

Review

Synthesis of artificial lymphoid tissues with immunological function

Yuka Kobayashi and Takeshi Watanabe

Graduate School of Medicine, Kyoto University, Yoshida-konoe machi, Sakyo-ku,
Kyoto 606-8501 JAPAN

Corresponding Author: Dr Takeshi Watanabe, Graduate School of Medicine, Kyoto
University. Yoshida-konoe machi, Sakyo-ku, Kyoto 606-8501 JAPAN
Email: wtakeshi@ak.med.kyoto-u.ac.jp

Abstract:

The ability to generate functional artificial lymphoid tissue to induce specific immunity at ectopic sites would offer a potential breakthrough for treatment of diseases such as cancer and severe infection using immunotherapy. Artificial lymphoid tissue would also offer an informative tool to further study lymphoid tissue development and function in vivo. Here we review the process of secondary and tertiary lymphoid organization – for which an understanding will be essential for artificial lymphoid tissue synthesis. Using this knowledge, we consider the combination of cell types, soluble factors and scaffold properties that will enable proper accumulation and organization of lymphocytes into tissue grafts. Recent successes with trials for the in vivo induction of artificial lymphoid tissue are also considered.

Lymphoid tissue formation and induction of immunity

The adaptive immune system responds to antigens to produce various outcomes such as tolerance, antigen-specific immunity and immunological memory. These immune responses are, in most cases, supported by secondary lymphoid organs (SLO), such as lymph nodes (LN), Peyer's patches (PP) and the spleen, as well as tertiary lymphoid organs (TLO). In contrast to SLO, which develop during embryogenesis^{1,2}, TLO form in the postnatal period in response to antigenic stimuli³. Although TLO development is initiated by antigenic stimuli, the process partially recapitulates development of SLO during embryogenesis. For example, both SLO and TLO structures include segregated T and B cell accumulations, CD11c+ dendritic cell (DC) clusters, follicular dendritic cell (FDCs) networks, and high endothelial venules (HEV)^{3,4}. TLO formation occurs at ectopic sites and often accompanies the onset of acute and chronic inflammation, infection, autoimmune diseases and tumorigenesis, to provide a highly localized immune response against microbial-derived or autoantigens. Examples of TLO include lymphoid tissue formation in the synovial membrane of rheumatoid arthritis⁵⁻⁷, aorta adventitia of atherosclerosis^{8,9} and salivary gland of Sjögren syndrome^{10,11}. TLOs have also been observed during organ transplant rejection, which raises the possibility that they contribute to T cell-dependent allograft rejection by serving as a site for local adaptive immune responses¹². To address this question, RIP-LT α , which offer a model for skin transplant were used. These mice express *Lta* (lymphotoxin α) under the control of the rat insulin promoter (RIP) and develop TLOs in the pancreatic islets, kidney and skin. RIP-LT α mouse skin grafts (containing TLO) were transplanted into allogeneic splenectomized aly/aly host mice, which lack all SLO. The skin allograft with TLO containing host-derived lymphocytes was rejected. Thus, the TLOs support the

activation of naïve T cells to effector and memory T cells. The findings provide rationale for developing strategies to artificially synthesize lymphoid tissues at ectopic local sites, where a strong and specific immune response is desired. To synthesize functional artificial lymphoid tissues (aLT) at ectopic sites in vivo or in vitro, the regulated processes of SLO and TLO formation must be precisely understood. It will also be important to make use of advances in tissue engineering, with respect to both new technologies and biomaterials. Here, we provide an overview of SLO and TLO development, and then discuss trials to date for the in vivo induction of aLT.

Lymphoid tissue-inducer (LTi) cells and lymphoid organ formation

The physiological processes that drive SLO development have been well studied^{1,2,13,14}. During embryogenesis, lymphoid tissue formation is dependent on the interaction between hematopoietic CD45⁺ CD3⁻ CD4⁺ LT α 1 β 2⁺ LTi cells and VCAM-1⁺ ICAM-1⁺ LT β R⁺ stromal lymphoid tissue organizer (LTo) cells^{1,13,15}. LTi-like cells also contribute to lymphoid organogenesis in the adult¹⁶. LTi cells express a variety of factors important for organogenesis including the surface molecules IL-7R α , common gamma chain, CXCR5, RANKL (TRANCE) and RANK, and transcriptional factors such as RORC and Id2¹⁶⁻²². Since both the mature embryonic LTi cells and adult LTi cells are recruited and maintained at the lymphoid tissue anlagen (during embryogenesis) and at sites of chronic inflammation or infection (in the adult)¹⁸, they require the capacity to migrate through the vasculature. Indeed, whole embryonic spleens implanted under the renal subcapsular space of adult mice induce lymphoid tissue formation through accumulation of host-derived T cells, B cells and DCs. Host LTi cells are also recruited, which leads to stimulation of the grafted LTo cells and the production of CCL21 which

attracts naïve T cells and DCs ²³. These findings suggest that the circulating adult LTi cells migrate into the grafts and interact with embryonic stromal cells to organize splenic white pulp formation ²³. In human fetal tissues, LTi cells have been identified as lineage- RORC+ IL-7R α + hematopoietic cells. They are able to stimulate the expression of VCAM-1 and ICAM-1 on human mesenchymal stem cells *in vitro*, through expression of LT α 1 β 2, which triggers LT β R signaling in LTo cells ²⁴.

LTo in SLO and TLO

In contrast to LTi cells, the VCAM-1+ ICAM-1+ LT β R+ LTo stromal cells are adherent cells that are non-hematopoietic and can be categorized according to heterogeneous functions ²⁵. In SLO and TLO, two important stromal cells play a specialized role in lymphoid tissue formation: the FDC in B cell follicles and the fibroblastic reticular cells (FRC) in the T cell area. Their precise immunological contributions have been detailed in a recent review ²⁵. In the T cell area, FRC networks, and their conduit system, form three-dimensional structures that provide tracks for T cell migration, which is regulated by CCR7. FRCs are the source of survival factors such as CCL19, IL-6 and IL-7, as well as mediators such as VEGF²⁶ and retinoic acid (RA) that is produced by the enzyme RALDH2 ^{27,28}. RA leads to homing of activated T cells through activation of DCs ²⁹. VEGF induces proliferation of endothelial cells and maintains blood vessels²⁵. The conduit system is sheathed and interconnected by FRC and is also linked to lymphatic vessels with fluid flow at draining lymph nodes (LN). Using three dimensional LN T cell areas that were constructed *in vitro* using FRC clones cultured together with type I collagen and matrigel, it was shown that fluid flow in the *in vitro* structures was required for CCL21 secretion from FRC, which enhanced the organization of FRC networks ³⁰. *In vivo*

analysis by the same authors also indicated that blocking lymph flow in peripheral LN decreased CCL21 and CCL19 gene expression in FRC. These data suggest that under conditions of increased lymph flow, for example during inflammation, FRC networks increase in complexity, which could activate immune cell trafficking, lymph sampling and induction of immune responses³⁰. In inflammation foci, it has been shown that local resident fibroblasts³¹ and aortic smooth muscle cells^{8,9} give rise to LTo-like stromal cells, which express chemokines such as CXCL13, CCL19 and CCL21 and recapitulate SLO formation after triggering of the LT β R signaling pathway. Very recently, it was reported that lymph node FRC ectopically express and directly present peripheral tissue antigen (PTA) to naïve T cells under steady-state as well as inflammatory conditions³². Another report also documented that lymph node-resident lymphatic endothelial cells directly present PTA to T cells and mediate peripheral tolerance in an Aire-independent manner³³. These reports suggest that diverse stromal cell types exist that constitutively express PTA, and that lymph node stromal cells are involved in the maintenance of tolerance to self-antigens in the periphery. Thus, for the purpose of generating aLT with different immunological functions, including regulatory activity, it will be crucial to clarify the stromal cell heterogeneity in lymphoid organs.

Maintaining a microenvironment for lymphoid tissues formation

The initiation of lymphoid organ formation is triggered by the interaction of lymphotoxin $\alpha 1\beta 2$ (LT $\alpha 1\beta 2$) expressed on LTi cells with lymphotoxin- β receptor (LT β R) on the LTo cells at the lymphoid anlagen, which leads to activation of the NF- κ B signaling pathway. LT β R ligation activates expression of the adhesion molecules VCAM-1, ICAM-1 and MAdCAM-1, as well as lymphoid chemokines such as CCL19,

CCL21 and CXCL13^{34,35}. LT β R signaling events also promotes LTo cell expression of RANKL and IL-7, and binding of these ligands to their respective receptors on the newly recruited immature LTi cells acts to further induce LT α 1 β 2. This provides a positive feedback loop for lymphoid organ development at anlagen. Furthermore, the production of VEGF-C is induced in LTo cells, which contributes to lymphangiogenesis at this developmental stage³⁵, as shown in Figure-1A. LT β R associated signaling molecules also include TRAF2, TRAF3, the classical I κ B kinase complex containing IKK α , IKK β , IKK γ , and ELKs and NF- κ B-inducing kinase. Two recent studies suggest that TRAF3 is a dual-mode regulator acting downstream of LT β R to operate both canonical and non-canonical NF- κ B signaling pathways³⁶, and is critical for B cell homeostasis in SLO³⁷. Thus, LT β R signaling broadly contributes not only to lymphoid organ development but also to the maintenance of proper microenvironments wherein hematopoietic cells can home and interact. In influenza virus-infected mouse respiratory tracts, ectopic lymphoid tissues known as inducible bronchus-associated lymphoid tissues (iBALT) form through accumulation of lymphocytes and dendritic cells. CD11c+ DC depletion from the lung leads to the gradual disappearance of B220+ cells from the iBALT structure and a decrease of peanut agglutinin (PNA)-positive germinal center B cells, followed by diminution in the level of local immunoglobulin class switching. This suggests that CD11c+ DCs support maintenance of iBALT structure and function³⁸, indicating that CD11c+ DCs are involved in TLO organogenesis in addition to LTo stromal cells.

For proper lymphoid organogenesis, spatiotemporal regulation of the cells and molecules that localize and function in the lymphoid anlagen is required. Recently, it

has been reported that heparan sulfate proteoglycans (HSPGs) play an important role in lymphoid development³⁹. HSPGs are molecules that contribute to several regulatory mechanisms during tissue morphogenesis by specifically binding soluble factors. In this regard, a number of lymphoid chemokines contain HSPGs binding motifs^{40,41}. Heparan sulfate (HS) glucuronyl C5-epimerase (Glce) mutant mice, which lack proper HS modifications, suffer from splenic hypoplasia, peripheral LN deletion, blood vessel mis-location and excess branching around peripheral LNs³⁹. These results indicate that SLO development requires a tightly organized process that combines the signaling pathways required for accumulation of hematopoietic cells, together with suitable mechanisms for retention of both lymphoid cells and bioactive molecules within the developing lymphoid organ. It will be important to take these findings into account when generating synthetic immune tissues.

Contributions of chemokines to lymphoid tissue generation

Both SLO and TLO possess organized structures, which include T and B cell areas, DCs, FDC networks and vascular networks. To initiate SLO and TLO development and to sustain their function, lymphoid chemokines play important roles in the migration and proper positioning of these lymphoid tissue-associated cells [Figure 1B]. In mice, CCL19 and CCL21 expressed by FRC, and DCs attract and retain T cells within the appropriate area of the lymphoid tissues. Mice lacking both of CCL19 and CCL21, or mice lacking their receptor CCR7, retain LNs and PPs but exhibit defects in T cell trafficking to these lymphoid tissues, and in nasopharynx-associated lymphoid tissue maturation^{42,43}. By performing immunohistochemical analysis of human SLO and TLO in inflammatory lesions, it was demonstrated that CCL19 and CCL21 are both expressed by the smooth

muscle actin-positive stromal cells that are found in close proximity to HEVs, and CCL21 is also expressed by mature DCs and lymphatic vessels ⁴⁴. CXCL12, the ligand for CXCR4, also contributes to T cell trafficking into LNs and PPs in collaboration with CCR7 ligands ⁴³. Another important chemokine is CXCL13, which is expressed by stromal cells including FDCs. CXCL13 initiates lymphoid organ formation ⁴⁵, entrance of B cells into follicles, and increased expression of LT α 1 β 2 on B cells. CXCL13 further facilitates CXCL13 expression in B cells, establishing a positive feedback loop for B cell follicle homeostasis ⁴⁶. Mice deficient for both CXCL13 and IL-7R α lack most LNs, but CXCL13 is sufficient to recruit LTi cells to ectopic sites in vivo ⁴⁷. These results, along with the IL-7R α -mediated LT α 1 β 2 up-regulation on LTi cells, suggest that both CXCL13 and IL-7R α contribute to accumulation and activation of LTi cells respectively during the initial LT β R signaling-dependent stage in LN development ⁴⁷. It has been shown, however, that in E12.5-14.5 LN anlagen, CXCL13 expression in LTo cells is induced by retinoic acid RA in a LT β R signaling-independent manner. A possible source of RA appears to be cells of the nervous system around the LN anlagen ⁴⁵. In the SLO and TLO microenvironment, B cells circulate throughout B cell follicles to survey antigens. Once B cells engage antigens, the antigen-specific B cells increase CCR7 and CXCR5 expression, leading to their migration to the boundary between the B cell follicle and T cell zone, where B cells interact with antigen-specific T cells. Thus, controlled positioning of B cells in follicles is regulated by the finely-tuned responsiveness of B cells to chemokines such as CXCL13, CCL19 and CCL21 ⁴⁸. TLO formation was observed by ectopic expression of various chemokines such as CXCL12, CXCL13, CCL19 and CCL21 in transgenic mice ⁴⁹⁻⁵¹. Overexpression of each chemokine alone induced the complete formation of TLO. Ectopic expression of cytokines such as LT α β or IL-7 also formed TLO

^{52,53}. Thus, certain chemokines and cytokines might be important and useful for generating artificial lymphoid tissues.

Scaffolds for artificial lymphoid tissues

Tissue engineering has received much attention in the field of regenerative medicine and is undergoing continuous innovation. The characteristics of available biomaterials play an important role in tissue engineering strategies, and have allowed for the construction of a variety of tissues including blood vessels ^{54,55}, bone ^{56,57}, spinal cord ⁵⁸ and peripheral nervous system ^{58,59}. As a matter of course, this is now being applied to lymphoid tissues. Experimentally, to maintain the proper microenvironments at local sites, it will be necessary to attempt transplantation using scaffolds that: 1) contain the appropriate cell populations; 2) contribute to generation of lymphoid organs; 3) produce soluble factors – such as lymphoid chemokines and cytokines – that are expressed by stromal cells. A number of synthetic biomaterials are now able to duplicate the three dimensional microenvironments that are provided by natural extracellular matrices, such as fibrillar or non-fibrillar collagen, proteoglycans and matricellular proteins ^{60,61}. It has been demonstrated that structurally-engineered macroporous scaffolds, which combine poly ethylene glycol hydrogels with collagen, support T cell and DCs migration ⁶². Further advances are being made in this area ^{60,61}. The first *in vivo* synthesis of artificial lymphoid tissues was achieved using a porous biocompatible collagen matrix, prepared from bovine Achilles tendon (referred to as a collagen sponge) ⁶³. This matrix has a non-homogeneous pore size ranging from 50-300µm. A difficulty in tissue engineering is the achieving the ability to properly modulate or mimic, dynamic tissue microenvironments, for example to achieve

appropriate cell recruitment or correct concentration gradients of soluble factors. As a first step toward this goal, biomaterials were recently described that are able to release several soluble factors, not only uniformly, but also gradually with temporal differences⁶⁴. In this study, two growth factors were incorporated together into the same scaffolds by mixing PDGF-encapsulated polymer, lyophilized VEGF and non-treated polymer particles, so that the two growth factors were localized within distinct compartments. As a result, the scaffold could release the two factors with distinct kinetics and, after transplantation into subcutaneous tissue of Lewis rats, more mature vascular networks were structured than when using the scaffold which contained only a single growth factor⁶⁴. Two other recent studies have also reported that sustained⁶⁵ or spatiotemporal⁶⁶ delivery of growth factors can lead to formation of a mature vascular system. Thus, in order to efficiently generate artificial organized tissues, the scaffold microenvironment should be carefully constructed to be as close as possible to natural state.

Generating artificial lymphoid tissue *in vivo*

Generating functional artificial lymphoid tissues might be an important and beneficial method for making advances in immunotherapy, as well as for investigating the physiological functions of lymphoid tissues *in vivo*. For example, in the tumor microenvironment it is known that T cell immune responses are induced inside the tumor mass⁶⁷. This, however, is not sufficient for tumor destruction due to immunosuppressive mechanisms. This immunosuppression arises from an imbalance between activating and inhibitory signals that regulate immune cell function, as well as overproduction of immunosuppressive cytokines such as TGF β by the tumor mass^{68,69}.

Transplantation of aLT into the renal subcapsular space^{63,70} or close to the pathogenic site might help supplement anti-tumor immune responses. Another example where aLT might be beneficial to boost immune activity would be to counteract the atrophy of primary lymphoid organs and SLO which occurs during aging and which leads to immunosenescence^{71,72}.

So far, tissue engineering has been most widely studied in a rodent model using splenic tissue. Several reports have shown that not only embryonic and newborn splenic transplantation^{23,73}, but also tissue-engineered spleen (TES) generated from splenic multicellular components loaded on a biodegradable polymer scaffold⁷⁴, induce partial or sufficient immune responses to infection. These studies provide valuable information concerning the generation of artificial lymphoid tissues. However, this approach lacks versatility in a clinical setting where the use of biological material might not be feasible if tissue supply is limited. Optimized combinations of stromal or hematopoietic cells and soluble factors such as chemokines or cytokines captured on scaffolds will be required to achieve solid results with general versatility. To date, few studies have managed to combine structural formation of aLT with immune responsiveness. However, we transplanted a collagen sponge that contained the thymus-derived cell line TEL-2 that overexpressed LT α , stromal cells and bone marrow-derived DCs, into the mouse renal subcapsular space [Figure-2A]. In 2-3 weeks, it was observed that the graft behaved as an artificial lymph node (aLN), that possessed structures similar to secondary lymphoid tissues, such as T and B cell segregated clusters, FDC and FRC networks in B cell and T cell areas respectively, together with HEV-like structures. The aLNs induced a strong secondary immune response *in vivo*, and the high responsiveness was confirmed by the accumulation of

memory and effector T cells as well as memory B cells in the aLNs⁷⁰. When the aLNs were re-transplanted into SCID mice, and followed by immunization, a robust secondary immune response was induced^{63,70}.

In terms of an *in vitro* trial to create the human LN environment, it was demonstrated that antigen-specific B cell clusters could be observed in a bioreactor – an *ex vivo* culture device – containing a porous matrix with antigen-primed DCs⁷⁵. Recently, potentially useful cell lines of artificial antigen-presenting cells (aAPC) were established for humans⁷⁶. These cells possess the capacity to stimulate *ex vivo* or *in vivo* production of cytotoxic T cells from naïve T cells⁷⁶. aAPC have also been made by covalently coupling antigen peptide-MHC complex and the B7.1 co-stimulatory molecule to magnetic beads. *In vivo* treatment with the aAPC induced significant or complete reduction of tumors in mice⁷⁷. Based on this data, the aAPC might be an effective tool for tumor immunotherapy. The newly established aAPC might be also applicable for generating aLT. To apply artificial lymphoid tissues to human diseases, an *in vivo* human model would first have to be established, using knowledge from previous mouse experiments. There has been a much progress in the establishment and analysis of humanized mice⁷⁸⁻⁸⁰. NOD/SCID/IL-2 receptor γ chain^{null} mice reconstituted with human CD34+ hematopoietic stem cells develop a functional human hematopoietic and immune system. As a result, humanized mice are utilized as a human disease model. They can be experimentally manipulated and can be used to directly study infectious diseases, immunological disorders, as well as cancer therapy in humans⁸¹⁻⁸⁴. This model system could be a prime candidate for generating human aLT.

Concluding remarks

The idea of generating aLT, especially tissue that is functionally similar to SLO and TLO, is a novel strategy to trigger ectopic adaptive immune responses *in vivo*, as well as to study the functions of these tissues. Progress in this area might be applied to both locally and systemic treat severe infection, autoimmune diseases, tumors, for example. From this point of view, trials for construction of human aLT are at a preliminary stage, but could be imperative in the future.

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Figure legends

Figure 1: An overview of SLO and TLO formation

Left: During embryogenesis, SLO formation is triggered by the interaction between $LT\alpha 1\beta 2$ on LTi cells and $LT\beta R$ on LTo cells. $LT\beta R$ ligation leads to the expression of the adhesion molecules such as VCAM-1, ICAM-1 and MadCAM-1, lymphoid chemokines such as CXCL13, CCL19 and CCL21, RANKL and IL-7. RANKL and IL-7 receptor signaling on the recruited LTi cells further induces $LT\alpha 1\beta 2$ to form a positive feedback loop for lymphoid tissue formation. Induction of VEGF-C production by LTo cell promotes lymphangiogenesis. Thus, $LT\beta R$ signaling in LTo cells attracts and retains hematopoietic cells, and leads to vascularization through HEV and lymphatic vessels. Right: TLO arise during inflammation, infection and in some autoimmune diseases. There have been reported circumstantial evidences indicated that antigen stimulation and/or $LT\beta R$ signaling play an important role in TLO formation^{8,9,12}. At ectopic sites such as the synovial membrane or pancreas, specific stromal cell types produce chemokines in response to antigen stimulation. These stromal cells resemble LTo cells at lymphoid tissue anlagen of SLO and induce TLO formation. Both SLO and TLO have well-organized structures that include segregated T and B cell areas, DCs in T cell area, FDC networks in B cell follicles and vascular networks (lymphatic vessels and HEV). Migration and proper positioning of lymphoid tissue-associated cells is controlled by chemokines that essential for SLO and TLO development and function. CCL19 and CCL21 expressed by LTo and DCs attract and retain T cells within an appropriate area. On HEV, CCL21 facilitates migration of cells into lymphoid tissue. CXCL13 expressed by FDCs retains B cells in follicles.

Figure 2: Potential applications of artificial lymphoid tissue (aLT)

Transplant of a collagen sponge containing bone marrow-derived and antigen-pulsed DCs and VCAM-1+ LT β R+ LT α overexpressing stromal cells into the renal subcapsular space produces the artificial lymphoid tissue (aLT) three weeks later. The tissue grafts resembles LN and contains T cell and B cell clusters, FDC and FRC networks in B cell follicles and T cell areas respectively and HEV-like vascular structures⁶³. The aLT contains memory B and T cells, follicular helper T cells (Tfh) and other helper T cell subsets. The presence of these cell types was confirmed by re-transplanting the aLT into SCID mice and followed by immunization, which gave a robust secondary immune response [62, 69]. In these mice antigen-specific high-affinity IgG-producing B cells were present in the spleen, LN and BM. The antigen-specific IgG reached 3-7 mg/ml in the SCID mouse serum. Thus the aLT might be a suitable tool for the analysis of memory B cells and their niche, for characterization of Tfh and as an application to generate hybridomas secreting monoclonal high-affinity IgG class antibody with a high efficiency.

(B) aLT suppresses tumor growth in mice that have received tumor resection. When re-transplanted into SCID mice carrying the same tumors as in the original mice, aLT also suppress existing tumors. These data suggest that aLT may be applicable not only for enhancing antibody-mediated immune responses but also as a new strategy for cancer immunotherapy. In case of aLT use for immunotherapy, there might be a possibility that pathological lymphoid or myeloid cells co-opt the artificial lymphoid tissues and use the tissues to exacerbate the pathological response or induce unfavorable immune response(s) against the recipient. This point should be taken in consideration in a further trial for synthesis of artificial immune tissues/organs.

(C) aLT synthesized in the bacterial or viral antigen-immunized mice may also exhibit an

ability to prevent a further spreading of the infection.

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Figure-1

SLO formation

TLO formation



