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The Role of Ectopic Lymphoid Tissue in Allograft Rejection

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THE ROLE OF ECTOPIC LYMPHOID TISSUE IN ALLOGRAFT REJECTION

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THE ROLE OF ECTOPIC LYMPHOID TISSUE IN ALLOGRAFT REJECTION

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A b s t r a c t

The location of the immunologic response to an allograft is not known with certainty. However, organized collections of T cells, B cells and antigen presenting cells have been found in peripheral tissue, in close proximity to organs undergoing rejection. It is hypothesized that this tertiary lymphoid tissue may be a location in which activation of lymphocytes can occur, leading to rejection of an allograft. We report here that in a splenectomized *aly/aly* mouse, which is devoid of secondary lymphoid organs and will normally fail to reject an allograft, the presence of tertiary lymphoid organs is associated with graft rejection. We additionally find that tertiary lymphoid organs can act as lymph nodes, and can support effector and memory allograft rejection responses. It is demonstrated that ectopic lymphoid tissue in *aly/aly* mice will support the multiplication and transformation of transferred naïve CD4 and CD8 T cells into cells that display phenotypic markers characteristic of effector and memory lymphocytes. These results demonstrate that ectopic lymphoid tissue is associated with the loss of immunologic ignorance and is sufficient to enable graft rejection. This suggests that allograft rejection may take place within ectopic lymphoid tissue, and suggests that techniques to interfere with the development of this tissue might offer a therapeutic approach to preserving organ allografts.

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Lastly and most certainly not least, I wish to thank my wife, Susan, because when I worked in the lab, or read, or studied, or did anything at all connected to this research, the whirlwind of family life did not slow down, and neither did she.

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INTRODUCTION

Background of Problem

While organ and tissue transplantation holds great promise for the treatment of end-stage organ disease, immunological rejection of transplanted tissue remains a significant clinical challenge. In order to stave off rejection, transplant recipients must currently commit to a lifetime regimen of immunosuppressive drugs. While these pharmaceuticals greatly reduce the incidence and magnitude of the rejection process, their use comes with many drawbacks. The drugs suppress the entire systemic immune system, leaving patients open to opportunistic infection and malignancies. They are expensive, and most importantly, they fail to completely suppress long-term organ rejection.

Because of these substantial drawbacks of systemic immunologic suppression, any mechanism that would permit the immune system to remain disinterested in a specific antigen, such as a grafted organ, while maintaining the system's natural surveillance and disease suppression functions, would be highly desirable.

Acute rejection of an allograft depends on the responses of activated CD4 and CD8 T lymphocytes. Activation of naïve lymphocytes occurs when alloantigen is presented to a naïve lymphocyte by an antigen-presenting cell, along with coincident signaling through costimulatory receptors. Efforts to prevent rejection currently focus primarily on interruption of these signaling pathways in an effort to inhibit the subsequent activation and clonal expansion of alloreactive lymphocytes. It also important, however, to understand the anatomic context in which activation occurs, because if it is possible to interrupt the sites of

interaction of the cells involved in the adaptive immune response, such that foreign antigen is not presented to naïve T cells, it may be possible to exploit immunologic ignorance of grafted tissue, rather than tolerance, as a long-term mechanism permitting graft survival.

Immunologic Ignorance

Immunologic ignorance is a state in which the immune system remains quiescent because it is unaware that a particular antigen is present. In such a state, the immune system is not suppressed, but fully functional, and will freely respond to antigenic stimuli that are successfully presented. However, if the mechanism of antigen presentation is disrupted, then the immune system will remain oblivious to the presence of alloreactive tissue. As long as ignorance is preserved, foreign antigen, including transplanted tissue from unrelated donors, will remain free of efforts by the immune system to reject the tissue. However, if normal locations and mechanisms of immune stimulation are reestablished, ignorance will be broken, and allogeneic tissue will again be subjected to evaluation by the usual mechanisms of immune alloreactivity.

Zinkernagel has posited a general "antigen-localization dose time" model for immunologic interactions, which proposes that the localization of antigen with respect to lymphoid tissue and the time of interaction are prime determinants of the pattern of reactivity towards an antigen(1). Moderate quantity of antigen, newly presented to a naïve T cell by an antigen-presenting cell in the anatomic context of secondary lymphoid tissues will initiate a reaction. Antigens that do not reach lymphoid tissues, or do so in insufficient quantity or interact for too little time, will be ignored by the immune system. This model, when

applied to transplant antigens, implies that variation in the quantity or functional availability of tissue that facilitates interaction between antigen presenting cells and immunoreactive lymphocytes is a significant variable in determining the likelihood of reactivity. Precise understanding of the exact location of this immunological interaction is therefore of crucial importance if one is to successfully develop techniques to prevent the loss of ignorance, thereby facilitating the long-term survival of allografts.

The location of the alloimmune response

Preserving immunologic ignorance by interrupting the communication lines between the various parts of the adaptive immune response requires knowledge of where these interactions take place within the immune system. Most research to date has focused on the transport of antigen to secondary lymphoid tissue.

In 1968, Barker and Billingham demonstrated that skin grafts were rejected much more quickly when implanted into a recipient site that allowed lymphatic outflow, compared to placement in a vascular bed without lymphatic drainage (2). The presumptive mechanism behind this phenomenon is that secondary lymphoid tissue provides a permissive context for direct recognition of alloantigens that are presented by graft-derived antigen presenting cells to naïve T lymphocytes, which reside in these peripheral lymph nodes. Activated T lymphocytes then respond swiftly against graft tissue.

Consistent with this model, it was demonstrated in 2000 that a mouse devoid of secondary lymphoid tissue would indefinitely accept a vascularized allograft (3). Because the lymphocytes from these mice were able to mount an effective response if transferred to

another animal with intact lymphoid organs, it was concluded that the inability to reject the allograft in the lymphoid-deficient animal was due to immunologic ignorance, maintained by the inability of antigen presenting cells and potentially reactive lymphocytes to interact. This suggests that secondary lymphoid tissue is essential for the interaction between antigen-presenting cells and reactive antigen-specific lymphocytes.

Tertiary Lymphoid Tissue

There remains an additional possible lymphoid site for the activation of alloreactive lymphocytes. In addition to the embryonically derived lymphatic system, mucosal lymphoid tissue and the spleen (together, the traditional secondary lymphoid tissue) there is evidence that ectopic lymphoid tissue located in the peripheral tissues, known as tertiary lymphoid tissue, is able to contribute to the development of an immune response against a wide array of antigens.

Tertiary lymphoid organs (TLO) are organized collections of T cells, B cells and antigen presenting cells that form in peripheral tissue, usually in association with chronic inflammation. This ectopic lymphoid tissue displays a wide range of anatomical organization, ranging from simple expression of lymphoid-related signaling molecules to full-fledged lymph node-like structures with characteristic high endothelial venules, germinal centers, and specific areas of B- and T cell localization.

Distribution of Tertiary Lymphoid Tissue

TLO have previously been shown to be present in multiple pathologic conditions, including autoimmunity, infection and neoplasia.

Tertiary lymphoid organs in autoimmune disease

TLO have been observed at the site of autoimmune processes such as Sjögren's disease, Graves' and Hashimoto's thyroiditis, and rheumatoid arthritis (4). Thyroid tissue from patients with both Graves' and Hashimoto's disease has been noted to display lymphoid accumulations with high grade architecture, including high endothelial venules near organized T cell areas, distinct B cell follicles, and plasma cell concentrations (5). These lymphoid follicles contain high levels of antibodies specific for thyroid antigens and are sites of high levels of recombinase gene expression (RAG1, RAG2), suggesting that affinity maturation of lymphocytes against thyroid antigen occurs within these TLO (6).

The localization of leukocyte subsets within the circulation and the tissues is determined by the differential expression of chemokines, integrins, and other adhesion and communication molecules, which enable lymphocytes to enter or leave specific tissues. Analysis of chemokine expression can provide an indication that a particular tissue is actively engaged in the recruitment of a particular type of leukocyte. The chronically inflamed joint in rheumatoid arthritis has been demonstrated to display elevated levels of B and T cell homing chemokines, such as CXCL13, CXCL12 and CCL19, constitutively, which suggests a mechanism for the organization of ectopic lymphoid tissue near the site of pathology (7). Synovial tissue in rheumatoid arthritis displays lymphoid-like accumulations of CD4 T cells within the inflamed synovial membrane. These collections have been demonstrated to be functional, as the germinal center-like structures in the synovial tissue support hypermutation and terminal B cell differentiation (8).

Similar structures have been found in Sjögren's syndrome, an autoimmune disease characterized by autoantibody assault on salivary gland tissue. As in the case of rheumatoid arthritis, ectopic expression of T and B cell chemokines (CXCL12 and CXCL13 respectively) and formation of germinal center-like structures has been noted within affected salivary tissue (9). It has been further demonstrated that these germinal centers produce characteristic Anti-Ro and Anti-La autoantibodies, suggesting that local antibody production contributes to the pathology of the disease (10).

Tertiary lymphoid organs in infection and neoplasia

TLO have been noted in multiple organs during infectious challenge. In response to infection or inflammation, it has been shown that germinal center-like tissue appears in the lungs of infected mice (11). This lymphoid tissue is known as inducible bronchus-associated lymphoid tissue, or iBALT. This tissue expresses the lymphoid chemokines BLC (CXCL13) and SLC (CCL21), and on histologic examination, distinct T- and B cell areas are found within iBALT. In transgenic mice that lack other components of the peripheral lymphoid system (lymph nodes, spleen, Peyer's patches), iBALT has been shown to be sufficient to mount a functional immune response against viral infection, showing that TLO can fully support immune reactions without depending on native secondary lymphoid organs.

There have been multiple findings of TLO in the context of bacterial infection. Liver-specific peripheral immune tissue has been noted during hepatic granulomatous infection with *Propionibacterium actes* (12). TLO in murine small intestine are functional sites for the production of antigen specific IgA following bacterial challenge (13). Vessels with

histologic and biochemical similarity to lymphoid tissue have been described in gastric mucosa in correlation with *Helicobacter pylori* infection, and were noted to recede with antibiotic eradication of the microbe (14).

In invasive ductal carcinoma of the breast, ectopic germinal centers in intratumoral follicles support affinity maturation of B cells, with enhanced reactivity of follicular antibodies toward tumor antigens, suggesting a beneficial autoimmune response generated within TLO against malignant tissue (15).

Development of Lymphoid tissue

Lymphoid neogenesis occurring in peripheral tissue is believed to recapitulate the development of lymphoid tissue that takes place during organogenesis (16). Because of the parallels between fetal lymphogenesis and ectopic lymphoid neogenesis, and the importance of the transgenic models with germ-line defects in lymphogeneic pathways, the development of lymph nodes during embryogenesis will be briefly reviewed.

Fetal lymphogenesis

In the developing fetus, lymphogenesis occurs early in organogenesis, soon after the development of the vasculature. In murine development, this occurs on embryonic day 11 when buds of endothelial cells begin to project off venous conduits, near sites of signaling molecules.

Development of lymph nodes critically depends on interaction between hematopoietic "inducer" cells and stromal cells, which display both the adhesion molecule VCAM1 and the lymphotoxin-β receptor (LTβR). Both of these cells are present in early lymph node

anlagen. The inducer cells bear both the LTβR ligand $LT\alpha_1\beta_2$ and the integrin $\alpha_4\beta_1$ that is able to bind to the VCAM1 adhesion molecule on stromal cells described above. Once bound to VCAM1, $LT\alpha_1\beta_2$ mediated signaling of the LTβR on the stromal cell can take place.

THE LYMPHOTOXIN-BETA RECEPTOR

Signaling through the lymphotoxin-β receptor is critical to the formation of lymph nodes, and can be accomplished by two members of the TNF-family, the ligands $LT\alpha_1\beta_2$ and LIGHT. Because LIGHT deficient mice do develop lymphoid tissue, it is presumed that $LT\alpha_1\beta_2$ plays a dominant role. Mice deficient in lymphotoxin alpha(LTα) do not develop lymph nodes or Peyer's patches (17)(18). In such mice, treatment of pregnant mice with agonist antibody that is capable of stimulating the LTβR will restore lymph node development in offspring, thus demonstrating the critical role of LTβR in the development of lymph nodes (19) Blockade of the LTβR in utero with soluble blocking antibody will prevent the formation of most lymph nodes; however, a subset of mucosal lymph nodes will still develop, indicating that the LTβR pathway is not the exclusive trigger for lymphogenesis (20).

The LTβR mediates signaling through two distinct NFκB related pathways (21). These pathways are relevant to this experiment because an important murine model of ignorance, the *aly/aly* mouse, involves a mutation in a component of these pathways. In the initial "classical" LTβR receptor signaling pathway, LTβR ligation induces activation of an IKK complex, initiating a signaling pathway that results in upregulation of adhesion molecules

ICAM1, VCAM1 and MAdCAM-1 (22). The increased synthesis of these adhesion molecules may lead to further inducer cell adherence, thereby exponentially increasing LTβR signaling through positive feedback from the increased population of inducer cells. The second "alternative" LTβR signaling pathway leads to NFκB-inducing kinase (NIK) activation, which triggers IKKα and subsequently induces upregulation of transcription of multiple chemokines that are critical for lymphoid cell homeostasis including SLC (CCL21), BLC (CXCL13), and ELC (CCL19) (23). The *aly/aly* mouse is the result of a point mutation in the NIK gene, which prevents activation of the alternative LTβR pathway. Because of this inhibition, the *aly/aly* mouse possesses neither lymph nodes nor Peyer's patches, in addition to other defects including the absence of mammary glands, and abnormal splenic and thymic architecture (24).

During the development of lymph nodes, other critical signaling molecules are expressed, including the addressins PNAd (peripheral node addressin) and MAdCAM-1(mucosal addressin cell adhesion molecule), as well as enzymes such as HEC-6ST, which is necessary to appropriately sulfate these glycoproteins (25). The presence of these molecules has proved useful for the identification and localization of ectopic lymphoid tissues.

Experimentally induced lymphoid neogenesis

Mice that transgenically express ligands of the LTβR or the TNF receptor (TNFR) will develop inflammatory lesions at the site of expression (16). Such lesions have been shown to display characteristics of organized lymphoid tissue. RIP-LTα transgenic mice, which

express lymphotoxin-α under the control of the rat insulin promoter, develop chronic inflammatory lesions in the skin, pancreas and kidney. Pancreatic and renal lesions have been shown to contain T and B lymphocytes, plasma cells, and antigen presenting cells, and are the site of expression of lymphoid homing chemokines such as secondary lymphoid chemokine (SLC) and B lymphocyte chemokine (BLC) (26). The lesions display anatomically delineated areas with resident cells arranged in a manner similar to a classic lymph node. Formation of these lesions is critically dependent on the presence of TNF receptor 1 (27). Skin lesions in RIP-LTα transgenic mice appear histologically similar to the pancreatic and renal lesions, however there remains a need for further characterization of the contents of intradermal lesions. Mice with transgenic expression of both LTα and LTβ show accumulations with even more distinct T and B cell compartmentalization and prominent follicular dendritic cell networks within ectopic lymphoid tissue (25). Furthermore, expression of characteristic lymph node molecules such as PNAd and HEC-6ST as well as lymphoid chemokines can be observed within the lesions.

Tertiary lymphoid organs and the alloimmune response

To date, the functional capacity of TLO in allograft rejection has not been conclusively demonstrated. It has previously been shown that an immune response to an allogeneic vascularized transplant is dependent on the presence of secondary lymphoid organs (3). This paper demonstrated that in a splenectomized *aly/aly* model, devoid of all secondary lymphoid tissue, vascularized cardiac allografts were accepted indefinitely. It was shown that despite the failure to reject the allograft, the host immune cells remained competent, and were able to mediate rejection either if lymphocytes were transferred to recipients with

intact lymphoid structures or if exogenously activated T cells were transferred to transplant recipients. Because the acceptance of the grafts occurred without immunosuppression, and coexisted with an otherwise functional immune system, immunologic ignorance of the allograft was presumably a consequence of the absence of secondary lymphoid tissue.

Lymph node-related adhesion molecules have been noted to be present at the sites of allograft rejection. In a study of human post-transplant endomyocardial biopsy samples, Toppila et al. demonstrate an increased level of expression of L-selectin ligands selectively during the acute rejection episodes (28). This ligand, which mediates lymphocyte extravasation into peripheral tissue, was not present in the absence of rejection, and the magnitude of its expression was correlated with the degree of rejection. Kirveskari, et al, similarly demonstrate the upregulation of L-selectin ligands on kidney endothelial tissue solely during episodes of acute rejection, also with a magnitude of expression correlated with the degree of rejection (29). The presence of this lymphocyte ligand suggests a mechanism for the incursion of lymphocytes into graft tissue, thereby facilitating rejection.

The possibility that there is an additional site of interaction between APCs and naïve T cells during graft rejection follows from the identification of organized lymphoid structures within murine cardiac allografts undergoing chronic rejection (30). In this study, lymphoneogenesis was invariably associated with rejection: all recipients with either tertiary lymphoid organs with discrete T and B cell zones and PNAd positive high endothelial venules(HEV), or only less organized $PNAd^{+}$ HEV, were found to have some

degree of rejection. Of the allografts with chronic rejection, 78% had identifiable lymphoid neogenesis. Allografts or syngeneic grafts that were not undergoing rejection did not have evidence of lymphoid neogenesis.

Recently, similar findings were found in human cardiac and kidney tissues that were explanted following chronic rejection. In all rejected organs, but not in control explants, germinal center-like structures were found that contained T lymphocytes and areas of proliferating B cells in close proximity to follicular dendritic cells (31). In a rodent transplant model by the same authors, areas of TLO found in the vascular endothelium of transplanted aortic tissue displayed HEV phenotypic markers and organized lymphoid anatomy. The cell population within the TLO changed over time from a CD8 T cell infiltrate consistent with an acute cytotoxic response to a CD4 T cell population that displayed cell surface markers characteristic of helper T cells that assist resident B cells.

This close linkage between allograft rejection and the presence of ectopic lymphoid tissue suggests that there is a functional role for tertiary lymphoid tissue in the alloimmune response. The current study creates an experimental model to prospectively test the relationship between ectopic lymphoid tissue and transplant rejection.

STATEMENT OF PURPOSE, SPECIFIC HYPOTHESIS AND SPECIFIC AIMS OF THE THESIS

The review above suggests that ectopic lymphoid tissue may play a role in the loss of immunologic ignorance of an allograft, and thus may be a site of interaction between antigen presenting cells and T cells that is critical for allograft rejection. Demonstrating that the presence of ectopic lymphoid tissue is associated with graft rejection, the absence of ectopic lymphoid tissue is associated with immunologic ignorance, and the transfer of ectopic lymphoid tissue can reconstitute an alloimmune response would, in a manner consistent with Koch's postulates, strongly suggest a functional role for ectopic lymphoid tissue in the rejection of tissue allografts. Understanding the capacity of specific tissue to play a contributory role in allograft rejection may prove significant in developing therapies designed to prevent rejection of transplanted organs and tissue.

The **specific hypothesis** of this thesis is that ectopic lymphoid tissue in transplanted skin acts as functional lymphoid tissue and is sufficient to break immunologic ignorance. This thesis aims to prove this hypothesis by demonstrating the loss of immunologic ignorance and the development of specific lymph node function following the introduction of intragraft TLO into a transplant model, and by assessing the growth and transformation of T cells in mice with transplants containing tertiary lymphoid tissue.

To explore the functional capacity of tertiary lymphoid organs in supporting the alloimmune response and the breakage of immunological ignorance, we compare here the survival of wildtype allogeneic skin grafts (allografts), syngeneic RIP-LTα skin grafts that

have ectopic lymphoid tissue transgenically expressed in the skin, and allogeneic RIP-LTα skin grafts, also with dermal ectopic lymphoid tissue, after transplantation to splenectomized *aly/aly* (*aly/aly*-spleen) mice without secondary lymphoid organs (figure 1).

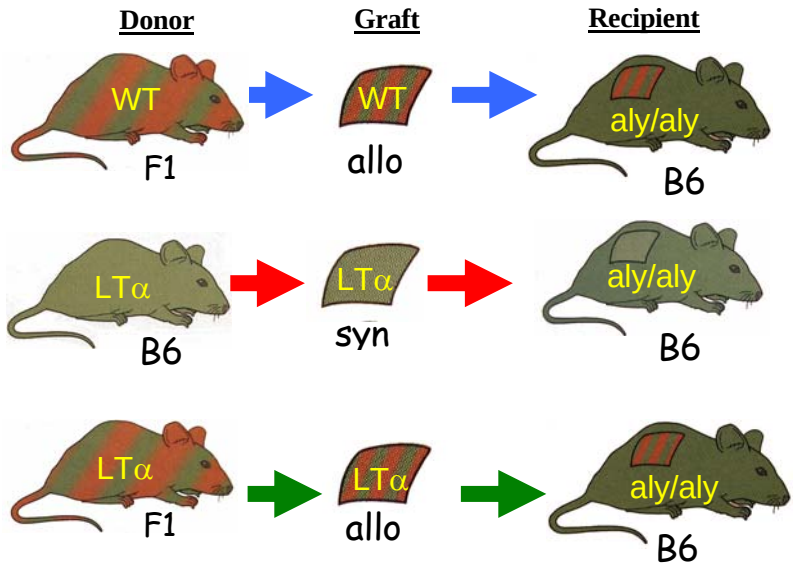

Figure 1 The experimental groups. Donor tissue is either allogeneic wild-type, syngeneic LTα transgenic or allogeneic LTα transgenic skin. All recipients are splenectomized *aly/aly* B6 mice.

It is expected that splenectomized *aly/aly* mice will remain ignorant of an allograft, consistent with prior published research. It is the **first aim** of this thesis to test whether the presence of ectopic lymphoid tissue in an allograft will be sufficient to break immunologic ignorance in this host and will lead to rejection of allografts that would otherwise remain accepted. Control syngeneic transplants, with or without intragraft TLO, should show no rejection.

If ectopic lymphoid organs actually function as lymph nodes, then it should be demonstrable that the presence of this tissue permits the development of the effector and

memory components of an allograft rejection response. The **second aim** of this thesis is to assess the response of a splenectomized *aly/aly* mouse to an allogeneic skin graft following simultaneous transplantation of allogeneic RIP-LTα skin, prior transplantation of allogeneic RIP-LTα skin, or prior transplantation of syngeneic RIP-LTα skin. As mentioned above, the splenectomized *aly/aly* mouse has been shown to accept allografts indefinitely, without developing an effector or memory response. If tertiary lymphoid tissue present in the RIP-LTα skin does indeed function as a lymph node, then one would expect to observe the establishment of an allospecific effector T cell response at the time of RIP-LTα skin transplant and generation of memory T cells, which are able to mediate the rejection of subsequent transplants.

The **third aim** of this thesis is to assess and quantify the actual development of T cells with effector and memory phenotypes in otherwise lymphoid-organ free mice that receive a transplant of allogeneic TLO-containing skin. If the hypothesis is true, then one should be able to transfer congenic naïve lymphocytes to *aly/aly* mice, and, following an allogeneic TLO-containing transplant, reharvest the same lymphocytes and observe an increase in expression of markers of effector and memory phenotypes on these cells.

METHODS

Mice: Wildtype (*wt*) C57BL/6 (B6, Thy1.2, H-2^b), B6.PL-Thy1a/Cy (B6.1.1, Thy1.1, H-2^b), and CB6F1/J (H-$2^{d/b}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). alymphoplasia (*aly/aly*) mice (Map3k14-/-, Thy1.2, H-2^b), were purchased from CLEA Japan (Osaka, Japan) and bred under specific pathogen free conditions. A RIP-LTα mouse colony (B6, Thy1.2, H-2^b) was originally obtained from Nancy Ruddle (Yale University) and maintained at the Yale Animal Resource Center. RIP-LTα (B6, Thy1.2, H-2^b) were crossed with BALB/cJ (Thy1.2, H-2^d) to generate RIP-LTα F1(CB6F1/J, H-$2^{b/d}$) mice.

Surgical Procedures:

SPLENECTOMY:

Splenectomy was performed under general anesthesia via a subcostal incision at least 1 week prior to transplant procedure. Completeness of splenectomy was verified post-mortem in every mouse.

SKIN TRANSPLANTATION:

Full thickness skin transplantation was performed as follows. The transplant was performed using general anesthesia. Abdominal skin, free of nevi or scarring, from donor mice was harvested and subcutaneous layers removed by gentle scraping underneath a cold saline bath. Recipient mice were 8-12 week old B6 *aly/aly* splenectomized mice. A 1.5-3.0 cm^2 graft bed was prepared on the dorsal flank of the recipient by excision of native skin. Donor skin was trimmed to fit the prepared bed, placed in situ and secured with skin

staples and bandages for seven days. Mice received acetaminophen in their drinking water for 3 days postoperatively for analgesia. Graft viability was monitored daily for the first seven days, after which grafts were monitored every other day. Rejection was defined as > 90% graft necrosis. Acceptance was defined as complete healing of the graft with evidence of hair growth.

Lymphocyte isolation and characterization

ISOLATION AND ADOPTIVE TRANSFER OF NAÏVE T CELLS:

Spleen and lymph node cells were harvested from Thy1.1^{+} B6 mice to obtain naïve T cells. Harvested cells were enriched for T cells using Nylon Wool Fiber Columns (Polysciences, Inc., Warrington, PA). Nylon wool purified T cells were then labeled with fluorochrome tagged antibodies against CD4, CD8 and CD44. These cells were sorted for naive CD4^{+}CD44^{lo} and CD8^{+}CD44^{lo} populations (> 98% purity) using a FACS Aria high-speed cell sorter.

Sorted naive CD4 or CD8 T cells (Thy1.1, H-2^{b}) were injected intravenously into the tail vein of splenectomized *aly/aly* (*aly/aly*-spleen, Thy1.2, H-2^{b}) recipients three days following skin transplantation. Adoptive hosts received 5 x 10^{6} sorted cells.

CELL HARVEST AFTER ADOPTIVE TRANSFER:

Mice were perfused with heparin (100 U/ml) at the time of sacrifice. Blood was removed by cardiac puncture and erythrocytes were lysed with pure water. The liver was perfused via the portal vein with 50 U/ml collagenase IV solution (Worthington Biochemical, Lakewood, NJ), removed and passed through a 70 μm strainer. The tissue suspension was incubated at 37ºC with 50 U/ml collagenase IV in RPMI 1640 plus 5% FCS for 30

minutes. Lymphocytes were then separated on a 25% Optiprep gradient (Accurate Chemical and Scientific Corp., Westbury, NY). Lungs were perfused with PBS via right ventricular cannulation, removed, finely macerated and incubated at 37ºC in 50 U/ml collagenase IV solution for 60 minutes. The digested tissue was passed through a 100μm strainer followed by centrifugation and RBC lysis. To obtain bone marrow cells, the femur and tibia were flushed with PBS and the cell suspension was centrifuged followed by RBC lysis. The skin was harvested, minced and digested at 37ºC for 2 hours in a solution containing 2.7mg/ml collagenase, 1mg/ml DNAse, 0.25mg/ml hyaluronidase. The digested tissue was passed through a 100μm strainer.

FLOW CYTOMETRY STAINING:

Flow cytometry was performed to phenotype naïve, effector and memory lymphocytes and to isolate congenic (Thy1.1^{+}) lymphocytes prior to and following adoptive transfer. Fluorochrome-conjugated antibodies were purchased from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA) or SouthernBiotech, (Birmingham, AL). Antibodies were against CD4 (RM4-5), CD8a (53-6.7), CD44 (Pgp-1), CD62L (MEL-14), and CD90.1 (OX-7) moieties. Flow cytometry was performed was done on FACS Aria or LSRII analyzers (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

IN VIVO PROLIFERATION ASSAY

Sorted Thy1.1^{+} CD4 and CD8 effector or memory T cells were labeled with CFSE (Molecular Probes, Eugene, OR) for 10 minutes at 37ºC, washed and adoptively transferred to either *aly/aly*-spleen or *wt* recipients (Thy1.2). Lymphocytes were harvested 15 or 60

days later from lymphoid and non-lymphoid tissues according to the protocol described above. Thy1.1^{+} lymphocytes were isolated by flow cytometry and the extent of cell proliferation was measured by CFSE dilution in the transferred cell population.

Delineation of work

The design and implementation of this research depended greatly on a team with diverse strengths, talents and experience. Preliminary research and alternative experimental strategies were investigated by Fadi Lakkis, Zenhua Dai, Michael Reel and Isam Nasr. Experimental methodology was designed by Fadi Lakkis, Isam Nasr and Michael Reel. Murine surgery was performed by Isam Nasr and Michael Reel. Graft survival was monitored by Isam Nasr. Isam Nasr and Michael Reel accomplished lymphocyte harvesting, with technical assistance provided by Lakkis Lab members. Flow cytometry was performed primarily by Martin Oberbarnscheidt. Statistical analysis was performed by Isam Nasr and Michael Reel. Preparation of figures to incorporate graphic elements constructed by Isam Nasr.

RESULTS

Finding 1: Tertiary Lymphoid Organs are associated with graft rejection

It has previously been demonstrated that a splenectomized *aly/aly* mouse will indefinitely accept a vascularized allograft (3), where it was found that the acceptance was due to immunologic ignorance of the graft. To explore the hypothesis that tertiary lymphoid tissue is sufficient to break such immunologic ignorance, it is necessary to show that in an otherwise ignorant model, the singular addition of tertiary lymphoid tissue leads to rejection. In order to demonstrate this, skin transplants from transgenic RIP-LTα mice were grafted onto *aly/aly* splenectomized mice. RIP-LTα mice express lymphotoxin-α under control of the rat insulin promoter, and display markedly increased expression of LTα in the pancreas, skin and the kidney. The expression of LTα in these areas leads to the local development of ectopic lymphoid tissue, similar in nature to that found in a lymph node, and notably similar to the tertiary lymphoid tissue found at the sites of allograft rejection in wild-type animals receiving vascularized allografts. Transplant of allogeneic RIP-LTα skin accomplishes both the establishment of ectopic lymphoid tissue as well as the implantation of antigenic tissue. Transplantation of syngeneic LTα-expressing donor skin is used to control for inflammatory stimuli occurring as a consequence of either the surgical transplant procedure or the expression of LTα.

Three different experimental skin transplant conditions were examined. In all cases, skin transplant recipients were splenectomized *aly/aly* B6 mice. In case 1, the donor mouse was

a wild-type F1 generation BALB/c x B6 mouse, allogeneic to the B6 recipient. Consistent with previously reported research(3), all six *aly/aly* recipients indefinitely accepted allogeneic skin transplants. In case 2, the donor was a syngeneic B6 RIP-LTα transgenic mouse, which expressed LTα in the skin, including skin used for transplant. This syngeneic transplant was also accepted indefinitely (n = 6). Case 3 incorporated an allogeneic, F1 generation BALB/c x B6 transgenic RIP-LTα mouse as the donor. When skin from this donor, which contains tertiary lymphoid organs, was transplanted onto a B6 *aly/aly* recipient, the skin was rejected in a median of 18 days (n = 12) (figure 2).

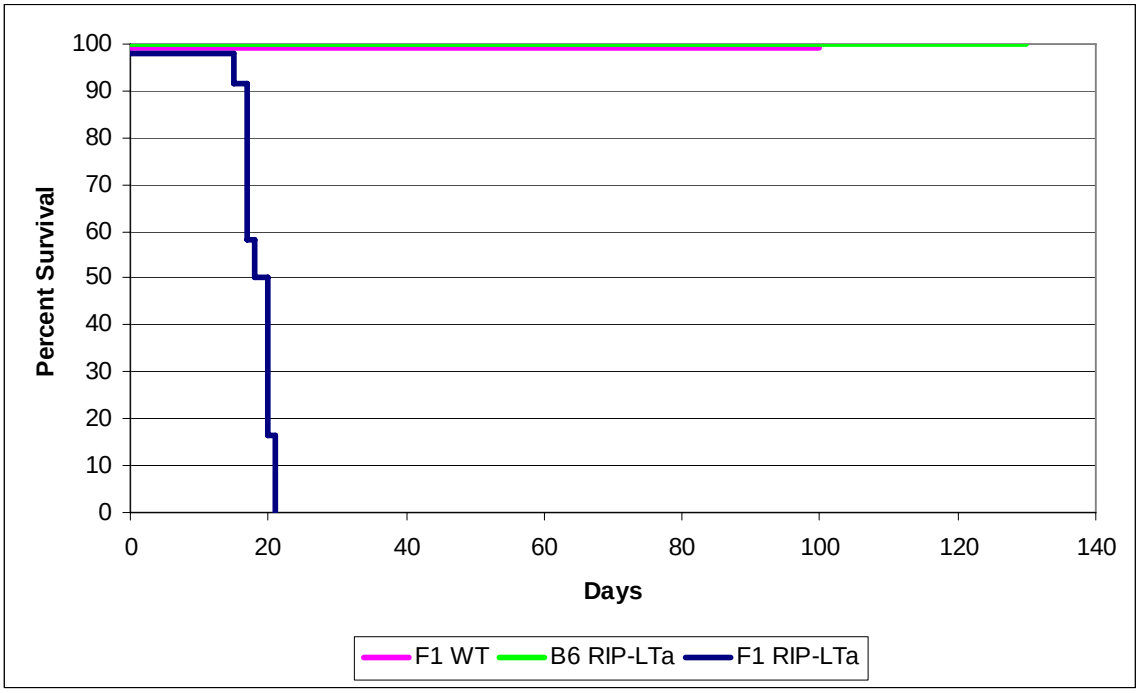

Figure 2 Differential survival of allografts containing tertiary lymphoid organs. Allogeneic wild-type skin and syngeneic LTα-expressing skin transplants are accepted indefinitely (n=6). Allogeneic skin expressing LTα rejects after a median of 18 days (n=12).

Finding 2: Tertiary Lymphoid Organs can function as a lymph node, and can enable effector and memory allograft rejection responses

The alloimmune response occurs in two phases. At the time of a transplant, naïve allospecific T cells that encounter antigen presenting cells bearing alloantigen receive costimulatory signals and become activated T cells. Clonal expansion of these activated T cells occurs, developing an effector response that may lead to the rejection of the allograft. Following the effector response, there is a dramatic contraction of reactive lymphocytes, during which the vast majority of the expanded reactive cell population undergo apoptosis. At the conclusion of this process, a select number of reactive cells remain in the circulation as memory T cells, which are able to mount a swift response in the periphery if antigen is encountered at a later time.

In the context of immunologic ignorance, however, neither the effector, nor memory response occurs, leading to acceptance of alloreactive tissue. One such cause of ignorance is a failure of antigen presenting cells to present antigen to naïve alloreactive lymphocytes. It is theorized that the immunologic ignorance of a splenectomized *aly/aly* mouse is a consequence of the lack of secondary lymphoid tissue, leading to a failure of interaction between antigen presenting cells and T lymphocytes. If this hypothesis is true, then transplanting tissue containing ectopic lymphoid tissue should be presumed to supply such a meeting site, and should be expected to permit rejection of allogeneic tissue.

To examine the ability of ectopic lymphoid tissue to permit an effector alloimmune response, a recipient splenectomized *aly/aly* mouse simultaneously receives two skin transplants. One graft comes from a wild-type F1 BALB/c x B6 mouse, which is allogeneic to the B6 *aly/aly*. As mentioned above, as a stand-alone transplant, this graft would normally be accepted indefinitely. The second graft, placed on the opposite flank of the recipient in the same surgical procedure, comes from an allogeneic F1 BALB/c x B6 RIP-LTα transgenic mouse. When this operation was performed on six recipients, both grafts were rejected in all animals. The median graft survival time was 52 days for the *wt* graft and 53 days for the F1 RIP-LTα graft (figure 3). Thus, the presence of a LTα expressing allograft precipitated the rejection of an otherwise ignored allograft. Surprisingly, the time to rejection was considerably longer when simultaneous transplants were performed (figure 3) than when a single transgenic transplant was performed (figure 2). Reasons for this delay in rejection are unclear, but may possibly be related to the aggregate quantity of grafted tissue that is rejected.

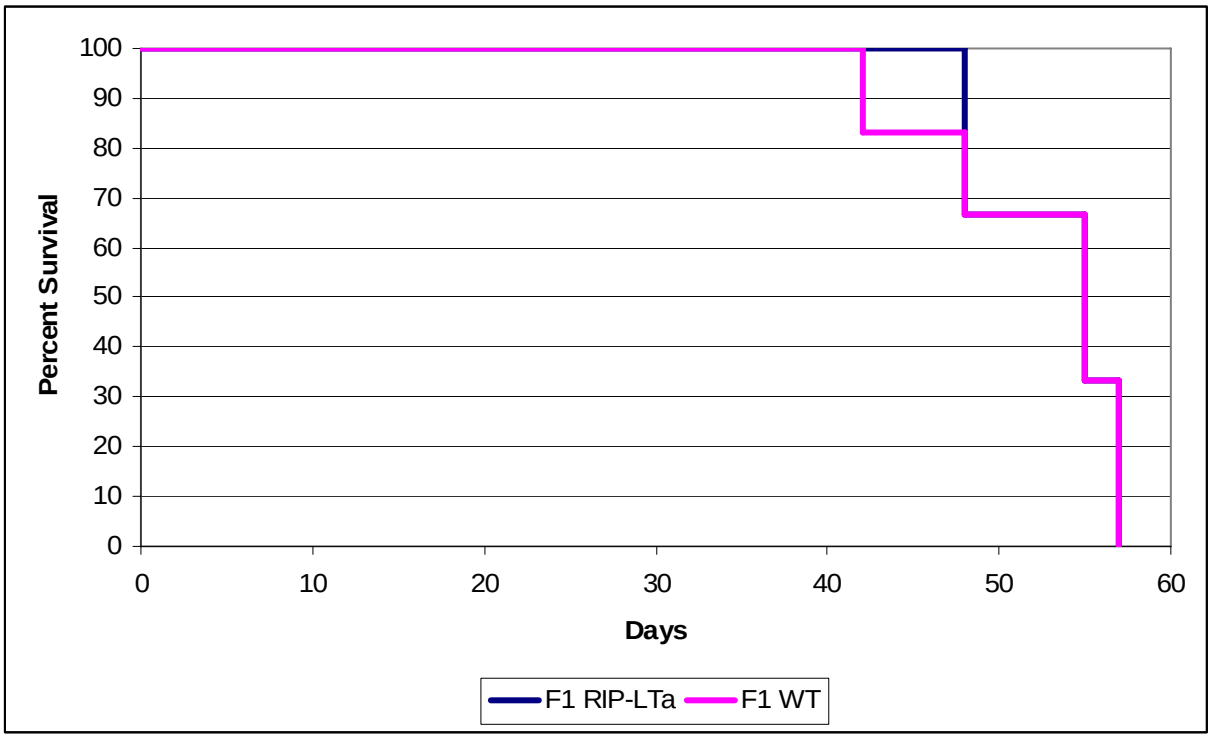

Figure 3 TLO permit generation of effector T cells. When LTα skin and *wt* allogeneic skin are simultaneously transplanted, the LTα skin allows the rejection of both grafts

To examine the ability of tertiary lymphoid tissue to spur a memory alloimmune response, 6 *aly/aly* recipients were given asynchronous allogeneic skin transplants. Initially, allogeneic grafts from F1 BALB/c x B6 RIP-LTα mice were placed, with rejection occurring shortly after (MST 21 days), consistent with the first experimental finding described above (figure 4). 50 days later, after the graft tissue had necrosed and been replaced by scar, a second transplant was performed, using wild-type allogeneic F1 BALB/c x B6 skin. Despite the lack of secondary or tertiary lymphoid tissue in this twice-transplanted *aly/aly* recipient, the second graft was also rejected, with a median survival time of 17 days, thus suggesting that antigen specific memory T cells that had been

generated during the initial transplant were still circulating and retained the functional capacity to reject subsequent allograft tissue.

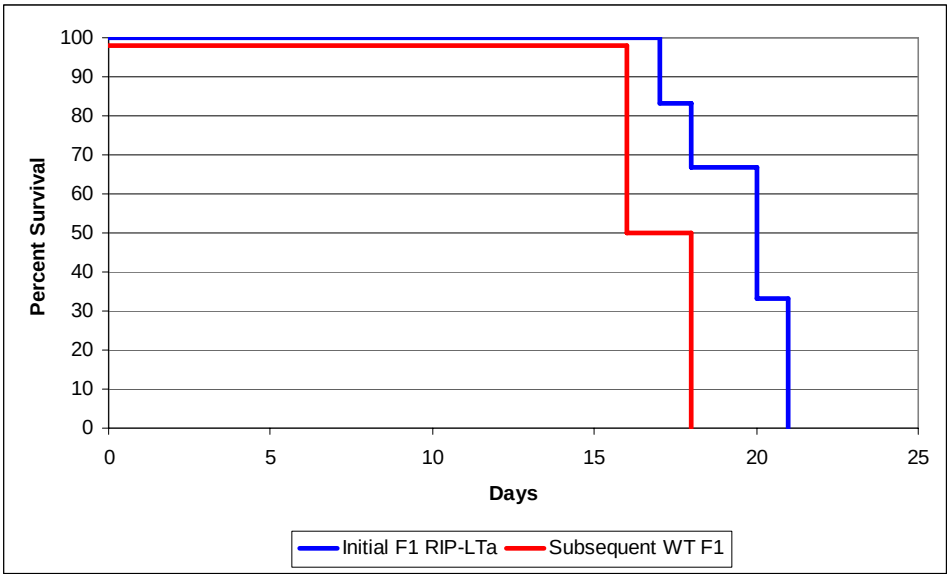

Figure 4 TLO permit generation of memory T cells. When allogeneic LTα skin is transplanted and allowed to reject, subsequent *wt* allogeneic grafts are rejected, rather than accepted as in Fig. 2.

In the final demonstration of the functional capacity of ectopic lymphoid tissue, six splenectomized *aly/aly* recipient mice again receive two asynchronous transplants. In this case, the initial transplant comes from a syngeneic B6 RIP-LTα donor. As in the control for the first experiment (figure 2), the syngeneic transplant is accepted, demonstrating again that RIP-LTα does not independently trigger graft rejection (figure 5). With an accepted syngeneic skin graft remaining in place, the animal receives a second, wild-type allogeneic F1 BALB/c x B6 skin graft on the opposite flank. The initial graft remains undisturbed. In this case, the new wild-type allograft is no longer accepted by the *aly/aly* recipient, but is

instead rejected in a median of 31 days, suggesting a functional role for the ectopic lymphoid tissue located in the syngeneic graft on the contralateral flank of the recipient.

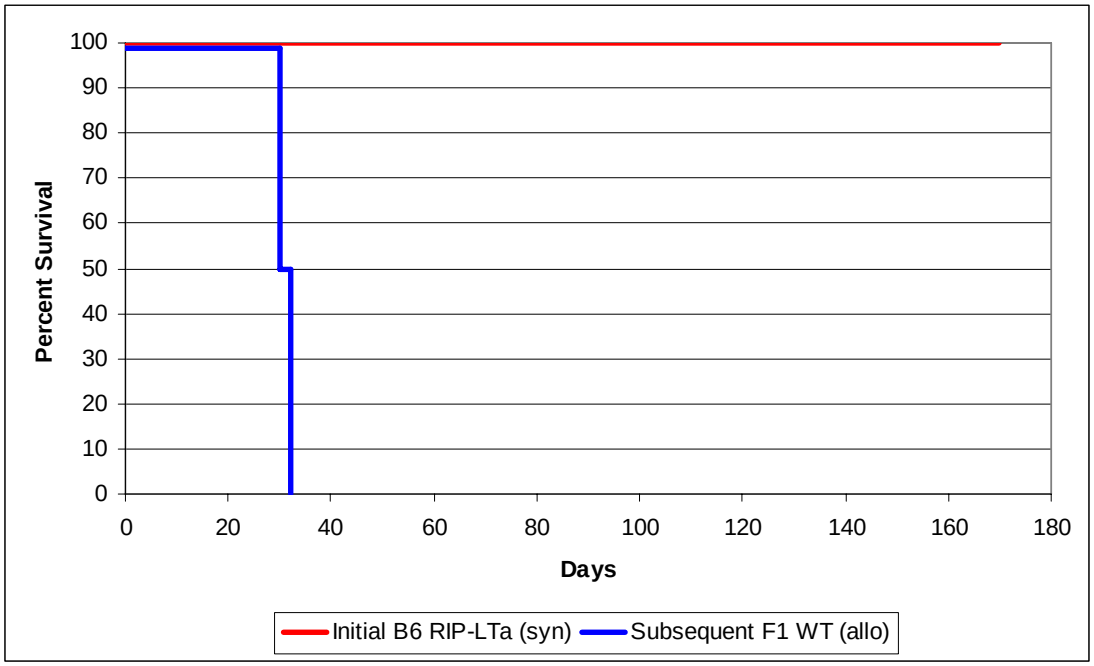

Figure 5. TLO function as a lymph node. Syngeneic LTα skin is transplanted and allowed to heal in. Subsequent grafts of allogeneic wild type skin are now rejected (n=6).

Finding 3: TLO permit development and multiplication of T cells that acquire effector and memory phenotypes

The preceding findings demonstrate on a macroscopic scale the tolerance-breaking effects of tertiary lymphoid organs in transplantation and demonstrate a tight correlation between the presence of ectopic lymphoid tissue and the rejection of an allograft. The third component to this thesis demonstrates on a cellular scale the change in the phenotype of T lymphocytes that occurs as a result of the presence of tertiary lymphoid organs.

If tertiary lymphoid organs act as lymph nodes then they must be able to permit the activation of naive lymphocytes and the development of T cells with effector and memory phenotypes. To assess whether tertiary lymphoid tissue could activate lymphocytes, naive T lymphocytes ($CD44^{low}$) were harvested from $Thy1.1^{+}$ mice. These lymphocytes are otherwise identical to lymphocytes from $Thy1.2^{+}$ wild-type mice, but can be specifically identified by the Thy1.1 antigen expressed on T cells. Experimental subjects were splenectomized *aly/aly* B6 mice that had three days previously received a skin transplant from an allogeneic F1 BALB/c x B6 RIP-LTα expressing mouse. Control animals were *aly/aly* splenectomized B6 mice that received allogeneic BALB/c x B6 wild-type skin or syngeneic B6 RIP-LTα skin. In each lymphocyte transfer, 5 million Thy 1.1^{+} lymphocytes were injected into the tail vein of the recipient mouse.

Recipients were divided into two groups based on the timing of the lymphocyte harvest. Lymphocytes from the first group were harvested at 15 days post-transfer in order to assess the development of effector T lymphocytes. Lymphocytes from the second group were harvested at 60 days, at which point the effector response would have been concluded, and lymphocyte levels returned to baseline, leaving only memory antigen-specific T lymphocytes.

At each harvest date, lymphocytes were retrieved from skin, lung, liver, bone marrow, and peripheral blood. Thy 1.1^{+} CD4 and CD8 T lymphocytes were isolated via flow cytometry, and the population was stratified based on expression of CD44 and CD62L, markers of T

lymphocyte activation. Lymphocytes were recoverable in small but identifiable quantities from all locations.

In experimental animals transplanted with allogeneic RIP-LTα skin, when harvested at the 15 day time point, Thy 1.1^+ lymphocytes displayed an increase in expression of CD44, a decrease in CD62L expression, and significant dilution of intracellular CFSE, consistent with expansion of the Thy 1.1^+ cell population and transformation from a naïve to an effector phenotype (figure 6). Control animals were transplanted with syngeneic RIP-LTα grafts. Thy 1.1^+ lymphocytes were recovered in only very minimal quantity. CFSE staining did not reflect rounds of cell division, and CD62L or CD44 expression did not demonstrate transformation of the naïve transferred cells into effector or memory lymphocyte phenotypes. Thy 1.1^+ lymphocytes were not recoverable from recipients of wild-type non-transgenic grafts, despite identical harvest techniques, suggesting that the absence of lymphoid tissue failed to permit survival or expansion of the transferred lymphocytes

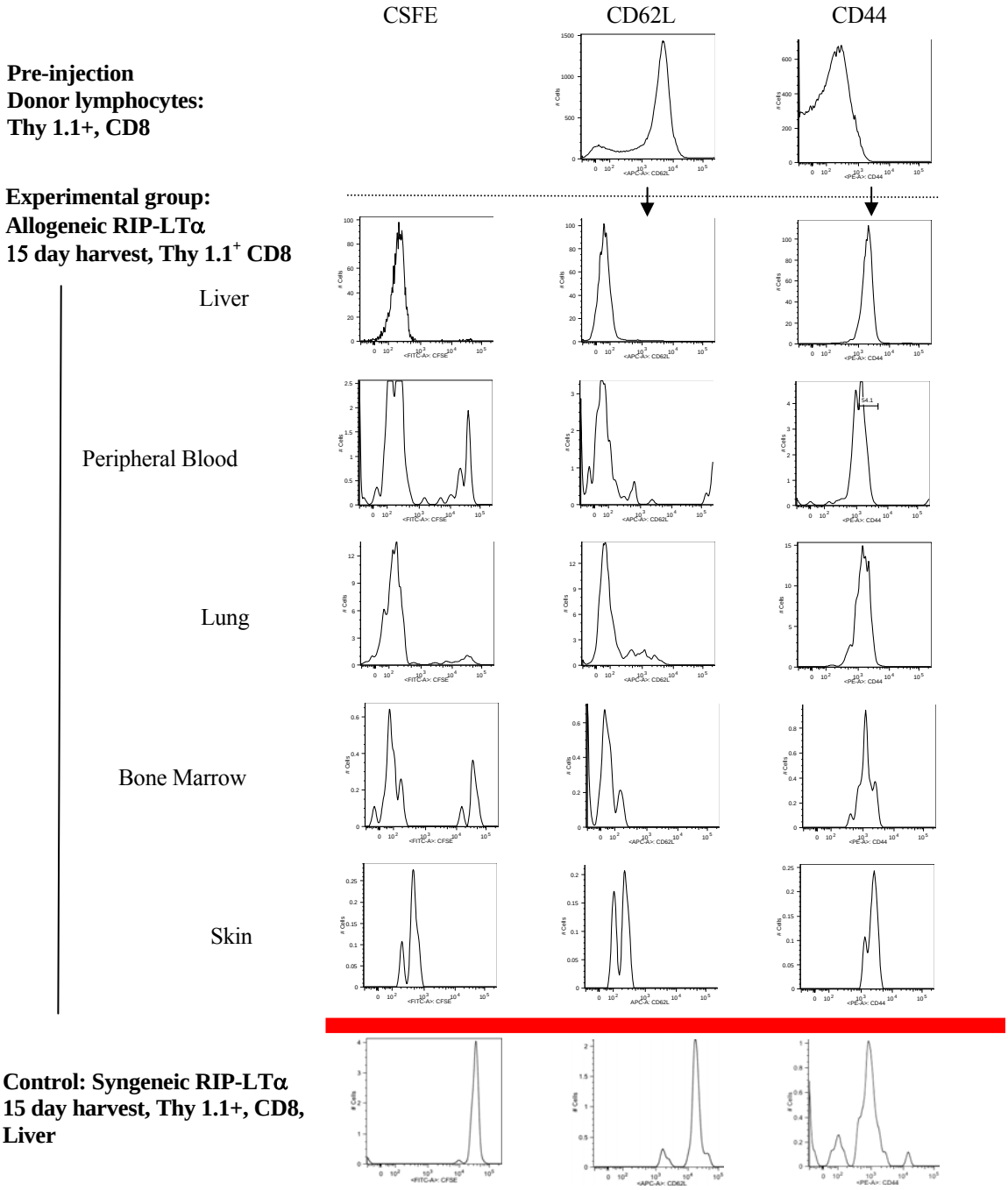

Figure 6. Generation of effector CD8 T cells in recipients of allogeneic LTα expressing grafts. Thy 1.1^+ transplanted T cells 15 days after injection show multiplication (by dilution of CFSE), an increase in CD44 and a decrease in CD62L expression, consistent with an effector phenotype. Control recipients, who received syngeneic LTα expressing grafts, show negligible expansion or phenotypic change.

When harvested at the 60 day time point, transferred Thy 1.1^+ lymphocytes harvested from liver specimens demonstrate an increase in expression of both CD62L and CD44, consistent with a transformation from a naïve phenotype to a central memory phenotype (figure 7). There is a single CD62L peak, consistent with the apoptotic elimination of the effector phenotype seen at the 15 day harvest point.

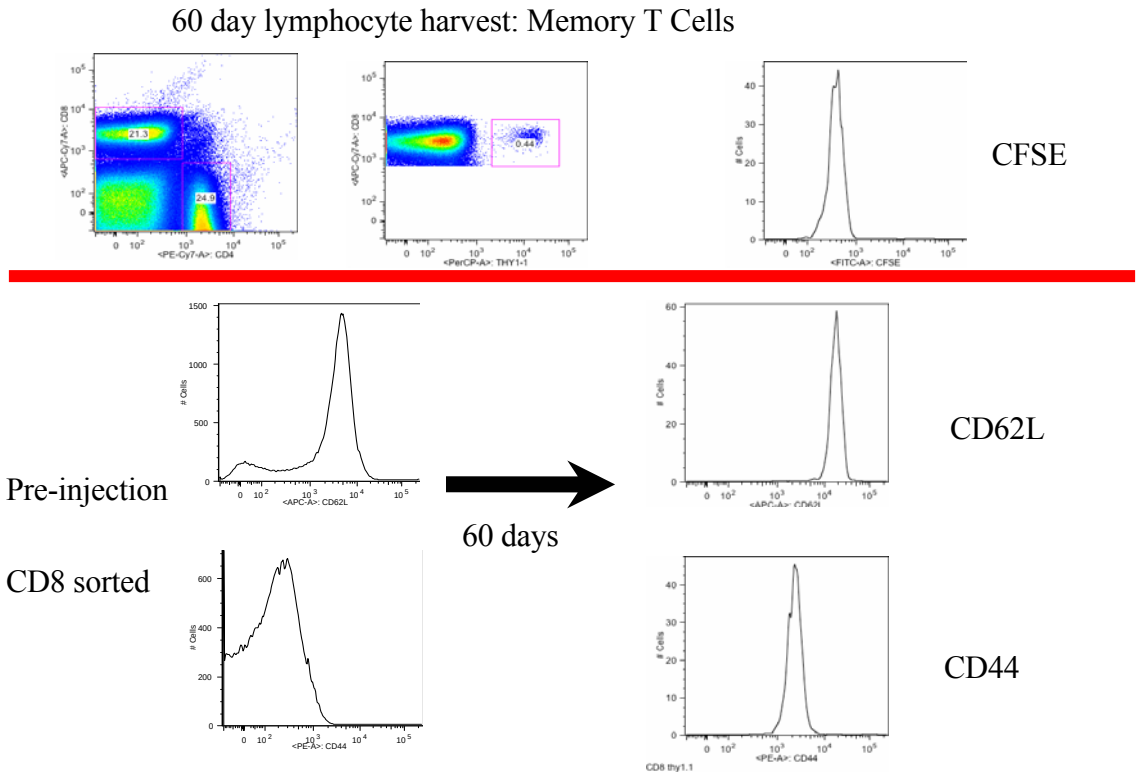

Figure. 7. Generation of memory CD8 T cells. Thy 1.1^+ cells are still able to be harvested from liver specimen 60 days after injection, and show phenotype consistent with memory T cells.

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DISCUSSION

This study demonstrates that rejection of an allograft in a mouse devoid of secondary lymphoid tissue occurs if there is simultaneous transplantation of ectopic lymphoid tissue as well as alloantigen. Immunogenic effects of inflammation related to the simple presence of the RIP-LTα transgene were controlled for by the inclusion of syngeneic transplants. These syngeneic grafts were not rejected, although they contained TLO. Thus, differential graft survival in mice that receive allogeneic transplants from RIP-LTα donors must be a function of the additional TLO.

We further characterized the functionality of this TLO by analyzing the differential ability of the tissue to support the development of effector and memory adaptive immune responses. When present, TLO were sufficient to endow a previously immunologically quiescent mouse with an operational rejection response, demonstrating the presence of effector T lymphocytes. Ectopic TLO also supported a delayed rejection capacity, consistent with the development of memory T cells. Lastly, we found that TLO provided an *aly/aly* mouse with a newfound ability to reject anatomically distant foreign tissue, demonstrating the ability of the ectopic tissue to support a systemic immune response.

These functional correlations were explored on the cellular level by the identification and quantitation of specific T lymphocytes with phenotypes characteristic of effector and memory T cells. CD62L is a selectin that facilitates, and is required for, lymphocyte entrance into in lymph nodes, by binding glycoproteins such as GlyCAM-1 or MAdCAM-1

on the endothelium of lymph node high endothelial venules. These glycoproteins are collectively referred to as peripheral node addressins (PNAd). CD62L is expressed on naïve lymphocytes, enabling their entry into lymph nodes to survey incoming APCs for activating antigen. If a T cell becomes activated, CD62L is downregulated, enabling egress from the lymph node into the circulation, for eventual traffic into a target organ where effector activity may take place. CD44 is another differential marker of activated T cells. The transition from $CD62L^{high}$ to $CD62L^{low}$ and from $CD44^{-}$ to $CD44^{+}$ observed here in mice receiving LTα expressing transplants is consistent with a loss of naïve phenotype for the transferred $Thy1.1^{+}$ lymphocytes. The dilution of CFSE is consistent with successive rounds of cellular division occurring in this cell population. Both of these events occurred only in those mice that received ectopic TLO, suggesting that the transplanted TLO was capable of providing a location in which this transformation could take place.

Our experiments confirm that TLO are sufficient to break immunologic ignorance, and demonstrate macroscopic and microscopic evidence of lymphocyte activation, allograft rejection and the formation of immunologic memory in the presence of TLO. These results are consistent with prior research that demonstrated an inability of mice devoid of secondary lymphoid tissue to reject allogeneic grafts (3). Interpretation of this prior research had been made more complicated following reports by other groups who found that in different models of mice without secondary lymphoid tissue, lymphotoxin-α or LTβR knockout mice, rejection of cardiac and skin tissue was delayed, rather than abrogated (32). Differences in the specific model used for as the lymphoid tissue-deficient model have been suggested to underlie the different conclusions of the two groups

regarding the necessity of secondary lymphoid tissue for an allograft response. Lymphocytes from *aly/aly* mice have previously been reported to display defects in homing to gastric-associated lymphoid tissue (33), suggesting that perhaps *aly/aly* lymphocytes have an inherently poor ability to enter ectopic lymphoid tissue. However, the results described herein suggest that the small amount of ectopic lymphoid tissue accompanying the allograft is traversable by recipient lymphocytes, as its presence correlates with rejection and the development of effector and memory lymphocyte phenotypes.

Alternative explanations for the differential rejection behavior following transplant of RIP-LTα ectopic lymphoid tissue are constrained by the failure of the control syngeneic RIP-LTα tissue to be rejected, and its ability to support rejection of foreign tissue. If there existed a factor inherent to RIP-LTα expression that is capable of inducing extralymphatic activation of recipient lymphocytes, then one would expect this factor to have induced rejection following a syngeneic RIP-LTα transplant. The absence of such syngeneic rejection supports the determination that it is the presence of the lymphoid tissue itself that endows the ability to reject allogeneic tissue.

Prior research has not specifically characterized the cellular components of the skin lesions found in RIP-LTα transgenic mice. While these lesions appear to have histological characteristics of lymphoid tissue, and are the sites of expression of lymphoid glycoproteins such as PNAd (unpublished data), in vivo demonstration of the lymphoid functionality of these accumulations has not been previously published. The evidence presented herein constitutes a functional demonstration of the lymph node-like abilities of

RIP-LTα skin. When transgenic RIP-LTα skin, containing the characteristic lymphoid structures, is transplanted, it empirically performs as a lymph node, which is consistent with its classification as functional tertiary lymphoid tissue.

Our results are consistent with prior research that has demonstrated that lymphoid tissue is found near the site of allografts undergoing rejection. Within the framework of Zinkernagel's localization-dose-time model of immunologic function, the formation of ectopic lymphoid tissue could be considered an additional mechanism for fostering the interaction between lymphoid-resident cells and APCs. Establishment of lymphoid tissue close to the site of antigen complements the more commonly appreciated traffic of antigen toward lymphoid tissue. The placement of lymphoid tissue close to the site of an allograft enhances the likelihood of an immunologic response, thereby leading to observed increased frequency of rejection when this tissue is present. This is also consistent with the presence of tertiary lymphoid tissue in close proximity to autoimmune activity. When lymphoid tissue is brought closer to antigens that normally would not be recognized by the immune system, ignorance is more likely to be broken, facilitating autoimmune response against normally non-immunogenic self targets.

Combined with knowledge of the pathways leading to lymphoid tissue development, these findings suggest therapeutic benefit from interventions designed to enhance or inhibit the development of tertiary lymphoid organs, in an effort to augment or suppress interaction of antigen-presentation to reactive lymphocytes. If local ectopic lymphoid tissue facilitates the development of autoimmune pathology, then interruption of lymphoid signaling

pathways underlying the growth and organization of ectopic tissue may be expected to lead to reduction of self-recognition and restraint of autoimmune assault. Such techniques have been attempted in murine models of autoimmune disease including colitis (34)(35), arthritis (36), and type 1 diabetes mellitus (IDDM) (37). In the case of arthritis and colitis, injection of lymphotoxin-β receptor specific immunoglobulin fusion protein (LTβR Ig) markedly reduces inflammation and physical symptoms in collagen-induced arthritis and multiple models of experimentally induced colitis. In IDDM, injection of LTβR Ig prevented the usual onset of diabetes in non-obese diabetic (NOD) mice, and was able to reverse islet destruction occurring in late stage autoimmune IDDM. Histologic analysis of pancreata from LTβR-Ig treated mice revealed marked decrease in the prevalence of lymphoid structures, suggesting that in this experiment, local lymphoid tissue played a role in the disease-causing immunologic response to the nearby islet tissue.

Not all such enhanced antigen recognition may be detrimental. It has been demonstrated that transgenic mice that are deficient in peripheral lymphoid tissue, yet express ectopic lymphoid tissue in the lung (iBALT) in response to an infectious challenge, are able to clear infection with a lesser degree of inflammation and damage to host tissue than wild-type mice (11). Many solid tumors display antigens that are distinct from normal tissue proteins, yet the immune system fails to respond and mount an attack against these tissues. It has been demonstrated that the cause of this failure to respond is immunologic ignorance of the tumor antigens, due to a lack of presentation of antigen to lymphocytes. If ignorance is broken, cytotoxic immune responses are actually quite effective in eliminating tumor tissue (38). In an early demonstration of possible therapeutic use of ectopic lymphoid

tissue, it has been demonstrated that if lymphotoxin-α is targeted to malignant tissue, the resultant development of ectopic lymphoid tissue allows an improved T lymphocyte response and, in murine models, has resulted in the complete eradication of pulmonary tumors (39).

Another potential route to inducing tolerance in autoimmune disease or transplantation is the development of a mechanism to target regulatory or suppressor T cells to ectopic lymphoid organs, thus exerting a local inhibitory effect without requiring systemic immunosuppression. In a murine model of cardiac allograft tolerance, regulatory T cells ($CD4^{+}CD25^{+}CD44^{high}CD62L^{high}$) were identified in peripheral lymph nodes of allograft recipients made tolerant by either of two different tolerogenic regimens. Suppression of lymph node homing by lymphocytes, induced by injection of anti-CD62L, prevented regulatory T cell residence in lymph nodes and abrogated this induced tolerance. When lymphocyte egress from lymph nodes was restricted by administration of FTY720, regulatory T cell residency in peripheral lymph nodes and allograft tolerance were restored (40). In a separate skin transplant assessment of regulatory T cell activity, transferred $CD4^{+}CD25^{+}$ cells were noted to attenuate proliferation of graft-specific effector cells, with marked reduction in CD4 replication occurring in draining lymph nodes (41). These studies suggest that in addition to enabling allograft rejection, lymph nodes are the site for the tolerance-promoting action of regulatory T cells, and that attention should be paid to both regulatory as well as effector T cells in determining the net effect that tertiary lymphoid organs may have upon target tissue.

There remain questions about the identity of the specific cells that prompt the induction of ectopic lymphoid tissue. Although multiple studies have confirmed the necessity of signaling through the NF-κB pathway, and in many cases the LTβR, in the development of both classic secondary lymphoid tissue as well as TLO, the source of the initiating cell remains unclear (42). Hematopoietic $CD4^{+}CD3^{-}$IL-7Rα^{hi} cells have been demonstrated to play a role in the embryonic development of lymph nodes and Peyer's patches, where they have been noted to chemotax toward the chemokines ELC, SLC and BLC (CXCL13) (43). Signaling through the IL-7Rα on these cells results in upregulation of $LT\alpha_1\beta_2$, suggesting a role as an inducer cell, activating the lymphogeneic pathways described earlier. In a connection to ectopic lymphoneogenesis, it has been shown that the pancreatic tissue in transgenic mice expressing a RIP-CXCL13 transgene contains a significant population of $CD4^{+}CD3^{-}$IL-7Rα^{hi} cells, suggesting that these cells may play a role in the formation of ectopic, as well as native, lymphoid tissue(44), however details of this pathway are yet to be fully elucidated.

There remains evidence that other cell types may also possess signaling mechanisms relevant to the development of TLO. It has been demonstrated in ex-vivo studies that activated, but not naïve, T cells express $LT\alpha_1\beta_2$ (45). LTβ expression is induced in B cells following antigen engagement and by chemokine (BLC) signaling, and it has been shown that the absence of LT expressing B lymphocytes results in the inability of isolated lymphoid follicles in the mucosal immune system to develop fully, suggesting that B cells participate in the development of mucosal lymphoid tissue (46). Complicating matters is the differential expression of chemokines by immune cells depending on their specific

anatomic location. It has been shown that while mature dendritic cells in lymph nodes strongly express the T cell chemoattractants SLC and ELC, mature dendritic cells residing in ectopic lymphoid tissue formed during chronic inflammation do not express these chemokines (47). Further research aimed at clarifying the nature of the inducer cell during T cell mediated pathology is important to formulating approaches to modulate the formation of tertiary lymphoid tissue.

Our data, which demonstrates that ectopic lymphoid tissue is sufficient to induce allograft rejection in an *aly/aly* mouse, support the theory that ectopic lymphoid tissue has the capacity to play a significant role in the alloimmune response. Further characterization of the site of the alloimmune response may allow for more precise targeting of anti-rejection therapy, thus improving the quality of life for those who rely on the failure of the immune system to reject precious transplanted organs.

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