study the mechanisms involved in the observed absence of graft-versus-host-disease associated with spleen transplantation.

6. Preclinical studies in nonhuman primates undergoing concomitant spleen and kidney transplantation as a strategy to induce tolerance.

7. Explore possibilities of a clinical trial of concomitant spleen and kidney transplantation.

8. Explore the further possibilities of the spleen as a source of adult stem cells with potential therapeutic value.

9. Further explore xenogeneic spleen transplantation in a pig-to-baboon transplantation model.

LIST OF ABBREVIATIONS

APC	antigen presenting cell
BFU-E	burst-forming unit-erythroid
BM	bone marrow
CAFC	cobblestone area-forming cell
CAV	chronic allograft vasculopathy
CFU	colony-forming unit
CFU-E	colony-forming unit-erythroid
CFU-GEMM	colony-forming unit-granulocyte-erythrocyte-macrophage-mega-karyocyte
CFU-GM	colony-forming unit-granulocyte-monocyte
cGy	centi-Gray
CML	cell-mediated lympholysis
CPP	cyclophosphamide
CyA	cyclosporine A
DST	donor-specific transfusion
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescein activated cell sorting
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
Gal	Galα1-3Gal
GC	germinal center
GVHD	graft-versus-host disease
HEV	high endothelial venule
HPC	hematopoietic progenitor cell
Ig	immunoglobulin
IgD	immunoglobulin D
IgG	immunoglobulin G
IgM	immunoglobulin M
ITN	Immune Tolerance Network
LC	lymphocyte corona
LF	lymphoid follicle
LN	lymph node
LPS	lipopolysaccharides
LTC-IC	long-term culture-initiating cell
MHC	Major Histocompatibility Complex
MLR	mixed leukocyte reactivity
MPS	mononuclear phagocytic system

MZ	marginal zone
OPSI	overwhelming post-splenectomy infections
PAA	pig allelic antigen
PALS	periarteriolar lymphocytic sheaths
PAS	periodic acid-Schiff
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PLHV-1	porcine lymphotropic herpesvirus-1
PSL	percent specific lysis
PTLD	post-transplant lymphoproliferative disease
SLA	Swine Leukocyte Antigen
SMA	α-smooth muscle actin
SMP	splanchnic mesodermal plate
SpTx	spleen transplantation
TI	thymic irradiation
TuI	TUNEL index
TI-2 antigens	T-cell independent antigens type 2
Tx	transplantation
WBI	whole body irradiation

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in de zin: Aan allen die hebben bijgedragen aan mijn persoonlijke en professionele ontwikkeling. De rest van de familie Dor-Ziere en alle "Grootjes en Okels" dank ik voor hun nimmer aflatende interesse in mij, mijn werk en het proefschrift: het is dan eindelijk af!

Prof Dr H.G. van Eijk, Beste Henk, jij was eigenlijk de eerste die bij mij de interesse in het basaal-wetenschappelijk onderzoek hebt aangewakkerd. Zo mondde een vraag van mij tijdens een practicum Chemische Pathologie uit in een heus onderzoeksproject! Ik wil je bedanken voor het destijds in mij gestelde vertrouwen en je enthousiasme. Onze gedeelde passie voor muziek en je schijnbaar onuitputtelijke CD collectie hebben daarnaast geleid tot veel mooie concerten en thuisluisterplezier.

Vrienden: Vriendschap is een kostbaar bezit. Ik krijg geen genoeg van de vele gezamenlijke etentjes, borrels, skireisjes, concerten, kampeertrips, verhuispartijen en discussies. Maar ook zijn we er voor elkaar in slechte tijden, en hoe! Enne…nu heb ik weer tijd ☺! Thanks for everything!

Mijn **paranimfen** en goede vrienden, **Dr Patrick Groenendijk** en **Martijn Verkade**: jullie vriendschap door de jaren heen is heel belangrijk voor me geweest (en nog!). We hebben samen al veel meegemaakt. Ieder van jullie weet op zijn eigen manier wat een promotieonderzoek inhoudt en kent de lusten maar ook zeker de lasten. Fantastisch dat jullie me op deze dag letterlijk terzijde willen staan. Martijn, collega, binnenkort samen in het SFG, ik kijk ernaar uit.

Ed en Thea, ik ben blij jullie als schoonouders te hebben. Dank voor alle begrip, steun en interesse de afgelopen jaren.

Cilia en Francisco: just married! Dank voor jullie interesse en steun. Veel geluk samen in jullie nieuwe huis! Veel sterkte met alle bureaucratie. Cilia, thanks for being my sister all these years. Dat ik nog lang je grote broer mag zijn!

Pa en Ma: voor een kind is het zo ongelofelijk bepalend in welk huis z'n wieg staat! Ik ben jullie enorm dankbaar voor de vele kansen die jullie me hebben geboden, soms ten koste van jullie zelf! Ik hoop dat jullie het gevoel hebben dat het allemaal niet voor niets is geweest. Ook gun ik jullie nog vele jaren, waarin zelf genieten voorop staat! Ik ben blij dat mijn wieg bij jullie stond…

Lieve Brenda: van alle mensen die hierboven staan genoemd, verdien jij absoluut de meeste dank. Al ruim 8 jaar ben jij mijn onvoorwaardelijke steun en toeverlaat, en ken jij als geen ander de "downsides". Samen opnieuw beginnen in Boston en later weer in Rotterdam was een onvergetelijke (levens)ervaring! Dat zit allemaal in dit boekje! Dank ook voor alle hulp en adviezen hierbij. En verder: "ook".

CURRICULUM VITAE AUCTORIS

Frank Johan Marinus Frederik Dor was born on April 15th, 1976 in Rotterdam, The Netherlands. From 1988-1994 he attended the Gymnasium Erasmianum in Rotterdam. After graduation, he studied Medicine at the Rijks Universitair Centrum Antwerpen, Belgium. In 1995, he started his medical studies at the Erasmus University Rotterdam (EUR), The Netherlands.
During the second year of medical school, he became interested in research and started a project at the department of Chemical Pathology (Prof. Dr H.G. van Eijk), leading to his first publications. Immunology became favorite; following electives at the department of Immunology (Prof. Dr R. Benner), he got involved in a research project on endocrine autoimmune diseases (Prof. Dr H.A. Drexhage). In 1998, he was offered the opportunity to work in the Royal Free Hospital in London, UK, for some months at the department of Chemical Pathology & Human Metabolism (Prof. Dr. A.F. Winder). In the same year he became a student research fellow at the departments of Cardiothoracic Surgery (Prof. Dr A.J.J.C. Bogers) and Transplantation (Prof. Dr W. Weimar) of the Erasmus MC Rotterdam, where he was involved in a research project investigating heart valve transplantation in a rat model until 2001.
In 1999, he received a Fujisawa-Grant for a clinical training in (Transplantation) Surgery at the Universitätsklinik Ulm, Germany (Dr M. Storck). Later, in 2000, he visited the Philipps Universitätsklinik Marburg for a clinical training in General and Vascular Surgery (Prof. Dr M. Rothmund / Prof. Dr M. Storck).
He received the Hippocrates Study Prize in 2000 because of "excellent scientific research fulfilled during the medical education".

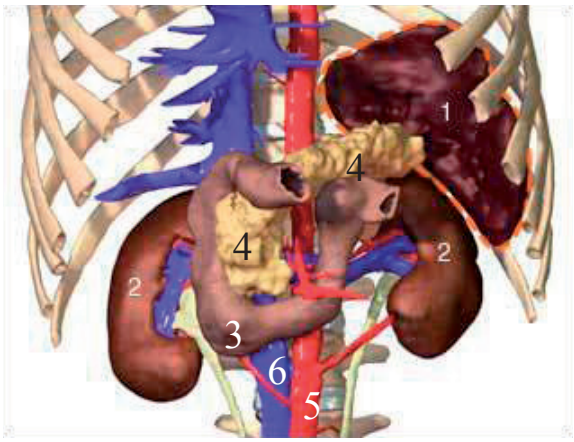
After graduating *cum laude* from Medical School in November 2001, Frank started his PhD project in January 2002 at the Transplantation Biology Research Center, Massachusetts General Hospital, Harvard Medical School, in Boston, USA (Prof. Dr D.K.C. Cooper / Prof. Dr D.H. Sachs). Here, he performed his experimental work leading to this dissertation until January 2004, with financial support of the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences (KNAW), the Prof. Michaël-van Vloten Fund, and the Netherland-America Foundation. During this period, he received a Trainee Award of the Basic Sciences Symposium 2003 of the Transplantation Society, and the Young Investigators Award of the American Transplant Congress 2004 (The American Society of Transplant Surgeons and The American Society of Transplantation). The Royal Netherlands Academy of Arts and Sciences (KNAW) awarded the final scientific report at the end of his Fellowship with the predicate *cum laude*.

Frank started his surgical residency at the Department of Surgery of the Erasmus MC Rotterdam (Prof. Dr J.N.M. IJzermans / Prof. Dr H.J. Bonjer) in April 2004 and continued his training in surgery at the Sint Franciscus Gasthuis Rotterdam (Dr C.H. Wittens) in April 2006. In April 2009, he will return to the Erasmus MC for his last year of surgical training.

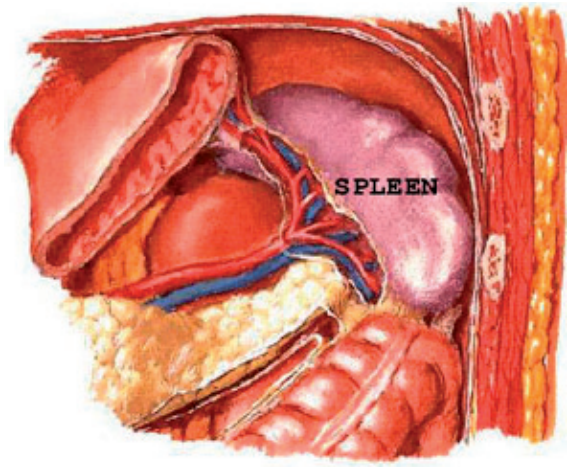
In 2005, Frank was elected as a member of the Editorial Boards of the "ASAIO Journal" (American Society for Artificial Internal Organs) and the "Internet Journal of Surgery". In the same year, he was appointed as a teacher at the Erasmus MC Educational Institute for ER nurses. He was appointed as Managing Editor of "Frontiers in Bioscience" in 2006, and is currently editing a special issue of the Journal, entitled "Transplantation: current developments and future directions".

He is a reviewer for the international journals "Xenotransplantation", the "American Journal of Transplantation", "Transplant International", "Artificial Organs", "The Journal of Immunology", the "International Journal of Surgery", the "Journal of Pathology", and "Clinical and Experimental Immunology".
Professional memberships include: The Transplantation Society, The American Society of Transplantation, The International Xenotransplantation Association, The European Society of Organ Transplantation, The Dutch Society of Surgery and The Dutch Society of Transplantation.

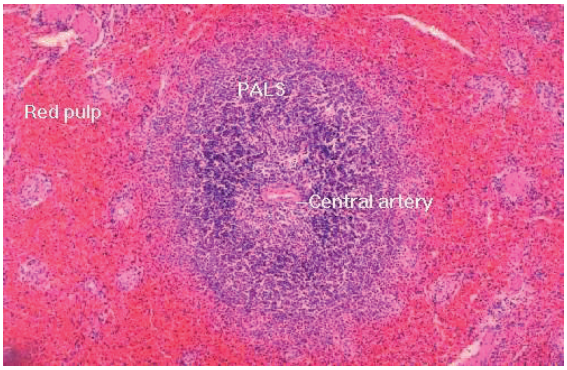
Every end is a new beginning…



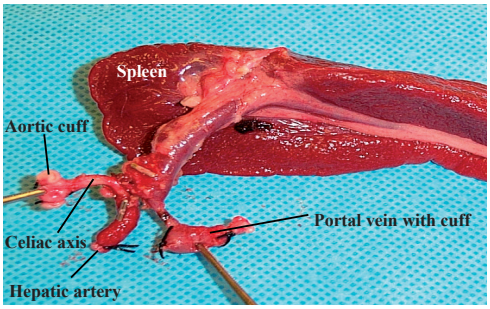
Chapter 1 Figure 1. Anatomical position of the human spleen.
1, spleen; 2, kidney; 3, duodenum; 4, pancreas; 5, abdominal aorta; 6, inferior vena cava.



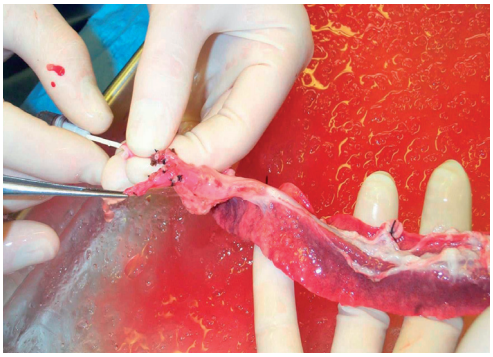
Chapter 1 Figure 2. Anatomy of the upper abdomen with respect to the spleen.



Chapter 1 Figure 3A. Cross section of the spleen with white and red pulp zones (Giemsa-stained).



A



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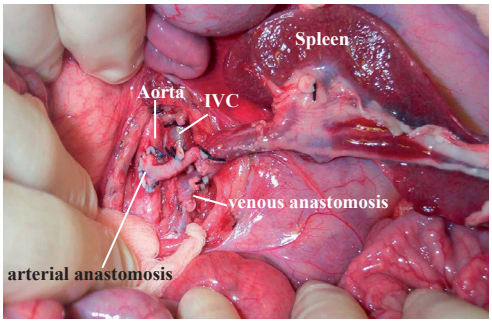
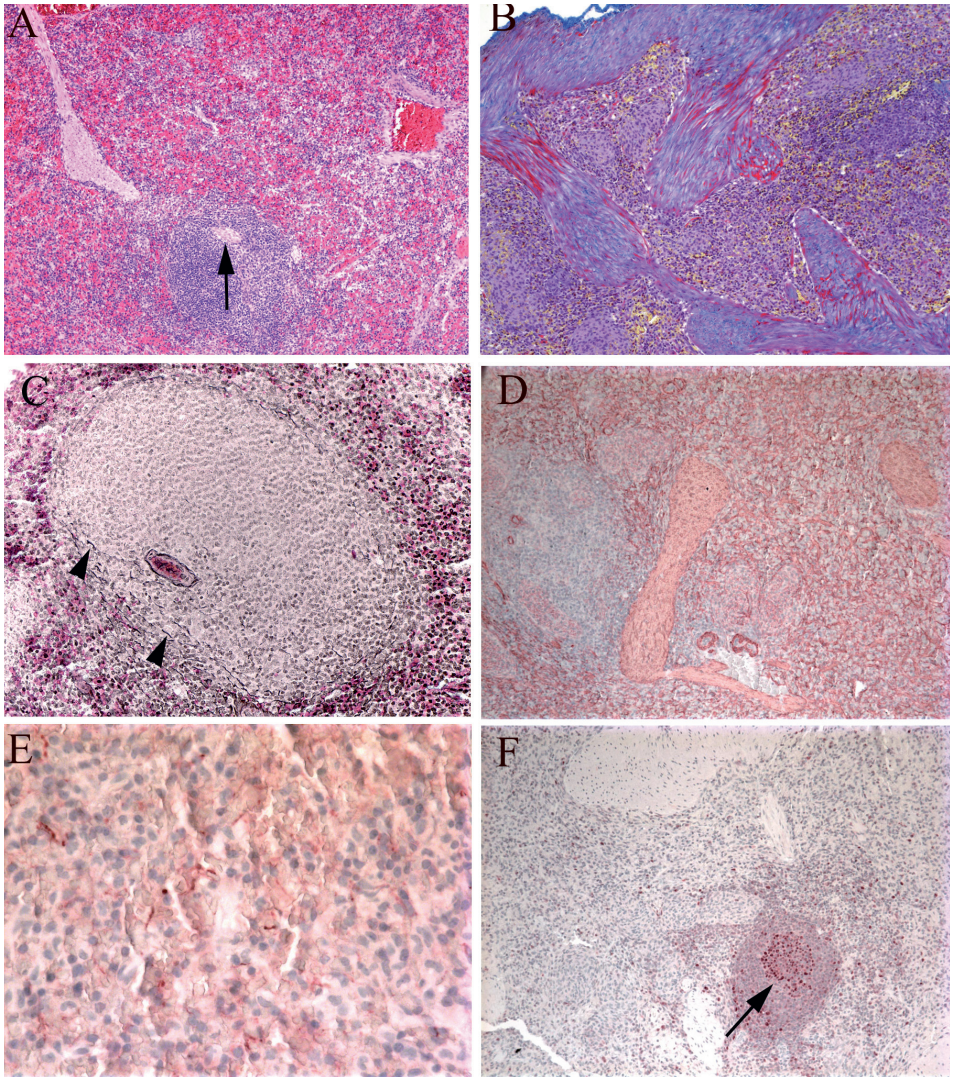


C

Chapter 4 Figure 1. Surgical technique of spleen transplantation in miniature swine.
(A) In the donor, excision of the spleen involves dissection of the splenic artery and celiac axis to the aorta and of the splenic vein to its confluence with the superior mesenteric vein to form the portal vein, which necessitates total pancreatectomy. Before transplantation into the recipient, the hepatic artery stump will be shortened.
(B) Flushing of the spleen graft with cold (4°C) electrolyte solution for kidney preservation trough the splenic artery, while placed in a tray of cold (4°C) saline, until the effluent from the splenic vein is clear.
(C) In the recipient, end-to-side anastomoses are performed between the cuff of the donor aorta to the distal abdominal aorta and between the cuff of the donor portal vein to the IVC.



Chapter 5 Figure 1. Normal histology of porcine spleen is illustrated with various staining techniques.

(A) Red pulp is predominant, and white pulp contains a small central artery (arrow), H&E

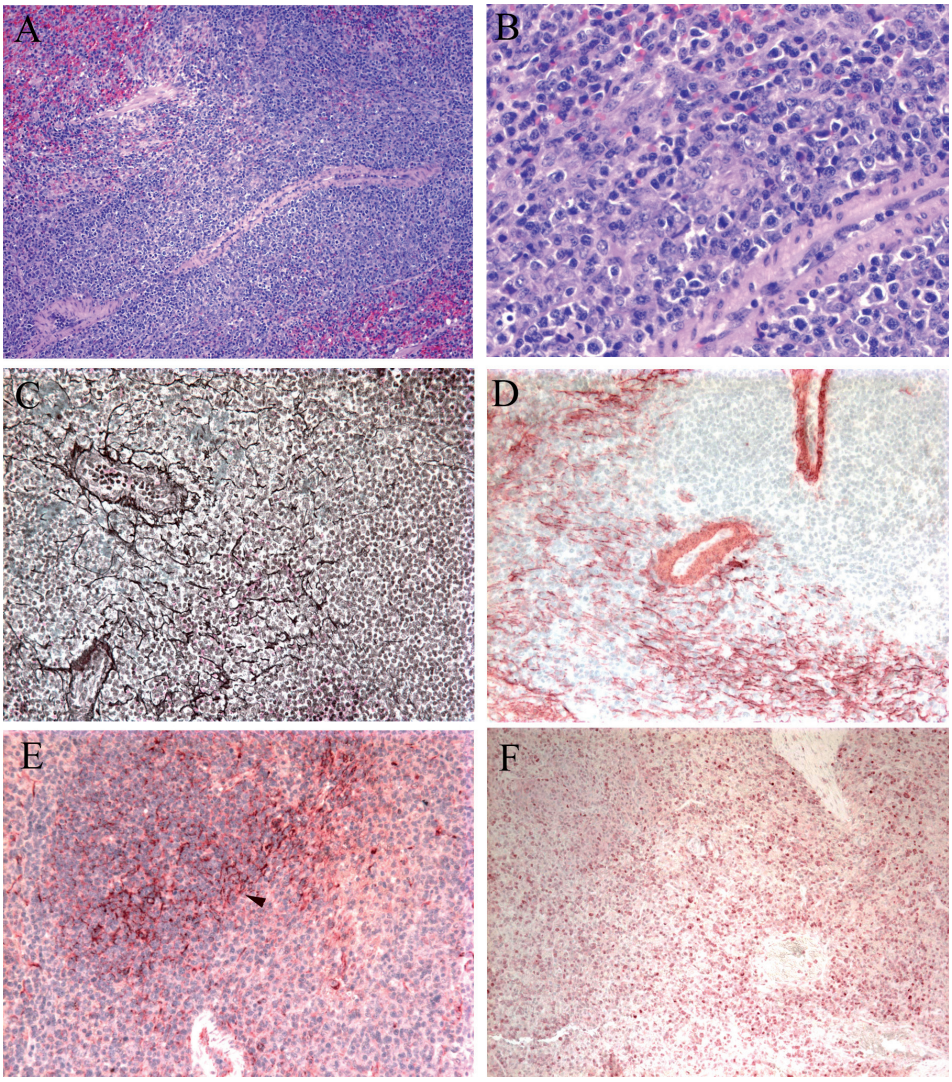
(B) Capsule and trabeculae, containing abundant blue-stained collagen, trichrome stain

(C) Scant collagen fibers (arrow heads) in periarteriolar lymphatic sheaths (PALS), reticulin stain

(D) Smooth muscle cells of trabeculae and of vessels and sinusoids in the white and red pulp express actin, SMA stain

(E) Red pulp, with delicately stained sinusoidal endothelial and/or smooth muscle cells, vimentin stain

(F) Normal cellular proliferative activity in red pulp and lymphoid follicle, with prevalence of staining in a germinal center (arrow), PCNA stain



Chapter 5 Figure 2. Histology associated with expanded PALS (#14867, Group 1, 15 days after SpTx) can be compared to baseline findings seen in Figure 1.

(A) Marked expansion of PALS around central artery, H&E

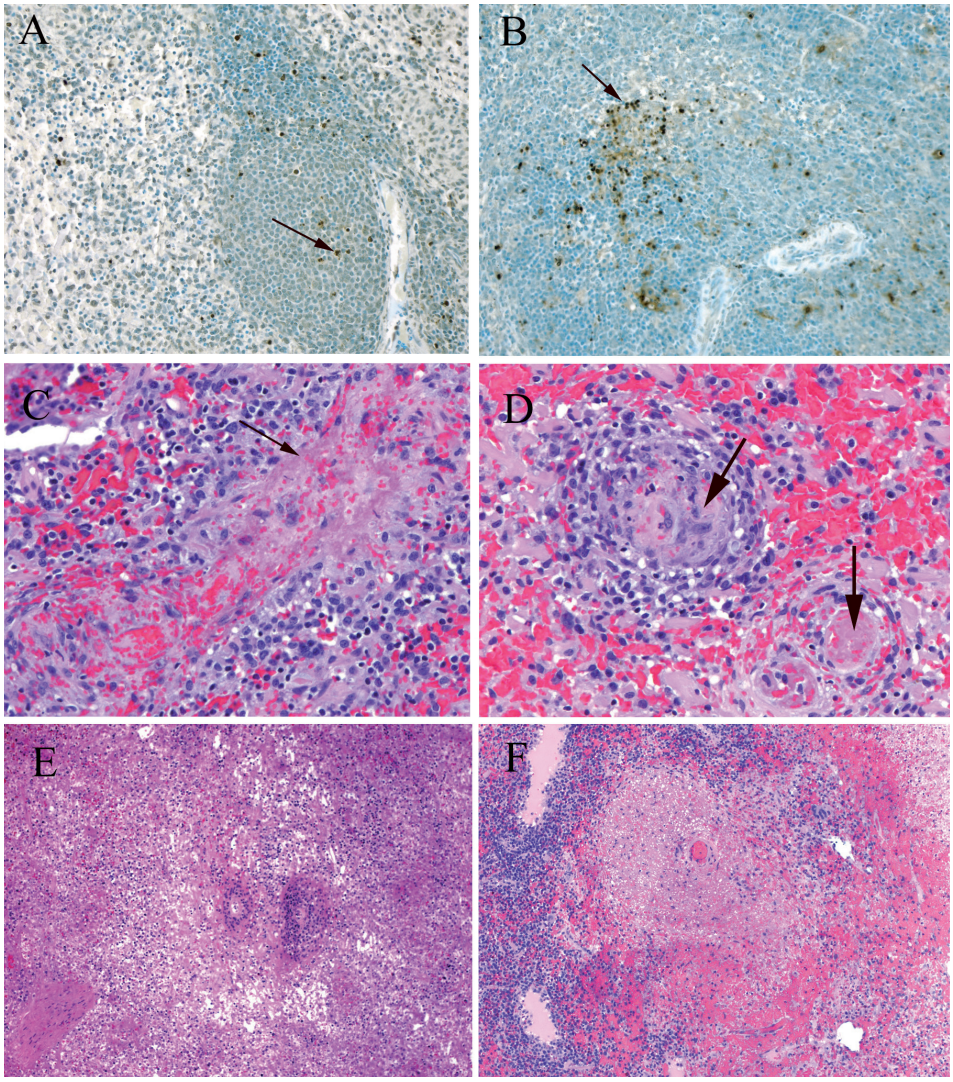
(B) High power view of pleomorphic, atypical lymphoid cells in the PALS, H&E

(C) Increased density of collagen fibers in PALS, reticulin stain

(D) Increased smooth muscle cell staining in red pulp adjacent to PALS, SMA stain

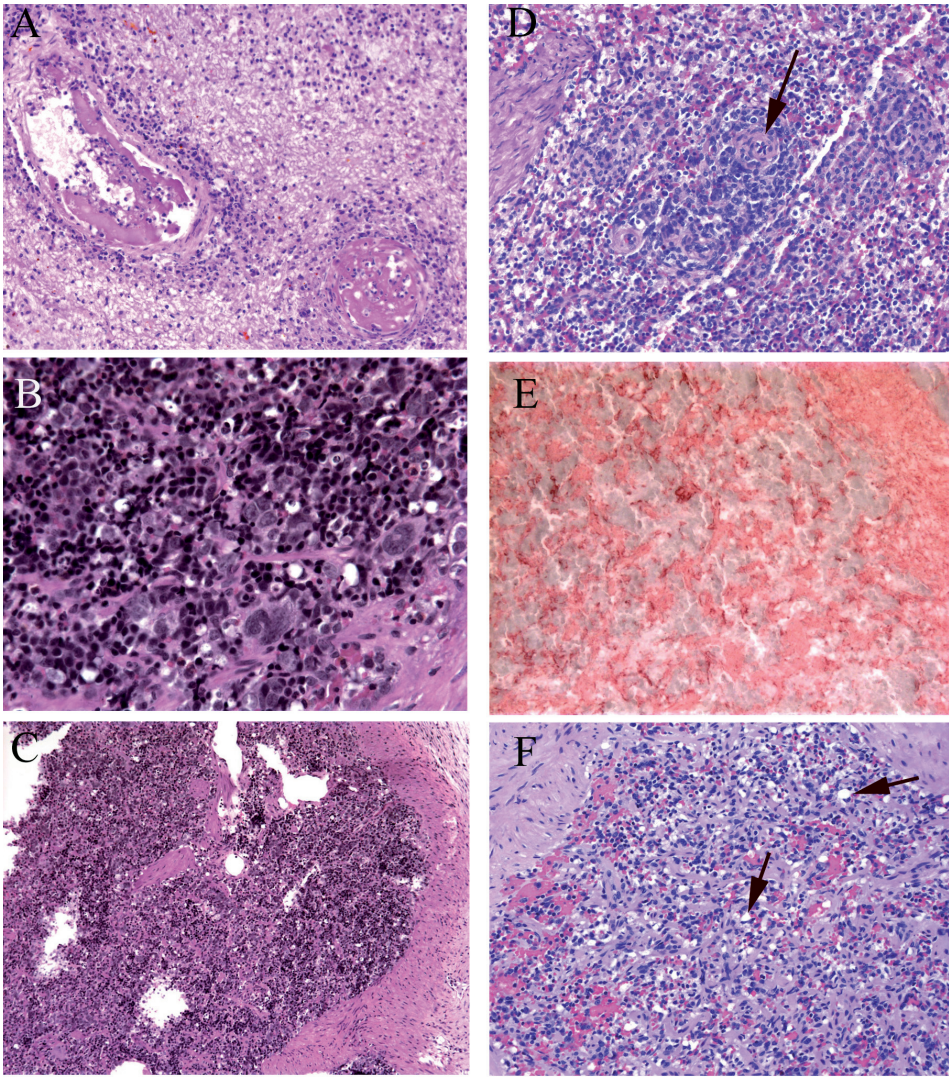
(E) Similar increase in staining of smooth muscle-like cells and/or endothelial cells (arrowhead) in expanded PALS, vimentin stain

(F) Increased proliferative activity associated with expanded PALS, PCNA stain



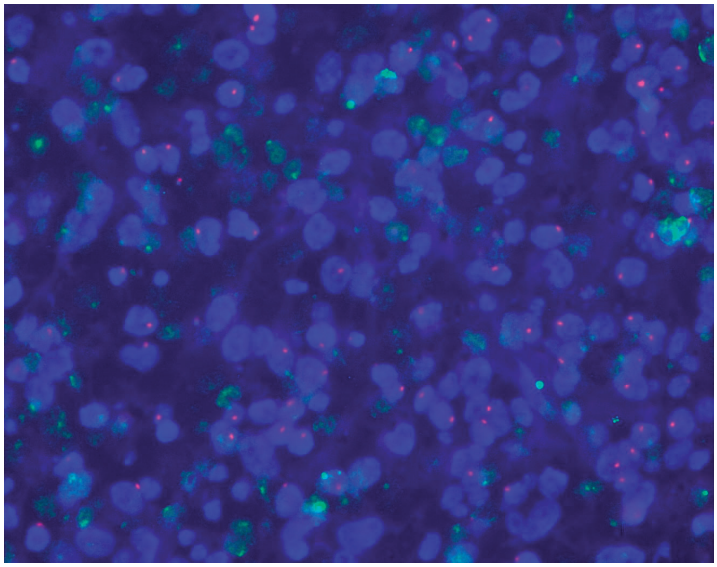
Chapter 5 Figure 4.

A TUNEL index at baseline on the day of SpTx (A) (#14867, Group 1) is increased more than two-fold 15 days after SpTx (B) (#14867, Group 1) before a subsequent biopsy showed histologic evidence of rejection (arrows mark TUNEL-positive cells). Spleen allograft necrosis varied morphologically from focal, fibrinoid necrosis of sinusoidal walls (C) (#14526, Group 1, 57 days after SpTx) (arrow), associated with fibrin thrombi in small vessels (D) (#14526, Group 1, 57 days after SpTx) (arrows), to diffuse liquefactive necrosis (E) (#14867, Group 1, 28 days after SpTx). Necrosis was usually associated with some hemorrhage (F) (#14867, Group 1, 28 days after SpTx).



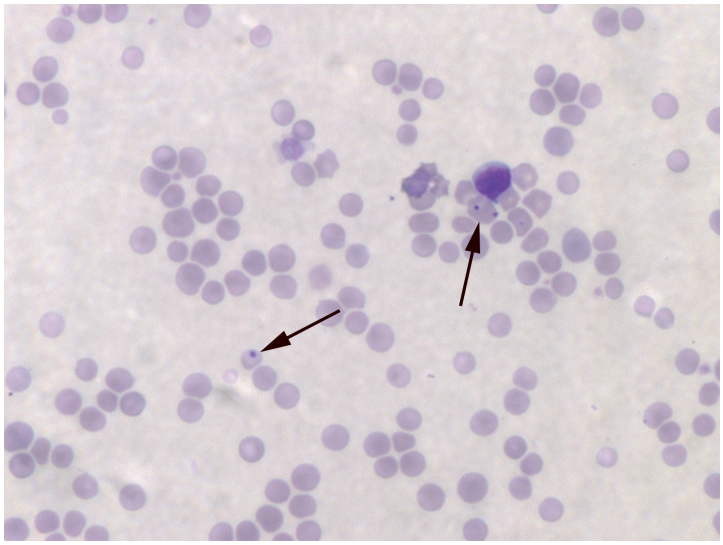
Chapter 5 Figure 5.

Other features of late rejection included acute vasculitis accompanying vessel wall necrosis (A) (#14646, Group 2, 28 days after SpTx), and, in one graft, extramedullary hematopoiesis (#14832, Group 2, 47 days after SpTx) (B and C). Histological changes associated with prolonged graft survival included atrophic white pulp (D) (#15051, Group 2, 57 days after SpTx) (arrow points to a central artery); increased SMA expression in red pulp (E) (#14526, Group 1, 160 days after SpTx), SMA stain; and an increase in vacuolated histiocytes (arrows) in red pulp (F) (#14526, Group 1, 160 days after SpTx).

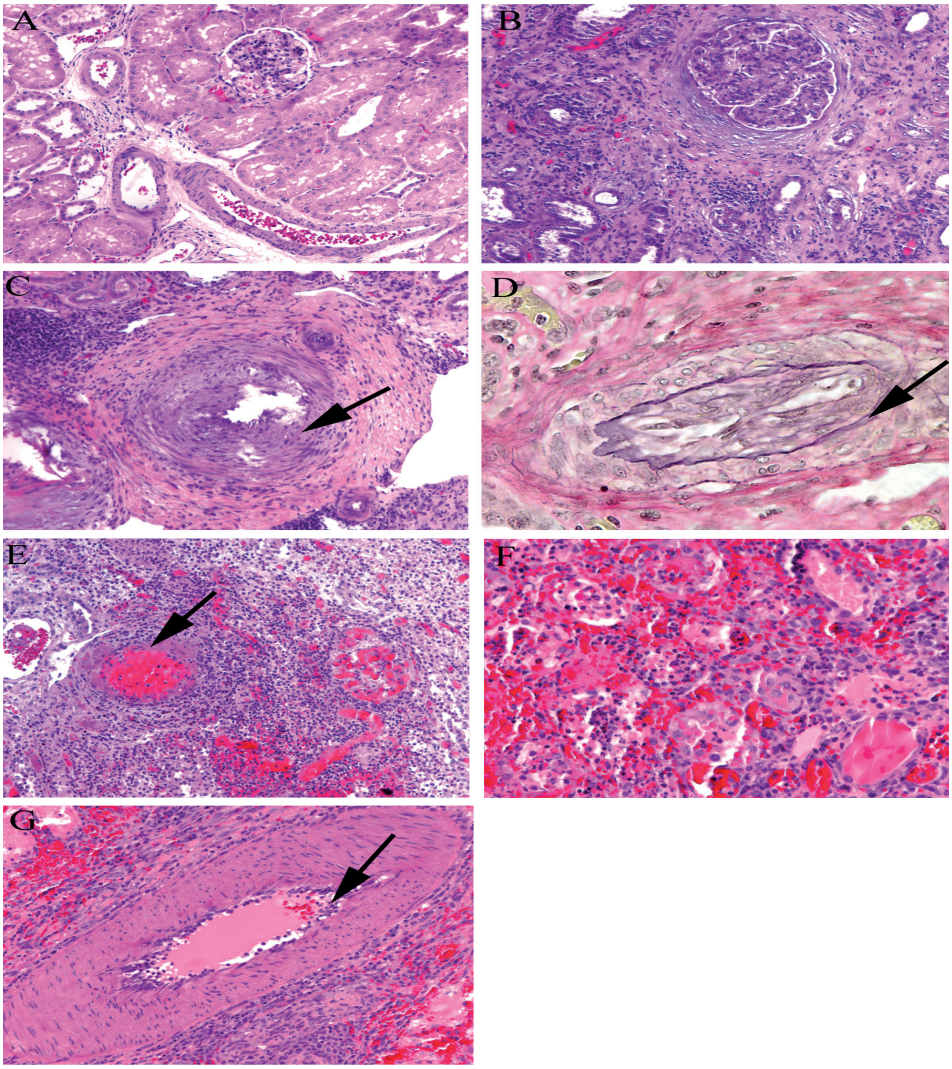


Chapter 5 Figure 7.

Section of spleen which was transplanted from a female donor into a male recipient (#14840, Group 2, 43 days after SpTx). A notable number of recipient-derived cells with Y chromosomes (red signal) has populated the graft at the time of expanded white pulp (day 43), FISH technique, x400.



Chapter 5 Figure 8. Red blood cells (arrows) contain Howell-Jolly bodies in a peripheral blood smear of a rejected (Grade 3) spleen allograft (#15311, Group 2, 75 days after SpTx, two weeks after spleen graft rejection (characterized by coagulative necrosis), Wright stain x640.



Chapter 6 Figure 7. Histopathology of kidney allografts in two Group B pigs tolerant to their spleen grafts and in two 'control' pigs (Groups A and C). Pig 15111 (tolerant to MHC class I-mismatched spleen graft, Group B); the kidney (MHC-matched to the spleen donor) showed (A) no features of rejection after almost 3 months (H&E x160). Pig 15410 (tolerant to full MHC-mismatched spleen graft, Group B); after 129 days the kidney showed (B) type 1 acute cellular rejection with an interstitial mononuclear cell infiltrate, tubulitis, and glomerulopathy in the absence of vasculitis (H&E x160); evidence of chronic rejection was manifested by interstitial fibrosis and tubular atrophy, as well as (C and D) occlusive arteriopathy; arrows indicate internal elastic lamina (H&E x200 and Verhoeff elastic stain x 640, respectively). Pig 15733 (Group A, that lost its full MHC-mismatched spleen graft from vascular pedicle compression on day 3) developed (E) acute cellular rejection of the kidney graft by day 15; arrow indicates fibrinoid necrosis of arterial wall (H&E x160). Pig 15330 (Group C) received a kidney graft from a donor MHC-matched to the donor of a blood transfusion given on day 0, and developed severe acute cellular and humoral rejection within 4 days, marked by (F) tubular necrosis, proteinaceous casts, and interstitial hemorrhage (H&E x320), as well as (G) endothelialitis (arrow) (H&E x200).