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Tissue Engineering the Thymus and Secondary Lymphoid Organs

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Abstract
Tissue engineering is making great strides to repair disease and damage in a wide range of tissues, many of which are high profile and well documented. However, the thymus and secondary lymphoid organs are tissues which have not received significant attention from the research community but are nonetheless important targets for tissue engineering based therapies. These organs are fundamental in developing and maintaining the mammalian immune system and create environments for antigen screening and pathogen removal. This review discusses the function of these organs with reference to tissue development, tissue resident progenitor cells and disease. Subsequently strategies utilised for tissue engineering and regeneration are discussed in conjunction with methods to replicate their function and alternative methods to generate progenitor cells.

Introduction

Tissue engineering is a multidisciplinary research field which aims to reproduce, repair or augment tissue function of whole or partial organs in individuals suffering disease or trauma. The field has roots in clinical medicine and employs the principles of engineering and materials chemistry with cell biology. This involves the selective use of cells, scaffold biomaterials, growth factors and mechanical stimulation in various combinations to recapitulate an organ *in vitro* or *in vivo*. It was first mentioned as a distinct area of research in the 1980's, before coming to prominence through Langer and Vacanti's seminal 1993 article *Tissue Engineering* (Skalak and Fox 1988; Langer and Vacanti 1993). Since this point thousands of research articles have been published on engineering or repairing practically every organ of the human body, with particular focus on musculoskeletal tissues (2201 publications in 2012), the heart (422) and skin (314). Two groups however, have received comparatively little attention; these are the primary and secondary lymphoid organs (SLO) (less than 10 specific articles in 2012). The primary lymphoid organs are composed of the bone marrow and the thymus, with the thymus being the major site of T cell generation, whilst the SLO comprise lymph nodes and lymphoid follicles in organs such as the spleen, tonsils and adenoids. As a group these organs generate and maintain the mammalian immune system and are fundamental to survival. Despite the comparative lack of effort advances have been made in attempting to regenerate these organs or recapitulate their functions and these will be discussed.

Thymus

The thymus is a primary lymphoid organ responsible for the production and dispersion to the periphery of lymphocytes, generically known as thymocytes or T cells due to their thymic origin (Miller 1961; Zuniga-Pflucker 2004). T cells develop in the thymus from haematopoietic stem cell (HSC) derived precursor cells which originate in the bone marrow and undergo differentiation, maturation and selection via a series of chemokine mediated cell-cell interactions in specific microenvironments (Figure 1) (Bommhardt *et al.* 2004; Gordon *et al.* 2004). Two main classes of T cell exist, CD8 cytotoxic T cells which destroy foreign antigens and CD4 helper T cells which mediate the immune response (Harty *et al.* 2000; Zhu and Paul 2008). Other cells produced by the thymus are regulatory T cells (TRegs), which play a major role in immunological self tolerance and negative control of the immune response and natural killer T cells (NKT cells) which are also involved in self tolerance as well as cytokine secretion (Vignali *et al.* 2008; Hegde *et al.* 2010).

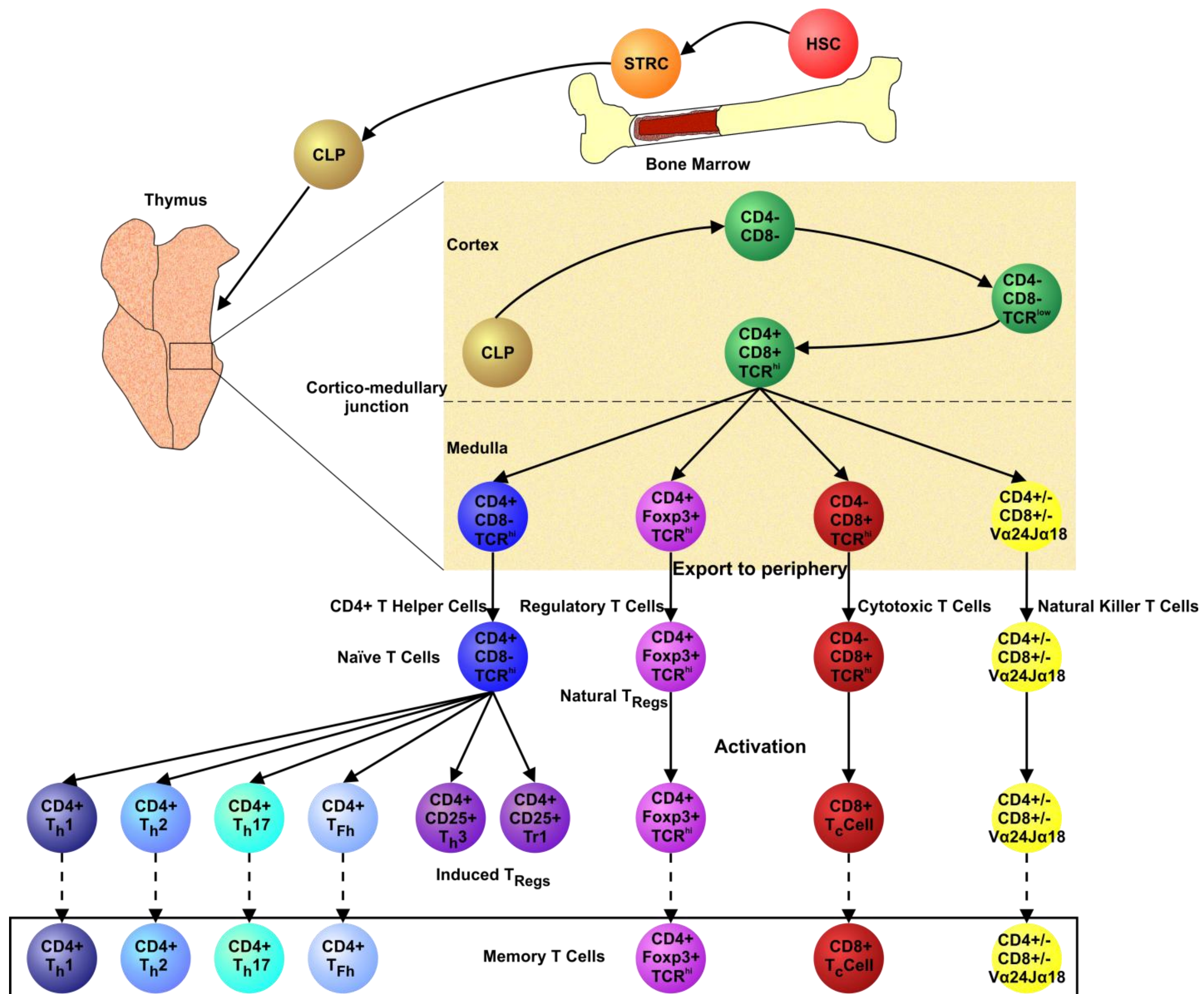

Figure 1. Differentiation of HSC derived precursor cells to multiple cell types within the thymus. Within the bone marrow of long bones, short term repopulating cells (STRC), derived from HSC differentiate to the common lymphoid progenitor (CLP) phenotype prior to entering the blood. CLPs subsequently migrate to the thymus, entering at the cortico-medullary junction, before undergoing a controlled series of molecular changes, the most significant being the expression of the T cell receptor (TCR), whilst passing through, and interacting with, the defined microarchitecture of the thymic cortex. Once the developing T cells express a TCR they migrate to the thymic medulla, at this stage they are known as double positive thymocytes because of their $CD4^+$ $CD8^+$ phenotype. In the medulla developing T cells are subject to positive and negative selection to remove both non-reactive and auto-reactive T cells. It is during this process that T cell lineage commitment is made and cells become either: $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, TRegs or NKT cells. The cells are subsequently exported to the periphery, usually to SLO such as the spleen, as naïve cells which become mature and activated upon interaction with a foreign antigen. $CD4^+$ T helper cells undergo a further round of differentiation upon activation to one of the four T helper cell phenotypes or to an induced TReg phenotype. Following activation most cells are lost, however a small subset of each cell type become memory T cells which recognise foreign antigens upon subsequent infection.

Thymus and T Cell Development

T cell development is a complex process involving the up and down regulation of many genes and the rearrangement of cell surface receptors and for this process to function efficiently a normal thymic stromal phenotype of cortex, medulla and subcapsular zone is required (Mohtashami and Zuniga-Pflucker 2006; Sitnicka 2009). This phenotype is important because each region has specific functions in thymocyte development and the epithelial cells which make up each region provide a framework upon which developing T cells reside (von Gaudecker *et al.* 1986; van Ewijk *et al.* 2000; Milicevic and Milicevic 2004). One potentially dangerous outcome of incorrect thymocyte development is the generation of autoreactive T cells which fail to recognise self antigens and attack the host's own tissue leading to potentially lethal conditions (Rodewald 2008). Therefore the thymus also functions to screen and remove potentially dangerous T cells, as well as ensuring T cells which are released to the periphery can recognise and react to foreign antigens (Zuniga-Pflucker 2004).

Correct differentiation of lymphocytes also has implications for the proper structural development of the thymus because of the need for cellular crosstalk between thymic epithelial cells and progenitor T cells. The absence of T cell progenitors has been shown to lead to abnormal cortical and medullary regions in cultured thymi (Anderson and Jenkinson 2001; Germeraad *et al.* 2003; Gill *et al.* 2003; Zuniga-Pflucker 2004; Gordon and Manley 2011). Disruption of the thymus structure by, for example, chemotherapy, has also been shown to affect T cell generation and patients with DiGeorge syndrome, where a deletion in chromosome 22q11 causes thymic aplasia or hypoplasia, have reduced functional T cells (Hendrickx and Dohring 1989; Greenberg 1993). However, recent findings have shown that the thymus is a dynamic environment with some potential inherent regenerative capacity after procedures such as thymectomy during juvenile cardiac surgery (Anderson *et al.* 2009; van Gent *et al.* 2011). This regenerative capacity is probably controlled by resident progenitor cells and it has been shown that a reduction in the initial progenitor pool reduces subsequent regenerative potential (Jenkinson *et al.* 2008).

Thymus Progenitor Cells

For several years thymic origin and the existence of a thymic epithelial progenitor cell was debated, with suggestions that the thymus had a dual origin and that cortex and medulla were formed by ectodermal and endodermal contributions respectively (Cordier and Haumont 1980). However, data presented in 2002 and 2006 disputed this theory and confirmed, in mice, the presence of a thymic epithelial progenitor cell of endodermal origin which expressed MTS24 and epithelial cell adhesion molecule 1 (EpCAM 1, CD326) and was capable of producing both cortical and medullary regions (Gill *et al.* 2002; Gordon *et al.* 2004; Bleul *et al.* 2006; Rossi *et al.* 2006). Further studies showed that the protein Placenta-expressed transcript-1 (Plet-1) interacts with the MTS24 marker during thymus organogenesis and can be utilised to study progenitor cell behaviour (Depreter *et al.* 2008). The presence of a thymic progenitor cell is an important finding as it suggests the possibility of stimulating endogenous thymic regeneration clinically and also because further study may elucidate the mechanisms of differentiation which can then be utilised in stem cell differentiation experiments.

Thymus Disease and Atrophy

One aspect of thymus biology where it varies with other organs is age related atrophy; which is the progressive replacement of stromal tissue with fatty deposits. This process leads to a reduction in overall thymus stromal volume and subsequently a reduction in its capacity to generate naïve T cells. In healthy individuals atrophy begins at puberty and persists throughout adulthood, however recent evidence suggests that chronic infections or autoimmune disorders can trigger an upregulation in tumour necrosis factor (TNF) levels, which may in turn accelerate the process meaning individuals can

become immunocompromised due to a reduction in thymopoiesis (Liepinsh *et al.* 2009).

Aside from pre-programmed atrophy and infection, there are several other instances whereby thymus function is impaired, either congenitally or by acquirement. The most striking example of a congenital condition is DiGeorge syndrome which is a multifaceted disorder resulting from a deletion in chromosome 22q11 causing thymic hypoplasia, leading to significantly reduced T cell generation and an increased susceptibility to infections (Greenberg 1993). The symptoms of DiGeorge syndrome vary greatly and as well as thymic hypoplasia can include cardiac and facial abnormalities, cleft palate and hypoparathyroidism. Another congenital condition that leads to thymic dysplasia is severe combined immunodeficiency syndrome (SCID), which can be caused by several different genetic factors and is characterised by a non-functional immune response leading to increased susceptibility to infections (Bosma *et al.* 1983). This lack of response is due to the incorrect development of lymphocyte precursor cells in the bone marrow, leading to a significant reduction in the number of precursor cells migrating to the thymus. This subsequently has negative impacts on thymus development and homeostasis and leads to thymus atrophy due to the importance of cellular crosstalk between thymic epithelial cells and developing T lymphocytes (Anderson and Jenkinson 2001; Poliani *et al.* 2009).

HIV infection is the most studied cause of acquired thymic atrophy and is due to the destruction of T cells within the thymus, leading to the loss of thymic cortex (Grody *et al.* 1985). In addition, various other chronic bacterial, fungal and parasitic infections can lead to thymus atrophy, again due to loss of T cells with the possible involvement of the TNF pathway (Savino 2006; Gruver and Sempowski 2008; Liepinsh *et al.* 2009). Many of these chronic infections cause severe depletion of circulating T cells, as well as thymus atrophy, meaning that individuals become immunocompromised because they are less capable of replacing peripheral T cells lost due to infection. In addition to infection induced atrophy is the phenomena of stress induced atrophy (Gruver and Sempowski 2008). In these instances environmental stresses such as malnutrition and emotional stress can lead to involution of the thymus with an associated reduction in lymphopoiesis, leading to individuals becoming immunocompromised during episodes of acute stress. The precise mechanism of this process is not fully understood but it is known to be reversible, highlighting the inherent regenerative capacity present within the thymus. For this reason studies to investigate the molecular signals behind this 'rebound' effect, and potentially harness it, are ongoing.

The above examples pertain to diseases where various factors lead to thymus atrophy with subsequent reductions in function; however there are also diseases where thymic hyperplasia or hypertrophy is associated with a disease. For example one of the symptoms of the neuromuscular autoimmune disorder Myasthenia Gravis (MG) is hyperplasia in the thymus medulla with alterations in cell patterning and differences in T cell rich areas compared to healthy controls (Bofill *et al.* 1985; Hofmann *et al.* 1987). The specific pathology of MG is a current area of research but evidence suggests that incorrect recruitment and development of lymphocytes to and within the thymus is a causative reason for the disease with thymectomy often used as a treatment for MG patients (Balandina *et al.* 2005; Spillane *et al.* 2013; Weiss *et al.* 2013). Often associated with MG is thymoma, a tumour of thymic epithelial cells which is again mostly treated by surgical resection (Kalhor and Moran 2012). Other instances where thymic hyperplasia has been shown are: thymic 'rebound' after acute stress, sarcoidosis and in many endocrinopathies such as Addison's disease (Hofmann *et al.* 1987).

Thymus Tissue Engineering
Attempts to engineer an artificial thymus have mostly used traditional tissue engineering approaches involving chemical scaffolds and cells liberated from isolated tissues, although newer methods involving decellularised organs show some potential. More simplistic methods to 'engineer' a thymus are foetal thymus organ culture (FTOC) and reaggregate thymus organ culture (RTOC) (Figure 2) (Anderson and Jenkinson 2007; White *et al.* 2008). These are both methods whereby the foetal thymus is ablated of T cell precursors prior to addition of HSC derived cells. This is as a whole organ or as a dissociated cell suspension and emerging T cells are assessed by their phenotype (Ueno *et al.* 2005). These methodologies are important for studying the function of the thymus, the cellular processes involved in T cell generation and the action of thymus epithelial progenitor cells. They are however limited as methods for replacing damaged thymus tissue due to the need to dissociate and recapitulate the tissue.

The action of thymic progenitor cells can be seen in studies where individuals with complete DiGeorge anomaly had cultured thymus tissue transplanted (Li *et al.* 2011). In these procedures functional thymus tissue developed at the site of transplantation, further investigation confirmed that this tissue contained histologically distinct regions and that the formation of this tissue was consistent with the action of a thymic epithelial progenitor cell. Alternative methodologies that can utilise progenitor cell action are decellularised organ scaffolds, stripped of native cells before repopulation with progenitor cells (Park and Woo 2012). These scaffolds hold promise for use in lymphoid tissue regeneration.

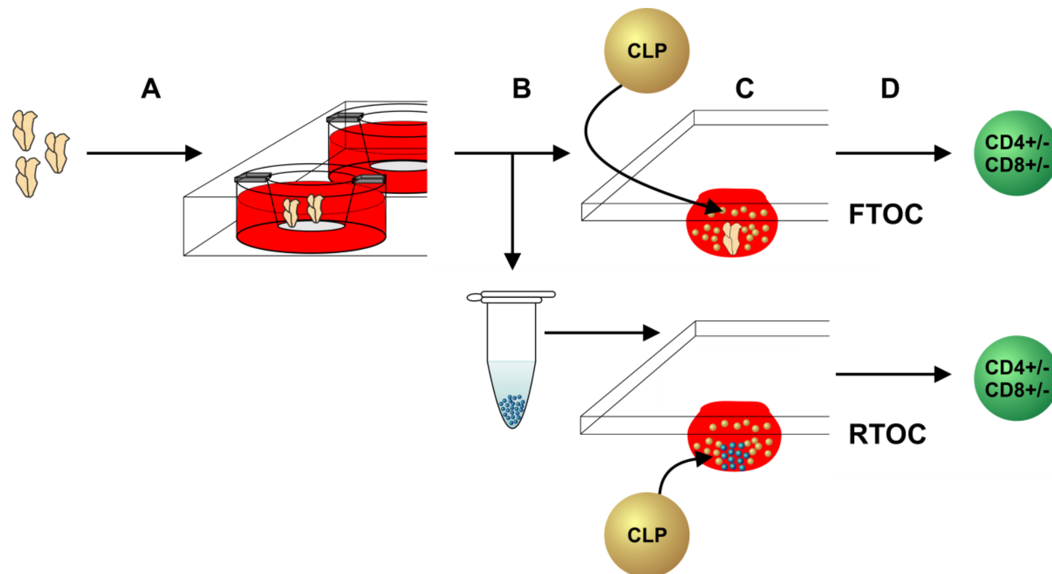

Figure 2. Formation of foetal and reaggregate thymus cultures.
Diagram developed from protocols described in Anderson and Jenkinson (2007). FTOC and RTOC cultures are established by culturing isolated embryonic thymi with 2'-deoxyguanosine to remove endogenous thymocytes (A). Treated thymi are subsequently either enzymatically digested for RTOC studies (B) or are placed directly into hanging drop cultures (FTOC). Following enzymatic digestion thymic cells for RTOC studies are also placed in hanging drop cultures. Whole or digested thymi are cultured in hanging drops in the presence of CLP cells or precursor thymocytes at various stages of differentiation (C). Subsequently thymocyte development can be assessed to understand thymus function and thymocyte colonisation of the thymus (D) (Anderson and Jenkinson 2007).

Studies which have utilised synthetic scaffolds to engineer a thymus have shown some promise in generating *in vitro* thymus tissue. In 2000 Poznansky *et al*. generated a thymic organoid *in vitro* using cultured thymic stromal cells on tantalum coated carbon scaffolds prior to the addition of lymphocyte precursor cells (Figure 3) (Poznansky *et al.* 2000). Following a two week culture period the emerging T cells were assessed phenotypically and were found to have mature $CD4^+$ and $CD8^+$ phenotypes, functional assessment also found evidence of a response to external challenge. From this study the importance of both scaffold porosity and pore size for cell attachment and development were shown and also the potential for the *in vitro* generation of T cells. Figure 4 shows a further example of an engineered thymic organoid, in this model embryonic mouse thymic epithelial cells, sorted for the expression of EpCAM-1, were cultured on electrospun poly-L-lactic acid scaffolds in a rotating bioreactor, though these constructs were not tested for functionality.

Mouse chamber model studies with foetal mouse thymus tissue, encapsulated within Matrigel and a silicone chamber prior to implantation into the inguinal fat pad of athymic mice, showed the potential to generate functional T cells within a cell-scaffold construct (Seach *et al.* 2010). These murine tissue engineered constructs were vascularised and viable 11 weeks after implantation; however human paediatric thymus tissue treated using the same experimental regimen showed more limited success. Despite the success of this model in generating functional T cells, its limited success with sustaining human thymus tissue makes this model more suitable for the study of lymphopoiesis.

Alternative strategies to repair the thymus are based on endogenous tissue regeneration. Studies of thymus cortical epithelial cells, sensitised to, and treated with diphtheria toxin showed cell death followed by endogenous regeneration after cessation of treatment with an associated restoration of T cell generation (Rode and Boehm 2012). This study highlights the latent regenerative capacity thymus epithelium possesses, however the molecular signals which guide this process must be elucidated before this functionality can be utilised in a clinical environment. One molecule involved in thymus regeneration is interleukin 22 (IL-22) which has been shown to be upregulated following thymus injury (Dudakov *et al.* 2012). This molecule was shown to enhance thymic regeneration when administered following irradiation, indicating its potential utility in

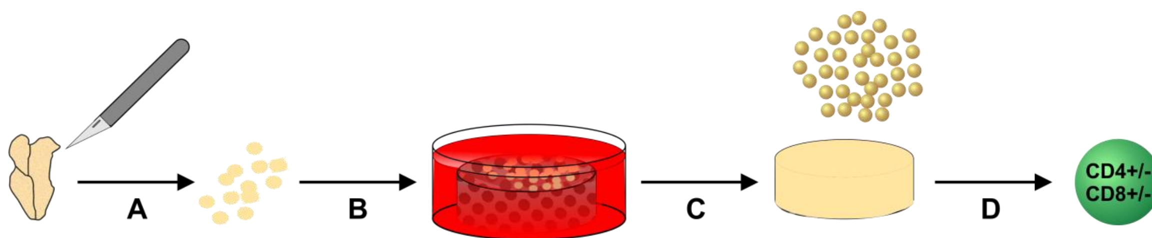

Figure 3. Development of an *in vitro* thymic organoid.
Protocol as described by Poznansky *et al*. (2000). Isolated thymi from adult mice were dissociated to give a tissue fragments (A), these fragments were subsequently cultured on the surface of carbon-based scaffolds (B) for two weeks until the thymic stromal cell coverage of the scaffold disc was approximately 80%. To this cell-scaffold construct lymphocyte progenitor cells were added (C) and over the subsequent 21 days any non-adherent cells were counted and phenotyped by flow cytometry (D) (Poznansky *et al.* 2000).

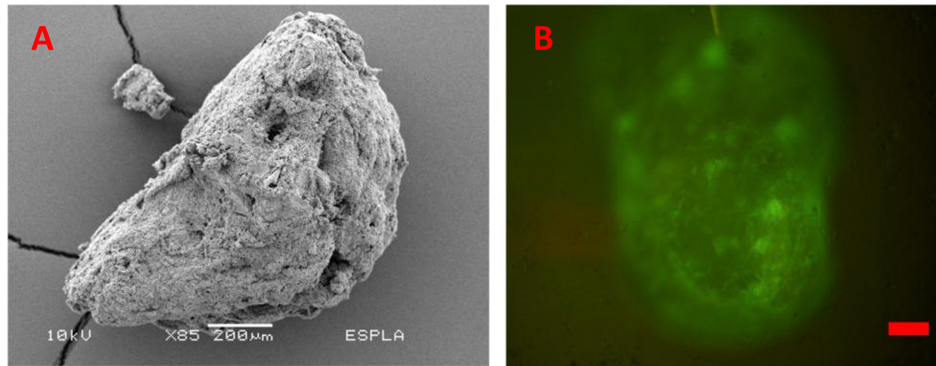

Figure 4. Examples of thymus tissue engineering.
(A) shows a scanning electron micrograph of EpCAM-1 sorted mouse embryonic thymic epithelial cells seeded to an electrospun poly-L-lactic acid scaffold and cultured for 7 days in a rotating wall vessel bioreactor, bar = 200 µm. (B) shows a fluorescent light micrograph of the same cells, labelled with cell tracker green, again seeded to an electrospun poly-L-lactic acid scaffold and cultured for 7 days, bar = 100 µm, modified from Tomlinson (2009).

thymus regeneration strategies. IL-7, cloned and expressed in conjunction with the β-chain of hepatocyte growth factor has also been shown to stimulate proliferation of thymic epithelium and consequently support T cell production (Jin *et al.* 2011). Use of molecular therapeutic agents to harness inherent thymus regeneration potential is an attractive strategy for thymus repair and replacement and it will be of interest to follow this field of study as progress is made towards clinical therapy.

Thymic Epithelial Cells from ES Cells
Despite the presence of thymic epithelial progenitor cells in the postnatal thymus there are problems associated with their use, not least the isolation of these cells by conventional cell sorting methodologies and the acquisition of matched donor tissue which has not undergone significant atrophy. For this reason there is interest being shown in methods to generate thymic epithelial cells and thymus progenitor cells from pluripotent stem cells for the restoration of thymus function. Initial work with mouse embryonic stem cells (mESC) showed differentiation to a thymic epithelial progenitor cell phenotype (Lai and Jin 2009). *In vivo* transplantation of these cells showed self-renewal, differentiation into epithelial cells, restoration of normal thymic architecture and enhancement of T cell generation. Subsequent mESC studies demonstrated the potential of this technique in enabling the generation of T cells in young and old recipients following allogeneic bone marrow transplant (Lai *et al.* 2011). These individuals showed no evidence of graft versus host disease and generated T cells were tolerant of self, mESC and transplanted bone marrow antigens. These results highlight the potential of this technique for the rapid reestablishment of the immune system following ablation therapy. Recent studies with mouse induced pluripotent stem cells (miPSC) have used chemically defined conditions for the generation of thymic epithelial progenitor cells that have the capacity to differentiate to medullary epithelial cells (Inami *et al.* 2011). Studies with human pluripotent stem cells are not yet as advanced as those involving mouse cells, however recent data with both human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) have described the differentiation of these cells to an anterior foregut endoderm phenotype (Green *et al.* 2011). This step is important in the production of human thymic progenitor and epithelial cells *in vitro*, as this is the germ layer from which these cells derive during *in vivo* organogenesis (Gordon *et al*. 2004).

Generation of T cells *in vitro*

Whilst the generation of a functional thymus from stem cells is an attractive option for many conditions, it is both technically challenging and, in many instances where immune reconstitution is needed, unnecessary. In these situations individuals may require an infusion of functional T cells to reestablish immune function following a procedure such as bone marrow transplant. For these purposes it may be preferable to generate a patient matched population of T cells *in vitro*, as opposed to an allogeneic cell transplant with the potential risks of donor mismatch.

One potential method for achieving this aim is co-culture of HSC with stromal cells over-expressing Notch ligands such as delta-like-1 and delta-like-4 which bind to the Notch-1 receptor on HSC (Karanu *et al.* 2001). Mouse stromal cell lines which have been engineered to overexpress these ligands, such as OP9-DL1 and S17-DL1, have been shown to induce lymphopoietic differentiation of HSC from a variety of tissue sources in co-culture, leading to the establishment of mature phenotypes similar to that of T cells derived from the thymus (De Smedt *et al.* 2004; Van Coppernolle *et al.* 2009; Mohtashami *et al.* 2010; Awong *et al.* 2011). Engineered overexpression of the delta-like-1 ligand by human thymic epithelial cells has recently been shown to induce lymphopoiesis from human cord blood and bone marrow derived HSC in a co-culture system, highlighting the importance of this ligand in lineage specification (Beaudette-Zlatanova *et al.* 2011). An alternative study with human HSC derived T cell precursors showed that human skin derived cells were capable of directing differentiation to a T cell phenotype (Clark *et al.* 2005). In this study skin derived keratinocytes and fibroblasts were seeded to a tantalum coated carbon matrix and co-cultured with lymphocyte precursors producing functionally mature T cells with a diverse receptor repertoire and evidence of self-tolerance. As well as cell culture models, some studies have attempted to utilise transgenic animal models, such as swine, to direct differentiation of human bone marrow derived HSC leading to the generation human T cells which respond to human antigen presenting cells (Ogle *et al.* 2009).

Methods which utilise the differentiation of ESC to generate T cells have also been investigated, with different factors being used to induce differentiation. The thymic peptides thymosin alpha-1 and thymopeptides have been shown to induce sequential differentiation of mESC in an embryoid body system to CD4/CD8 T cells (Peng *et al.* 2008). Reaggregate thymic organ cultures have been used as a model system to study the differentiation of haematopoietic precursor cells derived from mESC co-cultured with OP9-DL1 stromal cells (de Pooter *et al.* 2003). These precursor cells were isolated from early stage differentiating mESC prior to introduction to the culture system and confirmed the ability of the thymus to induce differentiation of mESC derived precursor HSC. Further studies using OP9 cells in co-culture with hESC have also shown that hESC can be differentiated to a lymphocyte lineage under the influence of delta-like-1 ligand (Timmermans *et al.* 2009).

Generation of T cells for transplantation is a potential strategy for reconstitution of the immune system; it is also a potential method to induce tolerance to tissue engineered products, reducing the possibility of rejection. However this method also requires the presence of a functional thymus to continue the production of T cells which allow tolerance to the transplant, thereby highlighting the potential need to either engineer a functional thymus or find methods to reverse age related thymic atrophy (Seach *et al.* 2007).

Secondary Lymphoid Organs

SLO are fundamental constituents of the mammalian immune system comprising, for example, lymph nodes, spleen and tonsils. SLO are reservoirs for mature lymphocytes in the periphery of the body, away from sites of lymphopoiesis, and act as sites of lymphocyte activation upon antigen presentation (Cyster 1999). Generally SLO organogenesis depends on

interactions between lymphoid tissue inducer (LTi) cells and lymphoid tissue organiser (LTo) cells, although as yet no definitive SLO progenitor cell has been discovered (Glanville *et al.* 2009; Tan and Watanabe 2010).

As with the thymus, the structure of SLO is very important for tissue function, although in these instances this is due to the need to mount an efficient immune response to foreign antigens rather than the development of T cells (Mebius and Kraal 2005). This immune response is a complex chemokine driven process in which antigen presenting cells migrate through the SLO interacting with lymphocytes and potentially causing activation and thus an immune response (Cyster 2005). Structurally there are differences between SLO, with the spleen consisting of two main regions, the red pulp, involved in erythrocyte filtration, and the white pulp which contains the lymphocyte population with further subdivisions based upon localisation of T and B cells and antigen presentation (Tan and Watanabe 2010). Lymph node structure is similar to the white pulp and is composed of cortical and medullary regions which contain lymphocytes and antigen presenting cells, however they lack analogous regions to the red pulp (Cyster 2005). This also highlights a major difference between these two SLO, the spleen is presented by antigens from the blood, whereas the lymph nodes are supplied by lymphatic vessels.

Diseases of the Spleen and SLO

There are several conditions which lead to abnormal function of the spleen and SLO and potentially necessitate treatment or excision. Of the conditions which affect the spleen, splenomegaly, or the enlargement of the spleen, is the most common and can arise due to leukaemia and lymphoma. Other causes of splenomegaly include Epstein-Barr virus infection, sickle cell anaemia and malaria, leading to abnormal splenic function (Stuart and Nagel 2004). Following diagnosis of splenomegaly individuals may require a full or partial splenectomy to remove the enlarged area. Splenectomy may also be required in instances of trauma or rupture leading to internal bleeding, though use of this procedure is declining (Di Sabatino *et al.* 2011). As well as splenectomy, individuals can be asplenic and lack a functional spleen for several other reasons including rare congenital conditions or through acquired means such as sickle-cell anaemia. Several other diseases, such as coeliac disease and rheumatoid arthritis, can lead to individuals being hyposplenic and having reduced splenic function (Di Sabatino *et al.* 2011). Hyposplenia or asplenia can lead to complications with individuals being at greater risk of developing sepsis and having an increased susceptibility to infection. Additionally the risk of thrombus formation increases due to increased platelet circulation and individuals may not respond to vaccination as well due to the function of the spleen in harbouring mature T cells. There is also evidence to suggest that splenectomised patients are at greater risk of developing hyperglycemia and potentially diabetes (Ley *et al.* 2012).

Conditions which affect the lymph nodes are generally grouped together under the term lymphadenopathies due to the overall swelling of the tissue, however within this term there are distinct etiologies. The most common cause of lymphadenopathy is in response to infection and occurs due to T cell expansion within the lymph node and trafficking of T cells from the blood. Lymphadenitis is a form of lymphadenopathy which can also occur due to infection but is caused by microbiological infection within the lymph node leading to swelling. However, these conditions are common, do not require excision and are most often benign. Serious forms of lymphadenopathy where lymph nodes are abnormal in size, consistency or number are often due to primary or metastatic tumours where neoplastic proliferation leads to an increase in the size of the nodes (Ferrer 1998). Primary tumours, lymphoma and leukaemia occur when lymphocytes or macrophages undergo neoplastic proliferation within the lymph node. Metastatic tumours occur when

cancerous cells from tumours located in other organs infiltrate the lymphatic system and migrate to the lymph nodes leading to secondary tumour formation. In these instances lymphadenectomy can be required to remove the tumour, which can lead to complications such as lymphoedema or a lymphocele leading to tissue swelling around the excision site (Gould *et al.* 2001).

SLO Tissue Engineering

Attempts to recreate SLO have so far been fairly limited compared to other tissues; however there have been some interesting studies which have attempted to engineer these important organs, many of which are detailed by Tan and Watanabe, Hitchcock and Niklason and Kobayashi *et al.* (Hitchcock and Niklason 2008; Tan and Watanabe 2010; Kobayashi *et al.* 2011). Of the tissues generated one of the first studies was the engineering of generic SLO tissue organoids (Suematsu and Watanabe 2004). In this work thymus stromal cells were embedded into collagenous scaffolds prior to transplantation to a mouse kidney capsule. This led to the formation of structures with morphological similarities to SLO, such as areas of B and T cell organisation and follicular dendritic cell networks. It was also found that these lymphoid structures had functional similarities in the production of antibodies to introduced antigens. Subsequent studies developed artificial lymph nodes and showed that following transplantation to mice these structures were able to initiate a sustained immune response up to four weeks post-transplantation and that lymphoid cells from the nodes migrated to, and expanded in, the spleen and bone marrow (Okamoto *et al.* 2007).

Bioreactors have been designed for the culture of artificial lymph nodes *in vitro*, in one early study induced dendritic cells were seeded to an agarose-polyamide matrix and co-cultured with lymphocytes (Giese *et al.* 2006). Following a two-week culture period these lymphocytes showed clustering on the matrix and evidence of activation in response to stimulation. Indeed it has been shown that the use of three-dimensional bioreactors can maintain SLO stromal cells in culture and can partially recapitulate the structure, organisation and function of the organs *in vitro* demonstrating the potential for these systems in studying SLO and trying to recreate them for transplantation (Kuzin *et al.* 2011).

Some studies have attempted to develop scaffolds which support the generation of functional lymph node-like structures, such as an agarose-polyamide matrix (Giese *et al*. 2006). Other studies have utilised polyethylene glycol-based hydrogels with ordered macropores coated with extracellular matrix proteins to enhance dendritic cell attachment to, and lymphocyte migration through, the scaffold (Irvine *et al.* 2008). These scaffolds support interactions between dendritic cells and lymphocytes and can be functionalised so they contain cytokines and chemokines for sustained release during culture, giving them further utility. Further studies with macroporous scaffolds employed porous polyurethane scaffolds containing type I collagen and Matrigel to assess the importance of interstitial flow on immune cell migration and the correct development of lymph nodes *in vitro* (Tomei *et al.* 2009).

The work mentioned previously has examined lymph node generation or a generic SLO, however other studies have attempted to specifically engineer a spleen-like organ *in vitro*, prior to implantation and bacterial challenge (Grikscheit *et al.* 2008). In this work tissue fragments of juvenile rat spleen were seeded to tubular scaffolds of nonwoven polyglycolic acid sheets, sealed with poly-L-lactic acid (in chloroform solvent) before implantation to splenectomised rats. Following implantation pneumococcal sepsis was induced and rat survival was shown to be significantly greater in rats with tissue engineered spleen compared to spleen slices and untreated splenectomised rats. This work highlights the potential of using tissue engineered SLO compared to traditional methods and demonstrates the need for such

solutions, especially in younger immuno-compromised patients.

Many of the methods to reproduce SLO *in vitro* which have been detailed rely on fibroblastic cell lines or the recapitulation of SLO cells into a SLO-like structure with the assistance of scaffolds and bioreactors, however, alternative cell sources may be required for this work to progress clinically. Presently there are few protocols for the differentiation of stem cells to a SLO stromal cell phenotype. Recent work has identified adipocyte precursor cells present within the embryonic fat pad and lymph node stroma with the capacity to differentiate to lymph node stromal organiser cells, linking lymphoid development with adipose tissue (Bénézech *et al.* 2012). This offers the attractive possibility of utilising a patient's own adipose precursor cells to produce lymphoid stromal cells *in vitro* prior to implantation into the patient (Boehm 2012).

Conclusion

Recapitulation of functional lymphoid tissue is a challenging area within tissue engineering due to the complexity of the organs and the requirement for specific architectures. However, should these difficulties be overcome, then regeneration of lymphoid tissue could lead to great gains in several therapeutic areas (Cupedo *et al.* 2012). For individuals who have suffered loss of lymphoid organs through disease or surgical intervention, the possibility of organ replacement may lead to the reestablishment of immune function and likewise for those who have reduced circulating lymphocytes following disease. These applications are not the limit for engineered lymphoid tissue, it may be possible to utilise lymphoid tissue engineering in concert with other surgical interventions to reduce the possibility of immune rejection of transplanted allogeneic tissue or engineered tissue. Additionally there is great interest in using artificial lymphoid organs as a treatment for some forms of cancer. As such engineering of lymphoid tissues is an important area of research for a multitude of conditions and one which is beginning to increase in recognition.

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References

1. Anderson G and Jenkinson EJ. 2001. Lymphostromal interactions in thymic development and function. Nat. Rev. Immunol. **1**(1): 31-40.

2. Anderson G and Jenkinson EJ. 2007. Fetal Thymus Organ Culture. Cold Spring Harbor Protocols **2007**(8): pdb.prot4808.

3. Anderson G, Jenkinson EJ and Rodewald HR. 2009. A roadmap for thymic epithelial cell development. Eur. J. Immunol. **39**(7): 1694-1699.

4. Awong G, Herer E, La Motte-Mohs RN and Zuniga-Pflucker JC. 2011. Human CD8 T cells generated in vitro from hematopoietic stem cells are functionally mature. BMC Immunol. **12**: 22.

5. Balandina A, Lécart S, Dartevelle P, Saoudi A and Berrih-Aknin S. 2005. Functional defect of regulatory CD4+CD25+ T cells in the thymus of patients with autoimmune myasthenia gravis. Blood **105**(2): 735-741.

6. Beaudette-Zlatanova BC, Knight KL, Zhang S, Stiff PJ, Zuniga-Pflucker JC and Le PT. 2011. A human thymic epithelial cell culture system for the promotion of lymphopoiesis from hematopoietic stem cells. Exp. Hematol. **39**(5): 570-579.

7. Bénézech C, Mader E, Desanti G, Khan M, Nakamura K, White A, Ware Carl F, Anderson G and Caamaño Jorge H. 2012. Lymphotoxin-β receptor signaling through NF-κB2-RelB pathway reprograms adipocyte precursors as lymph node stromal cells. Immunity **37**(4): 721-734.

8. Bleul CC, Corbeaux T, Reuter A, Fisch P, Monting JS and Boehm T. 2006. Formation of a

functional thymus initiated by a postnatal epithelial progenitor cell. Nature **441**(7096): 992-996.

9. Boehm T. 2012. Caught in the act: Reprogramming of adipocytes into lymph-node stroma. Immunity **37**(4): 596-598.

10. Bofill M, Janossy G, Willcox N, Chilosi M, Trejdosiewicz LK and Newsom-Davis J. 1985. Microenvironments in the normal thymus and the thymus in myasthenia gravis. Am. J. Pathol. **119**(3): 462-473.

11. Bommhardt U, Beyer M, Hunig T and Reichardt HM. 2004. Molecular and cellular mechanisms of T cell development. Cell. Mol. Life Sci. **61**(3): 263-280.

12. Bosma GC, Custer RP and Bosma MJ. 1983. A severe combined immunodeficiency mutation in the mouse. Nature **301**(5900): 527-530.

13. Clark RA, Yamanaka K, Bai M, Dowgiert R and Kupper TS. 2005. Human skin cells support thymus-independent T cell development. J. Clin. Invest. **115**(11): 3239-3249.

14. Cordier AC and Haumont SM. 1980. Development of thymus, parathyroids, and ultimo-branchial bodies in NMRI and nude mice. Am. J. Anat. **157**(3): 227-263.

15. Cupedo T, Stroock A and Coles M. 2012. Application of tissue engineering to the immune system: development of artificial lymph nodes. Front. Immunol. **3**: 343.

16. Cyster JG. 1999. Chemokines and cell migration in secondary lymphoid organs. Science **286**(5447): 2098-2102.

17. Cyster JG. 2005. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu. Rev. Immunol. **23**(1): 127-159.

18. de Pooter RF, Cho SK, Carlyle JR and Zuniga-Pflucker JC. 2003. In vitro generation of T lymphocytes from embryonic stem cell-derived prehematopoietic progenitors. Blood **102**(5): 1649-1653.

19. De Smedt M, Hoebeke I and Plum J. 2004. Human bone marrow CD34+ progenitor cells mature to T cells on OP9-DL1 stromal cell line without thymus microenvironment. Blood Cells Mol. Dis. **33**(3): 227-232.

20. Depreter MG, Blair NF, Gaskell TL, Nowell CS, Davern K, Pagliocca A, Stenhouse FH, Farley AM, Fraser A, Vrana J, Robertson K, Morahan G, Tomlinson SR and Blackburn CC. 2008. Identification of Plet-1 as a specific marker of early thymic epithelial progenitor cells. Proc. Natl. Acad. Sci. U. S. A. **105**(3): 961-966.

21. Di Sabatino A, Carsetti R and Corazza GR. 2011. Post-splenectomy and hyposplenic states. The Lancet **378**(9785): 86-97.

22. Dudakov JA, Hanash AM, Jenq RR, Young LF, Ghosh A, Singer NV, West ML, Smith OM, Holland AM, Tsai JJ, Boyd RL and van den Brink MR. 2012. Interleukin-22 drives endogenous thymic regeneration in mice. Science **336**(6077): 91-95.

23. Ferrer R. 1998. Lymphadenopathy: Differential diagnosis and evaluation. Am. Fam. Physician **58**(6): 1313-1320.

24. Germeraad WT, Kawamoto H, Itoi M, Jiang Y, Amagai T, Katsura Y and van Ewijk W. 2003. Development of thymic microenvironments in vitro is oxygen-dependent and requires permanent presence of T-cell progenitors. J. Histochem. Cytochem. **51**(9): 1225-1235.

25. Giese C, Demmler CD, Ammer R, Hartmann S, Lubitz A, Miller L, Muller R and Marx U. 2006. A human lymph node in vitro--challenges and progress. Artif. Organs **30**(10): 803-808.

26. Gill J, Malin M, Hollander GA and Boyd R. 2002. Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells. Nat. Immunol. **3**(7): 635-642.

27. Gill J, Malin M, Sutherland J, Gray D, Hollander G and Boyd R. 2003. Thymic generation and regeneration. Immunol. Rev. **195**: 28-50.

28. Glanville SH, Bekiaris V, Jenkinson EJ, Lane PJ, Anderson G and Withers DR. 2009.

Transplantation of embryonic spleen tissue reveals a role for adult non-lymphoid cells in initiating lymphoid tissue organization. Eur. J. Immunol. **39**(1): 280-289.

29. Gordon J and Manley NR. 2011. Mechanisms of thymus organogenesis and morphogenesis. Development **138**(18): 3865-3878.

30. Gordon J, Wilson VA, Blair NF, Sheridan J, Farley A, Wilson L, Manley NR and Blackburn CC. 2004. Functional evidence for a single endodermal origin for the thymic epithelium. Nat. Immunol. **5**(5): 546-553.

31. Gould N, Kamelle S, Tillmanns T, Scribner D, Gold M, Walker J and Mannel R. 2001. Predictors of Complications after Inguinal Lymphadenectomy. Gynecol. Oncol. **82**(2): 329-332.

32. Green MD, Chen A, Nostro MC, d'Souza SL, Schaniel C, Lemischka IR, Gouon-Evans V, Keller G and Snoeck HW. 2011. Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. Nat. Biotechnol. **29**(3): 267-272.

33. Greenberg F. 1993. DiGeorge syndrome: An historical review of clinical and cytogenetic features. J. Med. Genet. **30**(10): 803-806.

34. Grikscheit TC, Sala FG, Ogilvie J, Bower KA, Ochoa ER, Alsberg E, Mooney D and Vacanti JP. 2008. Tissue-engineered spleen protects against overwhelming pneumococcal sepsis in a rodent model. J. Surg. Res. **149**(2): 214-218.

35. Grody WW, Fligiel S and Naeim F. 1985. Thymus involution in the acquired immunodeficiency syndrome. Am. J. Clin. Pathol. **84**(1): 85-95.

36. Gruver AL and Sempowski GD. 2008. Cytokines, leptin, and stress-induced thymic atrophy. J. Leukoc. Biol. **84**(4): 915-923.

37. Harty JT, Tvinnereim AR and White DW. 2000. CD8+ T cell effector mechanisms in resistance to infection. Annu. Rev. Immunol. **18**: 275-308.

38. Hegde S, Fox L, Wang X and Gumperz JE. 2010. Autoreactive natural killer T cells: promoting immune protection and immune tolerance through varied interactions with myeloid antigen-presenting cells. Immunology **130**(4): 471-483.

39. Hendrickx P and Dohring W. 1989. Thymic atrophy and rebound enlargement following chemotherapy for testicular cancer. Acta Radiol. **30**(3): 263-267.

40. Hitchcock T and Niklason L. 2008. Lymphatic tissue engineering: Progress and prospects. Ann. N. Y. Acad. Sci. **1131**: 44-49.

41. Hofmann WJ, Möller P and Otto HF. 1987. Thymic hyperplasia. Klin. Wochenschr. **65**(2): 49-52.

42. Inami Y, Yoshikai T, Ito S, Nishio N, Suzuki H, Sakurai H and Isobe K. 2011. Differentiation of induced pluripotent stem cells to thymic epithelial cells by phenotype. Immunol. Cell Biol. **89**(2): 314-321.

43. Irvine DJ, Stachowiak AN and Hori Y. 2008. Lymphoid tissue engineering: Invoking lymphoid tissue neogenesis in immunotherapy and models of immunity. Semin. Immunol. **20**(2): 137-146.

44. Jenkinson WE, Bacon A, White AJ, Anderson G and Jenkinson EJ. 2008. An epithelial progenitor pool regulates thymus growth. J. Immunol. **181**(9): 6101-6108.

45. Jin J, Goldschneider I and Lai L. 2011. In vivo administration of the recombinant IL-7/hepatocyte growth factor beta hybrid cytokine efficiently restores thymopoiesis and naive T cell generation in lethally irradiated mice after syngeneic bone marrow transplantation. J. Immunol. **186**(4): 1915-1922.

46. Kalhor N and Moran CA. 2012. Thymoma: current concepts. Oncology (Williston Park) **26**(10): 975-981.

47. Karanu FN, Murdoch B, Miyabayashi T, Ohno M, Koremoto M, Gallacher L, Wu D, Itoh A, Sakano S and Bhatia M. 2001. Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. Blood **97**(7): 1960-1967.

48. Kobayashi Y, Kato K and Watanabe T. 2011. Synthesis of functional artificial lymphoid tissues. Discov. Med. **12**(65): 351-362.

49. Kuzin I, Sun H, Moshkani S, Feng C, Mantalaris A, Wu JH and Bottaro A. 2011. Long-term immunologically competent human peripheral lymphoid tissue cultures in a 3D bioreactor. Biotechnol. Bioeng. **108**(6): 1430-1440.

50. Lai L, Cui C, Jin J, Hao Z, Zheng Q, Ying M, Boyd R and Zhao Y. 2011. Mouse embryonic stem cell-derived thymic epithelial cell progenitors enhance T-cell reconstitution after allogeneic bone marrow transplantation. Blood **118**(12): 3410-3418.

51. Lai L and Jin J. 2009. Generation of thymic epithelial cell progenitors by mouse embryonic stem cells. Stem Cells **27**(12): 3012-3020.

52. Langer R and Vacanti JP. 1993. Tissue Engineering. Science **260**(5110): 920-926.

53. Ley EJ, Singer MB, Clond MA, Johnson T, Bukur M, Chung R, Margulies DR and Salim A. 2012. Long-term effect of trauma splenectomy on blood glucose. J. Surg. Res. **177**(1): 152-156.

54. Li B, Li J, Devlin BH and Markert ML. 2011. Thymic microenvironment reconstitution after postnatal human thymus transplantation. Clin. Immunol. **140**(3): 244-259.

55. Liepinsh DJ, Kruglov AA, Galimov AR, Shakhov AN, Shebzukhov YV, Kuchmiy AA, Grivennikov SI, Tumanov AV, Drutskaya MS, Feigenbaum L, Kuprash DV and Nedospasov SA. 2009. Accelerated thymic atrophy as a result of elevated homeostatic expression of the genes encoded by the TNF/lymphotoxin cytokine locus. Eur. J. Immunol. **39**(10): 2906-2915.

56. Mebius RE and Kraal G. 2005. Structure and function of the spleen. Nat. Rev. Immunol. **5**(8): 606-616.

57. Milicevic NM and Milicevic Z. 2004. Thymus cell-cell interactions. Int. Rev. Cytol. **235**: 1-52.

58. Miller JF. 1961. Immunological function of the thymus. The Lancet **2**(7205): 748-749.

59. Mohtashami M, Shah DK, Nakase H, Kianizad K, Petrie HT and Zuniga-Pflucker JC. 2010. Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lympho-myeloid lineage commitment outcomes. J. Immunol. **185**(2): 867-876.

60. Mohtashami M and Zuniga-Pflucker JC. 2006. Three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development. J. Immunol. **176**(2): 730-734.

61. Ogle BM, Knudsen BE, Nishitai R, Ogata K and Platt JL. 2009. Toward development and production of human T cells in swine for potential use in adoptive T cell immunotherapy. Tissue Eng. Part A **15**(5): 1031-1040.

62. Okamoto N, Chihara R, Shimizu C, Nishimoto S and Watanabe T. 2007. Artificial lymph nodes induce potent secondary immune responses in naive and immunodeficient mice. J. Clin. Invest. **117**(4): 997-1007.

63. Park KM and Woo HM. 2012. Systemic decellularization for multi-organ scaffolds in rats. Transplant. Proc. **44**(4): 1151-1154.

64. Peng Y, Chen Z, Yu W, Zhou Q, Xu L, Mao FF, Huang G, Zhang X, Li S, Lahn BT and Xiang AP. 2008. Effects of thymic polypeptides on the thymopoiesis of mouse embryonic stem cells. Cell Biol. Int. **32**(10): 1265-1271.

65. Poliani PL, Vermi W and Facchetti F. 2009. Thymus microenvironment in human primary immunodeficiency diseases. Curr. Opin. Allergy Clin. Immunol. **9**(6): 489-495.

66. Poznansky MC, Evans RH, Foxall RB, Olszak IT, Piascik AH, Hartman KE, Brander C, Meyer TH, Pykett MJ, Chabner KT, Kalams SA, Rosenzweig M and Scadden DT. 2000. Efficient generation of human T cells from a tissue-engineered thymic organoid. Nat. Biotechnol. **18**(7): 729-734.

67. Rode I and Boehm T. 2012. Regenerative capacity of adult cortical thymic epithelial cells. Proc. Natl. Acad. Sci. U. S. A. **109**(9): 3463-3468.

68. Rodewald HR. 2008. Thymus organogenesis. Annu. Rev. Immunol. **26**: 355-388.

69. Rossi SW, Jenkinson WE, Anderson G and Jenkinson EJ. 2006. Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. Nature **441**(7096): 988-991.

70. Savino W. 2006. The thymus is a common target organ in infectious diseases. PLoS Pathog **2**(6): e62.

71. Seach N, Layton D, Lim J, Chidgey A and Boyd R. 2007. Thymic generation and regeneration: A new paradigm for establishing clinical tolerance of stem cell-based therapies. Curr. Opin. Biotechnol. **18**(5): 441-447.

72. Seach N, Mattesich M, Abberton K, Matsuda K, Tilkorn DJ, Rophael J, Boyd RL and Morrison WA. 2010. Vascularized tissue engineering mouse chamber model supports thymopoiesis of ectopic thymus tissue grafts. Tissue Eng. Part C Methods **16**(3): 543-551.

73. Sitnicka E. 2009. From the bone marrow to the thymus: The road map of early stages of T-cell development. Crit. Rev. Immunol. **29**(6): 487-530.

74. Skalak R and Fox CF 1988. Tissue engineering: proceedings of a workshop, held at Granlibakken, Lake Tahoe, California, February 26-29, 1988. New York, NY, Liss.

75. Spillane J, Hayward M, Hirsch NP, Taylor C, Kullmann DM and Howard RS. 2013. Thymectomy: role in the treatment of myasthenia gravis. J. Neurol.

76. Stuart MJ and Nagel RL. 2004. Sickle-cell disease. The Lancet **364**(9442): 1343-1360.

77. Suematsu S and Watanabe T. 2004. Generation of a synthetic lymphoid tissue-like organoid in mice. Nat. Biotechnol. **22**(12): 1539-1545.

78. Tan JK and Watanabe T (2010). Artificial engineering of secondary lymphoid organs. Advances in Immunology. F. W. Alt. San Diego, CA, Academic Press. **105:** 131-157.

79. Timmermans F, Velghe I, Vanwalleghem L, De Smedt M, Van Coppernolle S, Taghon T, Moore HD, Leclercq G, Langerak AW, Kerre T, Plum J and Vandekerckhove B. 2009. Generation of T cells from human embryonic stem cell-derived hematopoietic zones. J. Immunol. **182**(11): 6879-6888.

80. Tomei AA, Siegert S, Britschgi MR, Luther SA and Swartz MA. 2009. Fluid flow regulates stromal cell organization and CCL21 expression in a tissue-engineered lymph node microenvironment. J. Immunol. **183**(7): 4273-4283.

81. Tomlinson MJ. 2009. Strategies Toward Tissue Engineering a Murine Thymic Organoid *In Vitro*. Ph.D. Thesis. University of Nottingham, UK.

82. Ueno T, Liu C, Nitta T and Takahama Y. 2005. Development of T-lymphocytes in mouse fetal thymus organ culture. Methods Mol. Biol. **290**: 117-133.

83. Van Coppernolle S, Verstichel G, Timmermans F, Velghe I, Vermijlen D, De Smedt M, Leclercq G, Plum J, Taghon T, Vandekerckhove B and Kerre T. 2009. Functionally mature CD4 and CD8 TCRαβ cells are generated in OP9-DL1 cultures from human CD34+ hematopoietic cells. J. Immunol. **183**(8): 4859-4870.

84. van Ewijk W, Kawamoto H, Germeraad WT and Katsura Y. 2000. Developing thymocytes organize thymic microenvironments. Curr. Top. Microbiol. Immunol. **251**: 125-132.

85. van Gent R, Schadenberg AW, Otto SA, Nievelstein RA, Sieswerda GT, Haas F, Miedema F, Tesselaar K, Jansen NJ and Borghans JA. 2011. Long-term restoration of the human T-cell compartment after thymectomy during infancy: A role for thymic regeneration? Blood **118**(3): 627-634.

86. Vignali DA, Collison LW and Workman CJ. 2008. How regulatory T cells work. Nat. Rev. Immunol. **8**(7): 523-532.

87. von Gaudecker B, Steinmann GG, Hansmann ML, Harpprecht J, Milicevic NM and Muller-Hermelink HK. 1986. Immunohistochemical characterization of the thymic microenvironment. A light-microscopic and ultrastructural

immunocytochemical study. Cell Tissue Res. **244**(2): 403-412.

88. Weiss JM, Cufi P, Bismuth J, Eymard B, Fadel E, Berrih-Aknin S and Le Panse R. 2013. SDF-1/CXCL12 recruits B cells and antigen-presenting cells to the thymus of autoimmune myasthenia gravis patients. Immunobiology **218**(3): 373-381.

89. White A, Jenkinson E and Anderson G. 2008. Reaggregate thymus cultures. J. Vis. Exp.(18).

90. Zhu J and Paul WE. 2008. CD4 T cells: Fates, functions, and faults. Blood **112**(5): 1557-1569.

91. Zuniga-Pflucker JC. 2004. T-cell development made simple. Nat. Rev. Immunol. **4**(1): 67-72.