Review

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Posttransplant Tertiary Lymphoid Organs

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Abstract. Tertiary lymphoid organs (TLOs), also known as tertiary or ectopic lymphoid structures or tissues, are accumulations of lymphoid cells in sites other than canonical lymphoid organs, that arise through lymphoid neogenesis during chronic inflammation in autoimmunity, microbial infection, cancer, aging, and transplantation, the focus of this review. Lymph nodes and TLOs are compared regarding their cellular composition, organization, vascular components, and migratory signal regulation. These characteristics of posttransplant TLOs (PT-TLOs) are described with individual examples in a wide range of organs including heart, kidney, trachea, lung, artery, skin, leg, hand, and face, in many species including human, mouse, rat, and monkey. The requirements for induction and maintenance of TLOs include sustained exposure to autoantigens, alloantigens, tumor antigens, ischemic reperfusion, nephrotoxic agents, and aging. Several staging schemes have been put forth regarding their function in organ rejection. PT-TLOs most often are associated with organ rejection, but in some cases contribute to tolerance. The role of PT-TLOs in cancer is considered in the case of immunosuppression. Furthermore, TLOs can be associated with development of lymphomas. Challenges for PT-TLO research are considered regarding staging, imaging, and opportunities for their therapeutic manipulation to inhibit rejection and encourage tolerance.

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INTRODUCTION

Tertiary lymphoid organs (TLOs), also known as tertiary or ectopic lymphoid structures or tissues, are accumulations of lymphoid cells in sites other than canonical lymphoid organs that arise during chronic inflammation in autoimmunity, microbial infection, cancer, aging, organ damage, and transplantation through lymphoid neogenesis. In this review, we use the term, TLO, since this is the convention in transplantation, the emphasis of this article (they tend to be called tertiary lymphoid structures in the tumor literature). TLOs bear striking cellular and organizational similarities to secondary lymphoid organs (SLOs) such as lymph nodes (LNs), spleen, and Peyer's patches. Many excellent reviews have been written about TLOs and their relationship to lymphoid organs[1-7] and to transplantation.[1,8] Here, we concentrate on posttransplant TLOs (PT-TLOs) in allotransplantation and consider how they are similar or distinct from TLOs in other contexts, analyze their functions in facilitating rejection and/or maintenance of transplanted organs, and probe their roles in cancer.

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COMPARISON OF LNs AND TLOs

LNs (Figure 1), examples of SLOs (in contrast to primary lymphoid organs such as the thymus and bone marrow), are kidney bean shaped entities located in discrete and reproducible areas throughout the body. They are connected to each other by lymphatic vessels (LVs), are arranged like beads on a string, and consist of cells that are organized in a fashion to allow maximal interaction of antigen with naive T and B lymphocytes. They develop in locations that are predetermined before birth. Their structure is ideal for their function of maximizing interaction of antigen-inexperienced lymphocytes with cognate antigen, permitting proliferation and differentiation into effector T (T helper 1 [Th1], Th2, Th17, regulatory T cell [Treg], or B [cytokine, antigen presenting, antibody producing]) and memory cells. SLOs are relatively constant in that the structure itself remains, although it can undergo extensive remodeling during an immune response.[9] Naive L-selectin$^+$ T (C-C chemokine receptor type 7 [CCR7]$^+$) and B (CXC motif chemokine receptor [CXCR]4$^+$CXCR5$^+$CCR7$^+$) cells enter LNs via specialized blood vessels called high endothelial venules (HEVs) that express adhesion molecules such as intercellular adhesion molecule 1 and peripheral node addressin (PNAd) (recognized by MECA 79 antibody) in peripheral LNs and mucosal addressin cell adhesion molecule-1 (recognized by MECA 367 antibody) in mucosal LNs and Peyer's patches. CCR7$^+$ dendritic cells

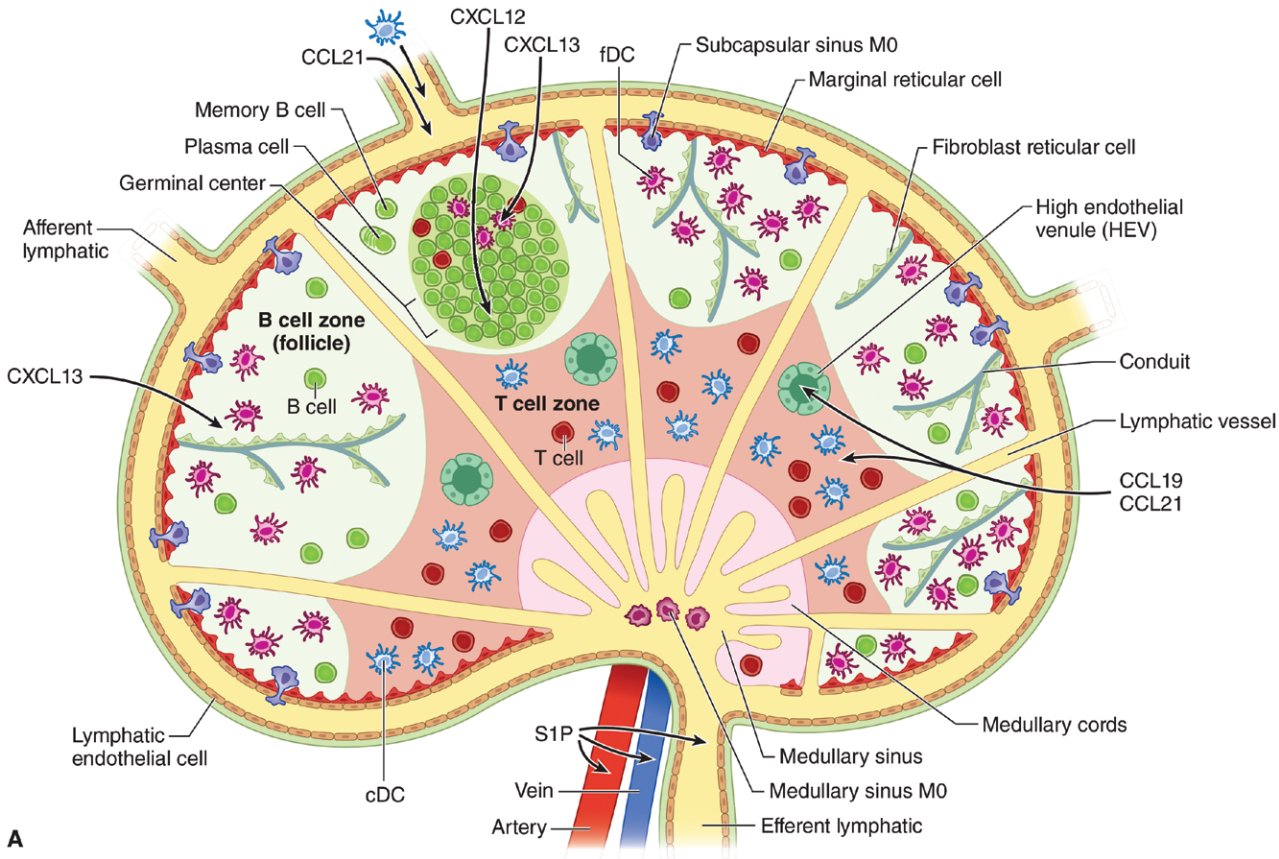

FIGURE 1. LN organization. The LN is organized into T- and B-cell zones with antigen-presenting cells, stromal cells forming conduits, high endothelial venules, and lymphatic vessels (reproduced with permission from Flajnik ME, Singh NJ, Holland SM. *Paul's Fundamental Immunology*. 8th ed. Wolters Kluwer; 2022). CCL, C-C chemokine ligand; cDC, classical dendritic cell; CXCL, CXC motif chemokine ligand; fDC, follicular dendritic cell; HEV, high endothelial venule; LN, lymph node; S1P, sphingosine 1-phosphate.

(DCs) carrying antigen enter through afferent LVs. T cells and DCs are directed to the paracortical region of LNs by C-C chemokine ligand (CCL) 19 and CCL21, the ligands for CCR7 that are produced by stromal cells, HEVs, and LVs; B cells are directed to the follicle by CXCL13, the ligand for CXCR5. A series of conduits (fibroblastic-lined channels) and LVs allows antigen and cells to percolate through the LN. B cells encounter soluble antigen and antigen-presenting follicular dendritic cells (FDCs) in the follicle where they can differentiate into memory cells or plasma cells through the benefit of activation-induced cytidine deaminase, which allows class switch recombination and hypermutation. Activated lymphocytes exit the LN through efferent LVs by their interaction with sphingosine 1-phosphate (S1P) expressed in that vessel. The LNs are connected one to another through their LVs, which join the thoracic duct. Entrance into the blood stream occurs through the left subclavian vein for the trunk and right subclavian vein for the head region.

The many examples of TLOs, including PT-TLOs (Figure 2), share several SLO characteristics, though there are some differences. Similarities include cellular composition, LVs, conduits, organization into T- and B-cell compartments, dictated by lymphoid chemokines, CCL19, CCL21, and CXCL13, and germinal centers (GCs) with expression of activation-induced cytidine deaminase.[7] In the case of PT-TLOs, allopeptides are presented to naive T cells by antigen presenting cells (APCs), including those of the host.[10] B cells recognize alloantigens and can differentiate into plasma cells producing alloantibody.[11] HEVs can be critical components of TLOs in that they allow the entrance of naive cells that can be activated by APCs, and some authors even use the presence of HEVs as a criterion for TLO identification (eg, rejecting murine cardiac allografts).[12]

Even the signals for cellular migration out of LNs, that is, the role of S1P, have been noted in TLOs in the nonobese diabetic (NOD) mouse model of type I diabetes, in which treatment with FTY720 (fingolimod), an S1P inhibitor prevents egress from the pancreatic TLOs with stabilization of the lymphoid accumulations of lymphoid accumulations and diabetes prevention.[13] FTY720 treatment of aged mice undergoing ischemic reperfusion injury also results in an increased size of renal TLOs, although an effect on kidney function was only seen if treatment was begun before their appearance.[14] It remains to be seen if PT-TLOs will also be sensitive to S1P inhibition. TLOs are considerably more plastic than SLOs in that they resolve if the impetus for their development is removed (see below). Such is the case in PT-TLOs of the transplanted heart once the rejection episode has been resolved,[15] in type 1 diabetes wherein the TLO resolves once the beta cells are destroyed,[13] and in TLOs in kidneys of aged mice after treatment with dexamethasone.[16]

An important difference between SLOs and TLOs is the absence of a capsule in the latter. Thus, TLOs lack the organizational structure of an LN with its subcapsular

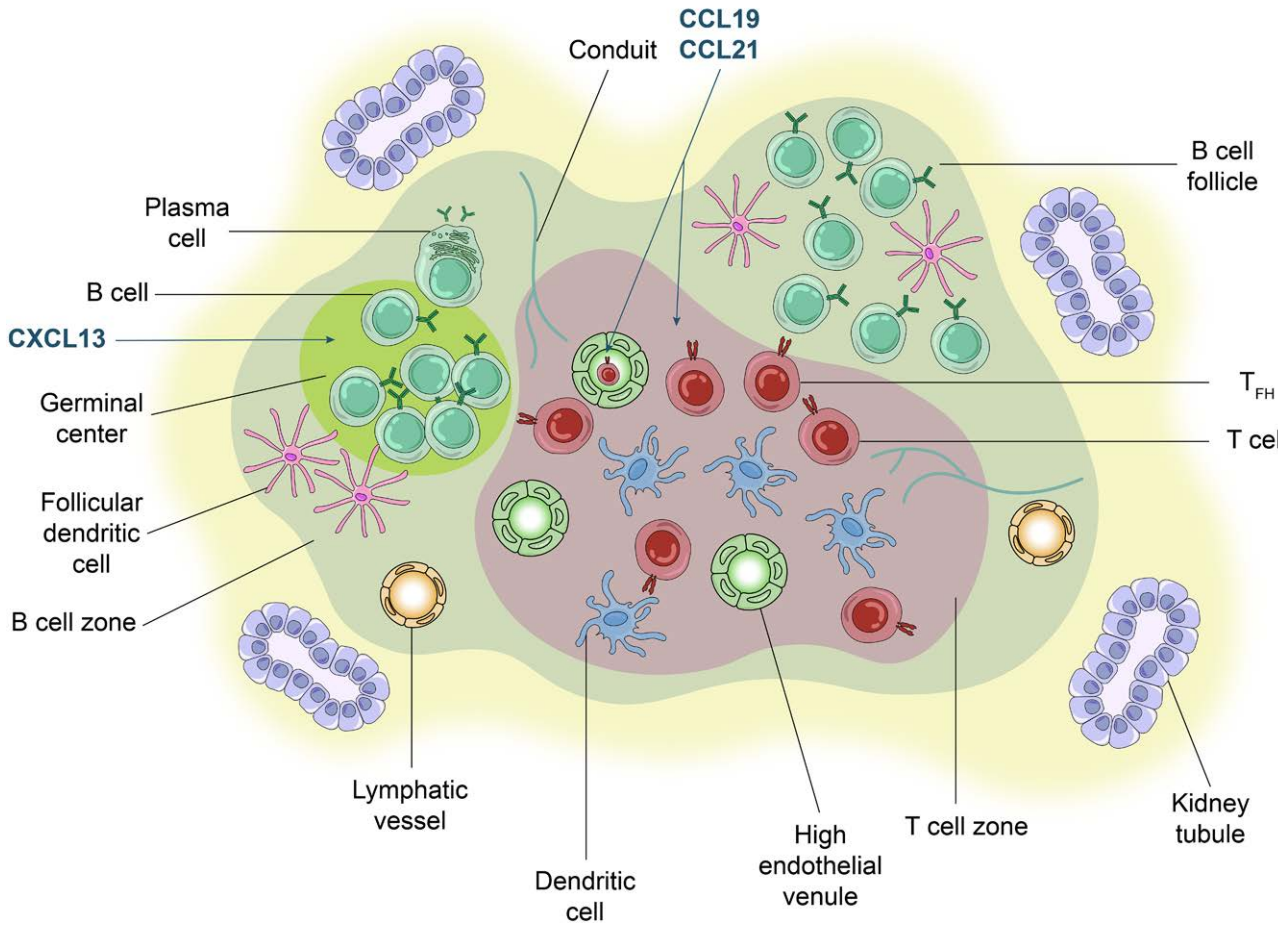

FIGURE 2. TLO organization. A diagram of an organized accumulation of lymphoid cells in the PT-TLO of a transplanted kidney. Note extensive similarities in cellular content and organization to that of a lymph node. This is a diagram of components that have been described in PT-TLOs. It is not meant to indicate that all features depicted are present in every PT-TLO. T cells can include effector T cells, resident memory, and regulatory T cells depending on the context and Tfh depending on the location. CCL, C-C chemokine ligand; CXCL, CXC motif chemokine ligand; PT-TLO, posttransplant tertiary lymphoid organ; T_{FH}, T follicular helper cell; TLO, tertiary lymphoid organ.

APCs. The absence of a capsule results in an intimate interaction with the microenvironment in which they are located—the transplanted organ, tumor, or organ undergoing an autoimmune response. How do individual TLOs in a particular rejecting organ interact with each other? Do cells circulate between one TLO and another in an individual organ in the manner that cells traffic from one LN to another via LVs, eventually rejoining the blood circulation, or are they confined to one area? How do they interact with nearby LNs? Is there movement from LN to TLO and back? In general, it is assumed that there is interaction between draining LNs and the organ in which a TLO resides. For example, in the case of type 1 diabetes, antigens are initially presented by migrating DCs from the islet to T cells in pancreatic LNs.[17] Once those T cells migrate back to the islet and set up conditions for a TLO with the development of HEVs that allow access of naive lymphocytes, is there migration of cells back to the pancreatic LN? The fact that unique T-cell receptor and B-cell receptor usage is apparent in TLOs compared with the peripheral circulation would suggest such interaction is minimal and may be limited to the draining LN. On the other hand, continual seeding of tumors (and likely their TLOs) is apparent from the observations of "stem like" precursor $CD8^+$ T cells in tumor (but not nondraining) LNs[18] and in tumor TLO HEVs.[19] These studies support the notion of transit between draining LNs and TLOs.

PARTICULAR CHARACTERISTICS OF POSTTRANSPLANT TLOs

Solid organ and skin transplantation have a long experimental and clinical history. Early forays into clinical organ replacement were plagued by immediate rejection, mediated by antibodies due to prior sensitization or by acute rejection mediated by CD8 T cells. With the advent of more precise tissue matching and immunosuppressant agents, greater success in solid organ transplantation has been realized. Even so, rejection can occur. When this does happen, TLOs can arise.

Rejecting heart and kidney grafts with $PNAd^+$ vessels (HEVs) were the earliest descriptions of posttransplant infiltrates that had characteristics of TLOs.[15,20] These vessels were associated with acute rejection; the cellular infiltrates were not further analyzed. Fully developed PT-TLOs were described by Baddoura et al[21] in acute, chronic, and mixed acute and chronically rejecting murine cardiac allografts. Although TLOs were seen in all instances of rejection, they were most frequently seen in chronic rejection and the authors conclude that TLOs correlate with that

process. These cellular accumulations had lymphoid follicles, T- and B-cell compartmentalization, GCs, and plump PNAd$^+$ HEVs. TLOs, although not invariably seen, have been described in many different transplanted organs. As noted above, PT-TLOs are most often associated with chronic rejection, but in some instances, HEVs have also been noted in acute rejection,[15,20,21] although in the case of rejecting heart transplants they correlate with the severity of rejection.[15] Just a few examples of PT-TLOs, in a variety of organs from multiple species are provided in Table 1 and include kidney, heart, lung, skin, aorta, and vascularized composite allotransplantation of hand and face.

LVs have been noted in many TLOs.[3] They were first described in CCR7$^+$ lymphocytic infiltrates in chronically rejecting human kidneys.[23] Lymphangiogenesis can occur as early as 10 d posttransplant and is not necessarily correlated with TLOs.[30] T follicular helper cell (Tfh) cells have been noted in acutely rejecting human kidneys[31] and Tfh, tissue resident memory cells, and plasma cells have been noted in the infiltrates of chronically rejecting mouse kidney grafts.[24] The process of effector to memory cell transition occurs in rejecting kidneys themselves,[24] consistent with the concept that the TLO itself acts as an immunocompetent site (see below). Host-derived DCs interacting with antigen-specific T cells invade the chronically rejecting kidney graft, setting up the process of activation of host T cells in the graft.[24,32]

INDUCTION AND MAINTENANCE OF TLOs

SLO development, which occurs in precise anatomical locations during ontogeny, is dictated by coordination of cytokines and chemokines, orchestrated by a group of cells that include fibroblast activation protein-α^{+}[33] stromal lymphoid tissue organizer (LTo) cells and CD4$^+$CD3$^-$ interleukin (IL)-7 receptor$^+$RAR-related orphan receptor gamma t$^+$ hematopoietic lymphoid tissue inducer (LTi) cells that express the lymphotoxin αβ (LTαβ) complex and drive expression of lymphoid chemokines.[7,34,35] TLOs, in contrast to SLOs, arise postnatally in the context of inflammation. The impetus for their origin, rather than the precise, highly coordinated dance of LTi and LTo cells, varies, but frequently includes the same cytokines and chemokines that drive SLO development. However, the nature of the stromal cells that act as organizers is variable, in part, depending on the tissue in which the TLO arises.[36] Fibroblast activation protein positive cells are present in adult LNs[33] and in salivary gland TLOs in Sjogren's syndrome[37] and it is likely that they and other stromal cells provide the function of LTo cells in PT-TLOs. Various stromal cells can substitute for LTo cells in PT-TLOs. Lymphatic endothelial cells provide CCL21 in chronically rejecting human kidney grafts[23]; *CXCL13*, *CCL21*, and *LTβR* are expressed in chronically rejecting kidney allografts.[38] CXCL13 is produced by CD68$^+$ (monocyte lineage) cells in rejecting heart transplants,[22] and fibroblasts produce CXCL13 and CCL19, serving as LTo cells in aged injured kidney TLOs.[39]

The mechanism of TLO induction, rather than the precise antigen-independent coordination of LTo and LTi cells is usually, but not always, the response of the immune system to sustained expression of an antigen and production of the same cytokines crucial for SLO development. The early experimental examples of TLOs were transgenic mice that ectopically expressed members of the LT family[40,41] or lymphoid chemokines.[42] Although LTi cells have been invoked in the formation of TLOs[43] and there is evidence for their continued existence[44-46] and ability to induce TLOs in the adult,[43] several other sources of LTαβ are available postnatally including T cells, B cells, natural killer cells,[47] and DCs,[48] which can substitute for classical LTi cells[1,49] in PT-TLOs. LTαβ has been invoked in TLO development via signaling through the lymphotoxin beta receptor (LTβR) in TLOs in the aged aorta[50] and cardiac allografts.[12] In the latter case, C57BL/6 recipients of bm12 cardiac allografts were treated with LTβR-immunoglobulin, which blocks LTβR signaling. There are slightly fewer lymphoid aggregates, T/B compartmentalization is lost, and there is a significant decrease in the number of HEVs and LVs. FDC networks are reduced, as is alloantibody production and grafts are maintained significantly longer than in control-treated mice. However, in a transgenic TLO model induced by lymphotoxin alpha (LTα), signaling occurs through the tumor necrosis factor receptor 1 with no requirement for lymphotoxin beta.[51] IL-17 producing cells that share some characteristics of LTi cells, including the transcription factor, RAR-related orphan receptor gamma t, have been noted in TLOs in chronically rejecting human kidney allografts[52] and can substitute for LTi cells. Thus, several instances indicate that the rigid requirements of SLO development are somewhat more flexible when it comes to TLOs in general including PT-TLOs.

TLOs differ from SLOs in that, in general, continuation of the impetus for their formation is required for their maintenance. As noted above, TLOs can arise as the result of T-cell recognition of a local antigen. In the case of cancer, it is a tumor antigen; in infection, a microbe; in PT-TLO, the impetus is the expression of an alloantigen in the transplanted organ. In many cases, continued expression of an inciting antigen is necessary for induction and maintenance of a TLO. Once the induction signal is removed, the TLO dissipates as noted above.

TLOs can be associated with acute kidney injury induced by ischemic reperfusion,[16] nephrotoxic agents,[39] or infection such as polyomavirus-induced nephritis in mice,[25] hepatitis C infection of human liver,[53] or latent cytomegalovirus infection of rat heart.[54] TLOs with T and B cells, HEVs, LVs, and fibroblasts expressing lymphoid chemokines CCL19, CCL21, and CXCL13 arise in the kidney following these insults, particularly in aged mice (12 mo old).[39] Inflammatory infiltrates in the renal cortex with some characteristics of TLOs, termed urinary tract-associated lymphoid structures, have been described in humans and in a noninfectious mouse model of chronic nephritis.[55] In this case, the authors implicate the contribution of urine to compromising urothelial barrier integrity and induction of lymphoid chemokines as contributing factors to these lymphoid accumulations. It is likely that these TLOs are associated with reactivity to an insult-exposed self-antigen or with molecular mimicry between the insult and a self-antigen.[56,57]

Aging is associated with "spontaneous" development of TLOs. Lymphoid accumulations with all the

TABLE 1.

TLOs in a variety of organs posttransplant and can be associated with acute and chronic rejection

Organ	Species	TLO characteristics	Notable features	Time posttransplant	Acute or chronic rejection	References
Heart	Human	HEVs	Early example of HEVs; other characteristics not noted	N.I.	Chronic	Toppila et al[15]
	Human	T/B compart, FDCs, HEVs, GCs, CXCL13, CXCR5	Early examples of human PT-TLO	N.I.	Chronic	Thaunat et al[11] and Di Carlo et al[22]
	Rat	T/B compart, HEVs	Early example of murine PT-TLO	Median: 95 d	Some acute, but mostly correlated with chronic	Baddoura et al[21]
	Mouse	T/B compart, FDCs, HEVs, LVs	Blocking LTβR blocks TLO	Identified in 3 of 5 at day 20 and all at days 40 and 100	Chronic	Motallebzadeh et al[12]
Kidney	Human	HEVs	Early example of HEVs; other characteristics not noted	N.I.	Acute	Kirveskari et al[20]
	Human	T/B compart, $CCR7^+$ T cells, DCs, GCs, CCL21, LVs	First description of LVs in PT-TLO	Biopsies with infiltrates correlate with rejection	Mainly chronic	Kerjaschki et al[23]
	Human	T/B compart, FDCs, HEVs, GCs	Early example of human PT-TLO	N.I.	Chronic	Thaunat et al[11]
	Mouse	Nodular infiltrates, tissue resident memory cells	TLO?	4 wk	Chronic	Abou-Daya et al[24]
	Mouse	CD4, CD8, Treg, DC, macrophages, HEV	Graft acceptance DBA/2 to C57BL/6	13.5 wk	Chronic	Brown et al[10]
	Mouse	Progression CD8, B, CD4, CD4, Treg, no HEVs	Graft acceptance DBA/2 to C57BL/6 TLO designation suggested	1–60 wk	Chronic	Rosales et al[25]
Trachea	Rat	T/B compart, HEVs		56 d	Chronic	Sato et al[26]
Lung	Human	T/B not compart, HEVs, no FDCs	Obliterative bronchiolitis	52.9 ± 10.4 mo	Chronic	Sato et al[26]
Artery	Rat	T/B compart, HEVS	Antibody	HEVs apparent at 10 d; later resolved	Acute	Thaunat et al[11]
	Mouse	GCs and multiple B-cell types	Transcriptomic analysis of inflammation progress	4 wk	Chronic	Cai et al[27]
Skin	Mouse	T/B compart, HEVs	Generation of effector and memory cells	N.A. transgenic model		Nasr et al[28]
Leg	Rat	Lymphocytes HEVs	Correlation with late rejection		Acute chronic	Hautz et al[29]
Hand	Human	T, B, HEVs	Correlation between PNAd staining and late rejection	All time points	Acute chronic	Hautz et al[29]
Face	Monkey	Lymphocytes HEVs		15, 36, and 332 d	Acute chronic	Hautz et al[29]

CCL, C-C chemokine ligand; CCR7, C-C chemokine receptor type 7; CD, cluster of differentiation; CXCL13, CXC motif chemokine ligand 13; CXCR5, CXC motif chemokine receptor 5; DC, dendritic cell; FDC, follicular dendritic cell; GC, germinal center; HEV, high endothelial venule; LTβR, lymphotoxin beta receptor; LV, lymphatic vessel; N.A., not applicable; N.I., not indicated; PNAd, peripheral node addressin; PT-TLO, posttransplant tertiary lymphoid organ; T/B compart, T/B compartmentalization; TLO, tertiary lymphoid organ; Treg, regulatory T cell.

characteristics of TLOs (T-, B-cell compartmentalization, HEVs, LVs) are found in the kidneys,[39] salivary glands,[58] and liver[59] of "super aged" mice (>23 mo old) without an overt stimulus. Fat-associated lymphoid clusters, a type of TLO that is apparent in young mice, becomes more prominent with age with an increase in aged adipose B cells and Nlrp3 inflammasome activation.[60] The stimuli that induce the various TLOs associated with aging are not completely understood and could be manifestations of autoimmune processes and/or responses to low-level chronic stress induced by external agents (microbes, tissue damage).

TLO STAGES

It is important to determine whether particular PT-TLOs are associated with acute rejection, and/or chronic rejection, or tolerance. As indicated in Table 1, most, but not all, are associated with chronic rejection. TLOs can be characterized by cellular composition and classified into stages that indicate their "maturity." Various authors attribute different characteristic to these stages and a consensus is yet to be reached regarding their classification. Staging schema have been put forward in autoimmunity in the pancreas of the NOD mouse,[13] atherosclerosis,[50,61] colorectal cancer,[62] and other tumors.[5] There are 3 stages of

pancreatic TLO development and dissolution in the NOD mouse, Initially, the cellular infiltrate is disorganized (stage I), then the cells organize into T- and B-cell compartments and HEVs appear as a typical mature TLO, with no apparent beta cell destruction (stage II). Later, the cells in the TLO are activated, the beta cells destroyed, and the TLO disappears to be replaced by a fibrotic accumulation (stage III). TLO maintenance depends on the presence of the beta cell antigen. Once it is removed, the TLO disperses. Sato et al[16] put forth a staging scheme for TLOs of human kidneys with pyelonephritis, those from young mice undergoing ischemic injury, or spontaneously developing TLOs in very old mice. Stage I consists of B cells and CXCL13, stage II includes B-cell infiltrates accompanied by FDCs, and stage III consists of organized GCs with FDCs. In this publication, the figures include compartmentalized T and B cells and HEVs, but the authors do not consider either of these characteristics in their staging scheme. Lee et al[63] used a slightly different staging scheme (I-TLOs lacking FDCs and GCs; II-TLOs with FDCs but no GC; and III-TLOs with FDCs and GCs was used recently to evaluate the correlation of TLOs and kidney rejection). Stage II, particularly the presence of FDCs, was associated with a less favorable outcome.

It is important to determine whether PT-TLOs are associated with acute rejection and/or chronic rejection or tolerance. In one of the earliest examples of PT-TLOs, HEV antibody staining intensity correlated with rejection grade.[15] Staining was most intense during early stages of rejection, remaining elevated throughout the episode and leveling off as rejection resolved. It seems logical that the Banff criteria for kidney rejection should be standardized to consider TLO staging as was the case in the study by Lee et al[63] in correlating TLO stage and Banff score.

FUNCTIONS OF PT-TLOs

Determination of the function of PT-TLOs and, in fact, all TLOS, will allow their manipulation when they are detrimental as in autoimmunity or helpful as in cancer. The key question is do they function in a manner like LNs in serving as sites of antigen presentation and generation of effector and memory cells and antibody-producing cells? Are PT-TLOs detrimental serving as sites of rejection? Or beneficial serving as sites of tolerance or even simply epiphenomena?

It is likely that certain PT-TLOs act as sites of antigen presentation and maturation of immune responses. They can have the cells (lymphocytes, APCs, stromal cells) vasculature (HEVs and LVs), and organization (T and B cells with respective APCs) to generate an immune response. Their HEVs can serve as entrance points for naive T and B cells, APCs, and sources of antigen either intrinsic to the TLO itself in its microenvironment (graft, autoimmune site, tumor) and LVs. Recipient APCs expressing donor peptides are present in transplanted kidneys.[10] There is much evidence from transgenic and autoimmune TLO models to support TLOs' ability to generate an immune response with evidence of antibody-producing plasma cells,[41,64] rearranged V genes and evidence of somatic hypermutation in the TLO,[65] distinct Vκ usage,[66] and evidence of distinct TLO B-cell clones.[67] Evidence that B-cell maturation occurs in PT-TLOs is apparent from the minimal overlap of B-cell alloantibody repertoires in human kidney grafts and peripheral blood[38] and the fact that B-cell clones in TLOs of rejecting human kidneys exhibit somatic mutations and differ from those in peripheral blood,[67] consistent with similar earlier studies of TLOs in transgenic models[41,64] and autoimmunity.[65,66] Nevertheless, the absence of defined dark and light zones typical of SLOs, the generation of autoantibodies,[1] and an apparent defect in the ability to expand Tfh cells in rejecting kidney lymphoid infiltrates[68] suggest that, although events typical of SLO GCs occur in TLOs, some differences exist.

T cells are important components of acute and chronic graft rejection, through their CD8 T cell–mediated cytotoxicity, their production of cytokines, such as LT, that contribute to TLO organization, and their provision of B-cell help. Determinant spreading, that is the generation of new T-cell reactivities, occurs in TLOs in autoimmunity.[69,70] Intramolecular and intermolecular determinant T-cell spreading also occurs in graft rejection[71] and is likely occurring directly in PT-TLOs, thus contributing to chronic inflammation and exacerbating graft rejection.

PT-TLOs likely serve as sites of T-cell antigen recognition and memory generation. Skin from rat insulin promoter-LTα mice that contains TLOs is rejected when transplanted to histoincompatible *aly/aly* mice that are totally devoid of lymphoid tissue, whereas skin from wild type mice is accepted.[28] Furthermore, naive lymphocytes transferred into *aly/aly* mice carrying rat insulin promoter-LTα skin TLOs differentiate into effector and memory cells[28] and in TLOs in renal allografts.[24] These studies and others demonstrate that TLOs in transplants "can" serve as sites of antigen presentation and differentiation, but it is unclear from these data whether they are the major sites of perpetuation of graft rejection. However, this is answered, the data are consistent with the interpretation that host APCs present antigen in TLOs and that both T-cell and B-cell differentiation occur at the local site.

TLOs likely can contribute to chronic organ transplant rejection[11] and are thus considered to be detrimental to graft survival. A considerable amount of literature demonstrates a correlation between TLOs and rejection in mice, rats, and humans. Alloantibody production at the graft site is one possible mechanism, as demonstrated in several studies including one with human kidney grafts.[38] An early study correlated B-cell infiltrates with human kidney rejection,[72] whereas another found no such correlation.[73] More recently, the staging scheme described by Sato et al[16] was used to evaluate progressive human kidney graft dysfunction.[63] Poor function correlated with highly differentiated TLOs, characterized as stage II, with T- and B-cell compartmentalization and FDCs (but not GCs).

The role of B cells in PT-TLOs has been a subject of continual scrutiny from the first studies that showed a local humoral response in rejecting rat aortas[11] and has been recently reviewed in some detail.[1] In general, as noted above, the emphasis has been on their role in alloantibody production and graft rejection, but B cells can also function as APCs and producers of cytokines, such as LT, which can contribute to TLO development and maintenance. On the other hand, B cells can produce IL-10, a suppressive cytokine, which has been reported to be a biomarker of enhanced renal graft survival.[74]

An alternative interpretation of the roles of PT-TLOs derives from analysis of a fully allogeneic murine renal graft model between DBA/2 donors and C57BL/6 recipients that results in variable acute rejection, chronic rejection, or indefinite acceptance.[10] TLOs with HEVs are seen and correlate with superior graft function (acceptance) whereas podoplanin$^+$ LVs are more likely seen in infiltrates of kidneys with poor function. Forhead box P3 protein (Foxp3) (a marker of Treg) is noted in kidney TLOs. Miyajima et al[75] also noted the presence of Foxp3 cells in the kidney TLOs in this model. Foxp3 depletion resulted in graft rejection, indicating that these Treg are functional. Treg are not unique to PT-TLOs, as they have been noted in prediabetic TLOs in NOD mice[76] and in tumor TLOs.[77] In fact, removal of the Treg results in tumor rejection, indicating their function. In a later study of tolerant DBA/2 kidney grafts in C57BL/6 mice the lymphoid accumulations are designated as Treg-rich organized lymphoid structures (TOLSs).[25] The TOLSs are located around an artery or arteriole, exhibit a progression from an early mixed CD8, B, CD4 cell, and CD4 Foxp3 infiltrate to a later predominance of Foxp3$^+$ cells. The authors posit that TOLSs differ from classic TLOs in that they lack T- and B-cell compartmentalization, GCs, and HEVs, and lack a requirement for LTβR signaling.[25] The absence of LTβR signaling is not unique to TOLSs, as this has been noted in other TLOs.[51,78] LTβR signaling is frequently,[9,40,79,80] but not always[81] required for PNAd$^+$ HEVs. The difference between the presence of HEVs in one study and their absence in another could be due to the time of analysis posttransplant (13.5 versus 60 wk) of the lymphoid accumulations. At the later time, the function of HEVs (ie, entrance of naive cells) might be less important. Thus far, the clinical significance of Treg in human PT-TLOs has not been determined, but it would be important to evaluate whether they are associated with long-term graft acceptance. At any rate, these studies demonstrate the importance of carefully categorizing the various kinds of lymphoid accumulations in grafts—those associated with acute rejection, chronic rejection, and tolerance to understand their differing functions to harness them for optimal clinical outcome.

THE ROLE OF PT-TLOs IN CANCER

The literature is replete with excellent articles and reviews concerning the roles of TLOs in cancer. These include summaries of early articles indicating that the higher the number of TLOS (as measured by HEVs and/or GCs), the more favorable the prognosis in breast cancer.[82] Later studies emphasized the role of intratumoral B cells in TLOs in cancer defense,[83-85] and a recent review summarizes the field in detail.[5] What role do TLOs play in malignancies in organ transplantation? It is well established that there is an increased incidence of several types of cancers after solid organ transplant, variously estimated between 4% and 18%.[86] These include colorectal cancer, nonmelanoma skin cancer, and non-Hodgkin's lymphoma. The conventional understanding of the increase in malignancies posttransplant is that the immunosuppressive regimens to prevent rejection also reduce overall immunosurveillance. Posttransplant patients with cancer have a poor prognosis. A comparison of tumors from transplant patients with those from nonimmunosuppressed patients with cancer showed a much-reduced number and size of TLOs[86]—pointing to an advantage of TLOs in tumor rejection, although likely a disadvantage in the transplanted organ where they can exacerbate the antigraft immune response.

Lymphoproliferative disorders can occur posttransplant. The pathogenesis of this complication of predominately proliferating B cells that can include B lymphomas has been attributed to 3 factors that reflect the fact that 50%–80% of these disorders are positive for Epstein-Barr virus (EBV). These include: a reduced defense after immunosuppressive therapies, the presence of EBV, and a derangement of molecular signaling and DNA repair mechanisms as a direct effect of immunosuppressant agents.[87] However, it is possible that the chronic immunostimulation and B-cell proliferation in PT-TLOs could also contribute to the development of such disorders and malignancies. This logic is consistent with the observation of an increase in lymphomas in individuals with Sjogren's syndrome,[88] an autoimmune condition of the salivary and lacrimal glands in which TLOs are a prominent feature. Similarly, there is an increase in gastric lymphomas in the chronic TLOs of *Helicobacter pylori* infection.[89] Is any aspect of posttransplant lymphoproliferative disorder attributable to the chronically proliferating and mutating B cells in the TLO in addition to the 3 factors noted above? The best evidence derives from non-EBV positive disorders. Support for such a role would derive from data from patients (or animals) with long-term grafts with TLOs and EBV-negative lymphomas. It is quite possible that the chronic B-cell proliferation and mutation in the TLOs could predispose to lymphomas even in the absence of overt immunosuppression, perhaps with well-matched donor recipient pairs.

CHALLENGES FOR PT-TLO RESEARCH

Staging Quantification

Although TLOs have been studied for many years, challenges and opportunities exist. One is the lack of a consensus regarding standardization of classification of the criteria for their identification and staging. As indicated above, various authors have put forth a variety of staging schemes, mostly based on immunohistochemical or fluorescent identification of cellular populations by their expression of various markers. Multiplex immunostaining combined with density and spatial distribution allows a quantitative approach to evaluating the nature of TLOs in primary and metastatic melanomas.[90] A recent publication presents an entirely different approach using hematoxylin and eosin staining and density assessment to evaluating TLOs in a variety of lung cancers.[91] The advantages of speed, minimal reagent use (eg, multiple antibodies), and automation could be useful clinically but lack the granular specificity of identification of TLO stages of maturation and function. Access to TLOs is another issue as their presence can be quite variable within the context of, for example, an individual organ undergoing rejection or responding to therapy. A biopsy can miss the full complement of TLOs in a large organ. The development of noninvasive biomarkers that could be detectable in serum or peripheral blood to assess the existence and nature of TLOs in, for example, a

transplanted organ would be useful in designing therapies to encourage or prevent them.

Imaging

Imaging TLOs, 3D in real time could address the nature of their interactions with the rest of the immune system and with other TLOs in the same organ, and reveal how they develop over time, and how they interact with and are influenced by their microenvironment. The increased vascular permeability in the inflammatory setting of TLOs can be exploited by using magnetic nanoparticle MRI. Early studies in NOD mice demonstrate in vivo in real time uptake of monocrystalline iron oxide nanoparticles by macrophages in inflammatory lesions.[92] A positive correlation of decreased inflammation with response to anti-CD3 therapy was apparent in these studies. This concept was further refined in studies of humans with type I diabetes.[93] It was possible with the use of ferumoxytol nanoparticle uptake by macrophages, to generate MRI 3D high-resolution pancreas maps. Although these studies did not evaluate the TLO per se, they provide insight in real time into the inflammatory process and should be readily applicable to the analysis of PT-TLOs.

Technical problems need to be solved to accomplish the goals of in vivo imaging. Several transgenic mouse lines including those with green fluorescent HEVs[94] and red fluorescent LVs[95] that have been used successfully in 2-photon microscopic analysis of LNs could be useful in such studies. One difficulty is the inaccessibility of target organs. Exteriorizing rejecting murine kidney transplants allowed in vivo imaging by 2-photon microscopy of interactions between DCs and antigen-specific T cells[24] and demonstrated that T resident memory cells do not circulate outside of the PT-TLO. These studies indicate that analysis of TLOs in real time is possible. Advanced intravital time-lapse microscopy was used to evaluate inflammation in the exteriorized pancreas of the NOD mouse over the course of several hours. By evaluating early (3–5 wk) or late (10–12 wk), insight was obtained into interactions between CD4 and CD8 cells with DCs and macrophages around the islet.[96] Interestingly, these authors did not observe any clear cellular organization or TLOs, possibly because of the fact they did not image B cells or HEVs. The time of peak TLO organization is crucial in their evaluation as indicated in previous studies.[13,97] At the present time, exteriorizing organs and evaluating multiple time points in the same animal is not practical for evaluating progression of PT-TLOs, although useful for short-term evaluation, and is not realistic for a clinical setting.

Manipulating TLOs Therapeutically

TLOs need to be accessed for therapeutic purposes, ideally without affecting the rest of the immune system. Once it has been determined that the TLO is detrimental to graft survival, it would be ideal to eliminate it or even switch it to a tolerogenic function. It was possible to prevent TLOs in a model of BALB/c mouse aortic segment transplantation into the carotid of C57BL/6 mice, where intimal hyperplasia resembles typical TLOs with Tfh, GCs, and plasma cells. Application of CCL21- and CXCR3-neutralizing antibodies to the local graft region at early times (before TLOs appear) significantly delays the progression of arteriosclerosis.[27] It would be interesting to determine if an increase in Treg in a PT-TLO could be generated by such a treatment. In a murine model of rheumatoid arthritis, IL-27 inhibits TLO development.[98] NOD mice develop a Sjögren's-like syndrome with TLOs in lacrimal and salivary glands. Treatment of such mice beginning at 9 wk with an LTβR-Fc fusion protein, which antagonizes LTαβ signaling, results in dramatic effects on the TLOs in salivary glands with reduction of lymphoid chemokines CXCL13 and CCL19, FDC networks, HEVs, and B and T cells.[99] LNs are affected only minimally, and salivary gland function is partially restored. LPR mice develop renal perivascular cell clusters of age with typical TLOs, with lymphoid chemokines, Tfh, and GCs. If the mice are treated with dexamethasone at 3 mo, when TLOs are in the process of developing, the lymphoid accumulations are drastically reduced in size as is the expression of lymphoid chemokines,[100] although the effect on the systemic immune response was not evaluated, and as noted above, dexamethasone treatment reduces TLOs in kidneys of aged mice.[16] Pretreatment of renal transplant patients with rituximab affects the composition of TLOs in that there are fewer B cells than in aged kidneys or those from patients with chronic kidney disease.[63] These studies, taken together, demonstrate that manipulation with external agents is possible, even when TLOs are well underway in their development, and provide hope for such treatments in inhibiting rejection and enhancing tolerance to transplanted organs.

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