REVIEW

Biomaterials Innovation for Next Generation *Ex Vivo* Immune Tissue Engineering

Ankur Singh^{1,2,3*}

¹Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, NY 14853, USA

²Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY 14853, USA

³Graduate Field Faculty of Immunology and Infectious Disease, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

*Prof. A. Singh

389 Kimball Hall, Cornell University, Ithaca, NY 14853, USA

Email: as2833@cornell.edu

Phone number: 607-255-2194

Fax: 607-255-2011

Abstract

Primary and secondary lymphoid organs are tissues that facilitate differentiation of B and T cells leading to the induction of adaptive immune responses. These organs are present in the body right from birth and are now also recognized as locations where self-reactive B and T cells can be eliminated during the selection process. Many insights into mechanism that control the process of immune cell development and maturation in response to infection come from the analysis of various gene-deficient mice that lack some or all lymphoid tissues. The complexity of such animal models limits our ability to modulate the parameters that control the process of immune cell development, differentiation, and immunomodulation. Engineering functional, living immune tissues using biomaterials can grant researchers the ability to reproduce immunological events with tunable parameters for more rapid development of immunotherapeutics, cell-based therapy, and enhance our understanding of fundamental biology as well as improve efforts in regenerative medicine. Here the author provides his review and perspective on the bioengineering of primary and secondary lymphoid tissues, and biomaterials innovation needed for the construction of these immune organs in tissue culture plates and on-chip.

1. Introduction

The primary and the secondary lymphoid organs of the immune system are body's one of the most multifaceted and dynamic tissues, which program the immune cells to fight against a vast range of maladies and maintain homeostasis. Often thought of as zonal, compartmentalized structures, immune cells within these lymphoid organs are constantly moving, as revealed by two photon microscopy of lymph nodes¹. The complexity of the immune tissue architectures and cellular compositions is such that various developmental niches offer distinct microenvironments adapted specifically for defined stages in immune cell development and activation²⁻⁴. There are two primary lymphatic organs: the red bone marrow and the thymus gland, and four secondary lymphoid tissues, lymph nodes, tonsils, Peyer's patch, and spleen. The thymus has a central role in the immune system, as it is crucially required for T cell development and repertoire selection. T cells are selected to recognize self-Major histocompatibility complex (MHC) molecules and discriminate between self and non-self-antigens during their development in the thymus. T cell and B cell responses to antigen occur in secondary lymphoid tissues, where cells of the immune system are organized in specialized microenvironments that provide signals for effective immune responses. Although considered controversial in past, a recent study has provided compelling results to categorize milky spot, located in the omentum of the peritoneal cavity, as secondary lymphoid structures rich in macrophages and B1 cells but lack dendritic cells^{5,6}.

To date, researchers have relied on live animal models^{7, 8} to understand immune cell development, functioning, screening of immunotherapies against diseases, and understanding the toxic

effects of new class of therapeutics. However in vivo approaches are costly with long turnaround times and do not allow for easy control over the parameters that modulate the dynamics of immune reaction because the amount of extracellular and intracellular signals presented in vivo cannot be easily and precisely tuned in terms of magnitude and persistence. On the other spectrum of ex vivo immune studies are the 2-D co-cultures and bead-based systems, which have shown reasonable utility in understanding cell-intrinsic immune processes⁹, but do not necessarily represent a lymphoid niche and offer less suitability to precisely study a large number of mechanistic questions in infection and immunity. There is an urgent unmet need to engineer functional, living immune tissues that are capable of inducing immune cell development and driving immune reactions ex vivo, at both innate and adaptive immunity levels. The author anticipates that the ability to drive immune reactions *ex vivo* will grant researchers the ability to reproduce immunological events with tunable parameters for more rapid development of high affinity antigen-specific antibody secreting cells, target-specific T cells, immunotherapeutics, vaccines, as well as allow us model the earliest steps of malignant transformation of cells in the immune system. Building ex vivo 3-D immune tissues can accelerate our path towards the treatment of various pathological conditions including infections^{10, 11}, cancers^{12, 13}, and autoimmune diseases^{11, 14}.

In this era of organ-on-chip and organoids, more and more studies are focused on developing whole organ or minimal functional units using natural or synthetic biomaterials. Despite their relatively modest size to other bigger organs such as kidney or brain, engineering functional, living immune tissues presents a formidable challenge because of the structural complexity of the lymphoid organ system, dynamic changes in extracellular matrix, staggering cell density and the constant movement of many of its constituent cells. Importantly, the biochemical signals between cells and their surrounding matrix are dynamic over multiple time (seconds–weeks) and length (nm–cm) scales, and these signals are also dependent on tissue mechanical properties. Spatio-temporal signaling and tissue stiffness are particularly important in cell fates¹⁵, which is regulated by the complex interplay of multiple parameters, including adhesive intercellular interactions, cytoskeletal and extracellular matrix remodeling, and gradients of agonists and antagonists. These factors individually and collectively vary as a function of spatial locale and temporal stages of development, differentiation, and activation of immune cells. In an analogous fashion, materials engineered to elicit targeted cellular responses must be similarly dynamic in their spatio-temporal presentation of bio-ligands and material mechanics for efficient development of robust end point immune response. With the emerging interest in immune tissue engineering, it has become important to understand the factors that influence the development, recruitment, interaction, and functioning of immune cells. Simplistically, these signals can be summarized into immune cell- immune cell interactions, immune cell- stromal cell interaction, soluble and sequestered signaling protein that enable autocrine-paracrine signaling, and extracellular matrix (**Figure 1**). Here the author provides his

review and perspective on the bioengineering of two lymphoid organ systems as examples, thymus and lymph nodes, and biomaterials innovation needed for the construction of these immune organs in tissue culture plates and on-chip.

2. Ex vivo intra-thymic microenvironments for engineering T cells

Generation, maintenance or replacement of a healthy, functional T cell population remains a critical aspect of immune-competence in patients with HIV/AIDS¹⁶⁻¹⁸, bone marrow transplants¹⁹, immunodeficiency disorders like Di George's Syndrome^{20, 21}, as well as cancer patients suffering T cell depletion with repetitive exposure to tumor suppressive therapies^{22, 23}. One of the major challenges in engineering a thymic tissue is the structural organization into its two morphologically and functionally distinct compartments: the cortex and the medulla (**Figure 2**). These compartments house two distinct populations of thymic epithelial cells (TECs): the cortical TECs (cTECs) and the medullary TECs (mTECs)^{4, 24}. Other thymic stromal cells include thymic fibroblasts, endothelial cells, as well as antigen presenting cells like macrophages and dendritic cells. For mechanistic understanding of thymus organogenesis, morphogenesis, and how stromal cells guide for T cell development and selection, readers can refer to reviews by Gordon and Manley²⁵ and Takahama²⁴.

While current microfabrication techniques and 3-D printing can enable mimicking structural assemblies, an important consideration is that this network of thymic cells provides both homing signals for the immigration of lymphocyte progenitors originated from the bone marrow (BM) and trophic factors necessary for the differentiation and maturation of thymocytes. In the thymic niche, stromal cells secrete specific cytokines, growth factors and provide inter-cellular signaling. One of the most notable among these stromal cell-stem cell signals is the Notch–Delta-like ligand interaction²⁶. Lymphoid precursors migrate from the bone marrow, enter the cortico-medullary junction of the thymus through blood vessels and then migrate into the cortex and other distinct subdivisions of the thymic architecture. These cells then undergo T cell lineage commitment and finally get converted into CD4+ and CD8+ single positive cells in the thymic medulla. Years of research from Zúñiga-Pflücker and others have shown that immobilized delta ligand (DLL-1) using stromal cell cultures, 2-D functionalization, or beads can induce early T cell progenitors²⁷⁻³¹. However, many of the existing 2-D studies required transfer of these pre-hematopoietic T cell subsets into re-aggregate thymic organ cultures (RTOCs) or reconstitution in mouse model for efficient T cell generation³². Therefore, it is apparent that a three-dimensional microarchitecture and perhaps extracellular matrix of the thymus is essential for such lineage differentiation. A recent attempt to develop 3-D thymic niche includes use of a human dermal skin 3-D co-culture system, where a 1.2-mm-thick viscose³³, nonwoven fibrous material was placed into porous filter inserts as scaffolds and fibroblasts were suspended in fibrinogen-containing fibrin gel³³.

Medullary TECs seeded onto this three-dimensional model preserves key features of mTECs: proliferation and terminal differentiation of $CD80^{lo}Aire^{-}$ mTECs into $CD80^{hi}Aire^{+}$ mTECs; responsiveness to Receptor activator of nuclear factor kappa-B ligand (RANKL); and sustained expression of FoxN1, Aire, and tissue-restricted genes in $CD80^{hi}$ mTECs. Whether these 3-D culture systems also support the earliest stages of mTEC differentiation from stem cells and the ability of these constructs to induce T cell differentiation remains to be tested ³³. More recently, three-dimensional scaffolds generated from decellularized mouse thymus were reported that can support TEC survival and maintain their characteristic molecular properties ³⁴. When transplanted into athymic nude mice, the bioengineered organoids effectively recruited lymphocyte progenitors and supported T lymphopoiesis in vivo (increasing numbers of $CD3+CD4+$ and $CD3+CD8+$ T cells were detected in circulation and secondary lymphoid organs). However, to date, no 2-D or 3-D approach has been able to fully recapitulate the function of a thymus and whether a standalone ex vivo 3-D thymic tissue can be engineered remains a standing question in the field. While these existing 3-D thymic immune tissues require implantation for complete T cell lymphopoiesis, they inform us of the extracellular matrix, porosity, chemical composition and gradient, as well as the mechanical properties of these tissues that could partially support the ex vivo process. Immunohistochemistry studies by Fan et al inform us that Collagen I, Collagen IV, fibronectin, and laminin are abundant in thymus and therefore may represent an essential component of biomaterials needed for thymic regeneration ³⁴. Since laminin-rich Matrigel and other collagen-based synthetic matrices have only supported limited differentiation of lymphocyte progenitors into T cells ^{35,36}, engineered matrices that present defined proportion of Collagen I, Collagen IV, fibronectin, and laminin as protein or peptides are needed to further understand the interaction of each component with T cells undergoing differentiation.

Likewise, ultrastructure characterization of native and decellularized thymus using scanning electron microscopy (SEM) ³⁴ shows the presence of 3-D meshwork within the parenchymal space composed of variety of fibers, including large bundle of Type I collagen associated with a variety of smaller fibers. Such structural and extracellular matrix components may be important in lymphocyte homing to implanted niches and induction of T cell lymphopoiesis. Nevertheless it appears there are micro-macro-scale changes in pore size of native versus decellularized extracellular matrix ³⁴ and further investigation into the role of porosity in ex vivo lymphopoiesis is needed. Therefore engineered biomaterials that capture the porosity and extracellular matrix presentation, preferably independent of each other, of the native thymus are needed. Independent control of biophysical parameters has been shown for other applications by Mooney et al ³⁷ and us ³⁸. We and others have previously demonstrated temporal control of extracellular matrix ligands in vitro ³⁹⁻⁴² and in vivo ³⁹, which may be importance in designing next-generation thymic organoids. Finally, the role of tissue's mechanical stiffness and

interstitial pressure forces may be critical for T cell development as well. Our current understanding of the biophysical properties of the thymic niche is simply a mechanically soft nature of the tissue. These parameters are not well understood and require thorough investigations to understand whether there are any mechanical gradients within the lymphoid tissue space.

Previous co-culture and bead studies have informed us of several key parameters that could be taken into consideration while designing a 3-D thymus niche. For example, density of Notch ligand plays an important role as low densities of Delta-1 can result in differentiation of lin-Sca1+ckit+ (LSK) hematopoietic progenitors into Sca1+ckit+ cells, positive for both early T cell progenitors (Thy1+CD25+) and B cell precursors (B220+CD43-/lo)⁴³. On the other hand, a high density of Delta-1 promotes differentiation into T lymphoid precursors. Therefore, design of ex vivo tissues could consider controlled ligand presentation as an important criterion for T cell development and differentiation, if a particular lineage is desirable. Another important parameter that emerges from studies by Kotov et al.⁴⁴ and Stachowiak et al.^{45, 46} appears to be the interconnectivity of pores within a scaffold. Using an inverse opal fabrication technique, Kotov et al. engineered notch functionalized 3-D inverted colloidal crystal scaffolds for ex vivo T cell development of human hematopoietic stem cells⁴⁴. In general, inverted colloidal crystal scaffolds comprise of highly uniform and size controllable pores, and the presence of interconnecting pores enhances oxygen and nutrient diffusion therefore providing optimum cellular development. The surface of hydrogel inverted colloidal crystal scaffolds was coated with layers of negatively charged clay nanoparticle dispersion and positively charged poly(diallyl dimethylammonium chloride) solution utilizing a layer-by-layer assembly method. The key interactions involved in these scaffolds included electrostatic, van der Waals, and hydrogen bonding between oppositely charged polyelectrolytes dissolved in water and allowed for simple electrostatic coating of Delta ligands than in uncharged hydrogels.

However one of the most challenging tasks in engineering a functional ex vivo thymus tissue appears to be the T cell selection process, which remains unaddressed. During T cell development, immature T cells are chosen to mature on the basis of the selection of their T cell receptor repertoire (Readers are suggested to see⁴⁷ for T cell receptor and stages of T cell development in thymus). T cell receptor binding to peptide–MHC can determine the survival fate of T cell under question and is dictated by the affinity of the interaction, as well as the maturation state of the T cell. Since recapitulating the selection process is a hallmark of T cell development, there is an urgent need to address this limitation in current ex vivo models. Biomaterials could be designed to present T cell selection-specific ligands to promote low-affinity interactions between the newly expressed T cell receptors and a diverse set of self-peptide–MHC molecules. This however presents an enormous challenge and likely will require key cell-

cell interactions. Another critical challenge in developing new biomaterials that enable T cell selection process is eliminating T cells with high affinity interactions by negative selection as they are potentially autoreactive. Such signals could be provided by functionalizing scaffolds with Fas Ligand that engages with Fas surface receptors on immune cells and results in apoptotic cell death, mediated by caspase activation. Fine tuning this process will enable ex vivo generation of “real” T cells that are capable of responding robustly to pathogen-derived peptides bound to the same major histocompatibility complex in the periphery. Because maintaining survival and growth of all primary cells that provide key signals for T cell selection is difficult, precise presentation of these ligands may aid in developing a functional thymus. The biomaterials innovation needed here may require spatio-temporal presentation of selection-specific ligands and can be achieved using a variety of bio-conjugate chemistries available to generate such designer tissues^{39, 41, 48}.

3. Ex vivo Lymph Node Tissues

Positioned throughout the lymphatic system to filter lymph, lymph nodes limit the spread of pathogens and facilitate the induction of the adaptive immune response⁴⁹⁻⁵³. In addition to the hallmark B, T, and dendritic cells, the lymphoid microenvironment consists of a spatially patterned extracellular matrix, follicular dendritic cells and follicular reticular cells to create the appropriate stromal matrix to allow immune cells to migrate and achieve their function. To achieve this function efficiently, strategically localized macrophages are also present to capture pathogens and limit pathogen spread.

In a reductionist fashion, the lymph node can be seen as a bioreactor with three zones (**Figure 2**) – an outer antigen-sampling zone, the B cell activation zone (B cell follicle), and the T cell activation zone^{8, 49, 50, 54-56}. These three zones result from dynamic migration processes and can effectively describe how the adaptive immune response is initiated. In the outer sampling zone, antigen-presenting cells, such as dendritic cells, bind to antigens that will be then be presented to the lymphocytes (**B and T cells**). Since the number of dendritic cells and the frequency of antigen-specific T cells are low, T cells migrate at rapid speeds to survey antigen-bound dendritic cells^{8, 54-56}. Upon recognition of an antigen, T cells are then activated and fulfill their effector functions in cellular-mediated immunity and in B cell activation. B cells can generally be activated by antigen presentation from dendritic cells or follicular helper T cells. In response to activation, B cells then form germinal centers to differentiate into antibody-secreting cells for the induction of humoral immunity. During the germinal center differentiation process, B cells undergo three main processes: affinity maturation, isotype switching, and terminal differentiation into antibody-secreting cells^{8, 54-56}. These first two steps are integral in producing high affinity antibodies capable of eliciting the proper physiological response. In affinity maturation, B cells first clonally expand and go through somatic hypermutation to **diversify** antibody binding before only B cells producing high-affinity

antibodies are selected to survive through anti-apoptotic signaling. Isotype switching then ensures the antibodies are able to elicit the proper physiological response by binding to the correct effector cells.

Similar to the complexity observed in a thymic niche, the lymph nodes are highly organized patterns and require a thorough understanding of distinct microenvironments within each compartment for creating accurate lymph node models. For inducing humoral immunity, specifically germinal center phenotype, presentation of T cell mediated CD40 Ligand remains indispensable. However simple presentation of this signal in 2-D space offers a modest induction of germinal center phenotype, and the cells are short lived^{57, 58}. Therefore, it is important to understand what other factors could provide survival and differentiation cues to these rare cells. For example, cell–extracellular matrix adhesion is tightly regulated in immune cells⁵⁹, and misregulated interactions often result in pathological conditions⁶⁰ such as developmental defects, wound healing deficiencies and tumor progression⁶¹. We have previously shown that integrins can function as pro-survival cells in malignant B and T cells^{61, 62}. T cell lymphoma tumors expressed integrin $\alpha\beta3$ at similar or higher levels than normal peripheral and tonsillar T cells⁶¹. Genetic or pharmacologic inhibition of integrin $\alpha\beta3$ decreased VEGF production and induced T cell lymphoma cell death in vitro and in human xenograft models⁶¹.

In general, densely packed populations of CD4 and CD8 single positive T cells reside in the T cell zone of the paracortex of lymph node (and in the periarteriolar lymphoid sheath of the spleen and the interfollicular zone of Peyer's patches). These T cells are surrounded by dendritic cells and a sponge-like scaffold of mesenchymal stromal cells called fibroblastic reticular cells (FRCs). While designing immune tissues, if FRCs are considered as stromal support then a rational choice of biomaterial will be parallel (type I and III) collagen fibres, which are known to span the T cell zone enabling formation of a 3-D network of FRC conduits (**Figure 2**; for biological understanding of these structures, see review from Junt et al²). The FRC conduits form a contiguous lumen with fluid channels around the high endothelial venules (HEVs) and function as a shortcut for interstitial and lymph flow from afferent lymphatics to the blood circulation via HEVs. Taken together, existing knowledge of T cell zone architecture and constituents inform us of possible ways to engineer structured 3-D immune tissues representing sub-anatomical zones of lymph nodes. Along these lines, Stachowiak et al. engineered an inverse opal hydrogel using colloidal crystal templating of polyethylene glycol hydrogel scaffolds infused with collagen^{45, 46}. The resulting hydrogel was macroporous promoted intra-scaffold migration of encapsulated T-cells and DCs, with T-cell migration dependent on the connecting pore size. Engineering such system can enable researchers better understand the process of B, T, and dendritic cell interactions, which in turn could allow for controlled modulation of immunity.

Recent studies have also shown the importance of integrin receptor interactions in cell-cell

interaction and cell-extracellular matrix in controlling the B cell phenotype and modulating intermediate steps in immune response. Various B cells signals, including the B cell receptor directly or indirectly influence integrin receptors on the same cell and subsequently increase integrin avidity for their respective ligands⁶³⁻⁶⁶. The $\alpha 4\beta 1$ integrin (often called Very Late Antigen-4 (VLA-4)) which binds vascular cell adhesion protein 1 (VCAM-1) on follicular dendritic cells facilitates B cell activation by low affinity antigen and affects the germinal center reaction⁶³. Within the lymphoid microenvironment, integrin $\alpha \nu \beta 3$ -binding Arg-Gly-Asp (RGD) motif is also presented by vitronectin in the germinal center niche of the B cell follicle^{63, 67}. Another key protein is laminin [alpha-5](#) within the marginal zone of B cell follicles and RGD has been shown to mediate the interaction between B cells and laminin⁶⁸. These studies suggest that distinct integrin–ligand interactions can have different influences on the germinal center-mediated humoral immunity. Based on these observations, we have engineered 3-D biomaterials-based immune tissues that present RGD ligands in addition to CD40L and, for the first time, induce 100-fold more germinal center phenotype^{57, 58} (compared to modest 10-20 fold germinal center cells with 2-D co-cultures) along with a robust increase in number of cells with antibody isotype class switching. Importantly, we and others have shown that the expression of integrin ligands is dynamic within B cell follicle sub-zones and B cell differentially regulate integrin receptor expression in these tissues^{63, 67, 69}. [The scaffold used here included gelatin re-enforced with polyionic two-dimensional silicate nanoparticles \(1 nm thick and 25 nm wide\)](#)^{57, 58}. In a recently published study, we demonstrated that upon immunization, B cells in mice undergo change in the expression of integrin receptor $\alpha 4\beta 1$ and $\alpha \nu \beta 3$ ⁶⁹. In order to understand the role of these integrin receptors and their corresponding ligands, there is a need for an engineered biomaterial that can be tailored to present pro-survival adhesive ligand of interest. This study demonstrated that germinal center-like phenotype was dependent on the integrin ligand subtype, density, and that the critical B cell receptor clustering on the surface was dependent on ligand subtype. Presentation of $\alpha 4\beta 1$ -binding ligands showed immunoglobulin (IgM) clustering on germinal center B cells, whereas $\alpha \nu \beta 3$ -binding RGD ligands showed diffused presence of IgM BCR. These findings highlight the need for modular, flexible, adaptable designer hydrogel/scaffolds which can address the dynamic changes in immune tissues.

Finally, unlike many organs in the body, such as cartilage, adipose tissues, heart, the role of mechanical cues in lymphoid tissues remain elusive. Few studies have addressed the question of fluid flow, shear stress, solid stress, tension and compression, which influence lymphoid organ development, function in health and disease, and malignant transformation⁷⁰⁻⁷⁴. Lymph node being a soft tissue has a distinct mechanical property profile of stress-strain measurement than those observed in classic mechanics. Therefore prior to biomaterials design, it is imperative that appropriate measurements are performed across both lymphoid tissue and the biomaterial-based ex vivo tissue. A recent study by

Rakesh Jain and colleagues modeled and determined solid stress and elastic energy to understand role of mechanical forces in lymph nodes and tumor mechanopathology⁷³. We recently quantified healthy and neoplastic lymph node (and spleen) tissue biomechanics using a micropipette aspiration technique which measures local tissue distention due to applied vacuum pressure, analogous to a uniaxial tensile test⁷⁵. This technique has been validated in past using finite element analysis for measuring soft tissue strain energy density^{76, 77}. We demonstrated that lymphoid tumors have increased tissue stiffness as compared to healthy lymph nodes and spleen. This study provides a quantitative measurement of lymphoid tissue mechanical properties during tumorigenesis, which otherwise remains a qualitative assessment common to the clinics where the enlargement of neoplastic lymphoid tissue and the palpable stiffness are typically used as an initial screen.

In summary, what would be important for future biomaterials research is developing immune tissues where ligand presentations could be dynamically controlled to understand spatio-temporal signaling and selection process. This requires engineering designer biomaterials, which are becoming more and more important for next-generation tissues engineering and developmental biology studies, as evident from a study recently published by Lutolf and colleagues⁷⁸. In addition, many current 3-D systems lack multiscale architecture, micro-nano-scale features (such as fluidics and topography), and tissue-tissue interfaces, such as the interface between vascular endothelium, lymphatic endothelium, and the lymphoid tissue, which are crucial to the function and trafficking cells and biomolecules. Integrating these would lead to development of functional, bench-top, higher-throughput systems that can revolutionize the field of immune-engineering. It is only recently that many features of zonal migrations have become evident; a physiologically relevant model is yet to be engineered to recapitulate these features. Biomaterials innovation in this area will likely involve integration of hydrogel scaffolds, microfluidic gradients, and whole tissue imaging.

Figures

Figure 1. Extracellular microenvironment as nature's biomaterial for immune cells. Schematic representation of paradigmatic cell-cell and cell-extracellular matrix interfaces in lymphoid tissues. A typical immune cell undergoing development or maturation/differentiation may interact with a neighboring immune cell or stromal cells by means of specific receptor-ligand interactions. These cells can further interact with surround native or cell secreted extracellular matrix by adhesive proteins such as integrins. The extracellular matrix is a hydrated protein- and proteoglycan-based gel network comprising soluble and physically bound signals as well as signals arising from cell-cell interactions. Specific binding of these signaling cues with cell-surface receptors induces complex intracellular signaling cascades that converge to regulate gene expression, establish cell phenotype and direct tissue formation, homeostasis, regeneration, and possibly activation. Lists of signals are not intended to be complete as indicated by ellipsis (...).

Figure 2. Future biomaterials engineering and functional building blocks of primary and secondary lymphoid tissues. **A)** Schematic representation of exemplary primary lymphoid organs – a thymus and lymph node. A thymus is compartmentalized into zones (cortex, medulla, etc.) and involves developmental stages for T cells to become single positive (SP) T cells from double negative T cells. A lymph node consists of *input channels for lymphocytes and antigen-presenting cells carrying antigens or pathogens*. Within the organ, there are antigen-sampling, T cell, and B cell zones. B cell zones form germinal centers in a T cell dependent manner for differentiation into memory and plasma cells. These zones are connected via distribution channels for the communication. In both organ/tissue types, chemokine gradients are well established however mechanical cues remain elusive. **B)** Schematic representation of possible materials innovation that could lead towards better engineering of 3-D immune tissues. Depending on the zonal distribution of cues, biomaterials that provide lymphoid-tissue specific extracellular matrix in singular or multi-modal designer form, integrated with biophysical and biochemical cues can result in improved

understanding of immune cell functioning. Of these include spatio-temporal signaling and micro-nano-patterning along with chemokine gradients and engineering immunomodulatory surfaces that not only provide a niche but further activate immune cells. FDC: follicular dendritic cell; FRC: Fibroblastic reticular cells; HEV: High endothelial venules; T_{FH} : Follicular T Helper Cells; cortical TECs (cTECs) and the medullary TECs (mTECs).

Acknowledgements

I acknowledge financial support from the National Science Foundation CAREER award (DMR-1554275 (A.S.)) for some of the results mentioned in this manuscript. The content is solely the responsibility of the author and does not necessarily represent the official view of the funding agency.

References

1. Cahalan, M.D. & Parker, I. Choreography of cell motility and interaction dynamics imaged by two-photon microscopy in lymphoid organs. *Annu Rev Immunol* **26**, 585-626 (2008).
2. Junt, T., Scandella, E. & Ludewig, B. Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. *Nature Reviews Immunology* **8**, 764-775 (2008).
3. Anderson, G., Lane, P.J. & Jenkinson, E.J. Generating intrathymic microenvironments to establish T-cell tolerance. *Nat Rev Immunol* **7**, 954-963 (2007).
4. Lucas, B. et al. Control of the thymic medulla and its influence on alphabetaT-cell development. *Immunol Rev* **271**, 23-37 (2016).
5. Mebius, R.E. Lymphoid organs for peritoneal cavity immune response: milky spots. *Immunity* **30**, 670-672 (2009).
6. Rangel-Moreno, J. et al. Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. *Immunity* **30**, 731-743 (2009).
7. Gitlin, A.D., Shulman, Z. & Nussenzweig, M.C. Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature* **509**, 637-640 (2014).
8. Shulman, Z. et al. T follicular helper cell dynamics in germinal centers. *Science* **341**, 673-677 (2013).
9. Roh, K.H. & Roy, K. Engineering approaches for regeneration of T lymphopoiesis. *Biomater Res* **20**, 20 (2016).
10. Shi, W. et al. Transcriptional profiling of mouse B cell terminal differentiation defines a signature for antibody-secreting plasma cells. *Nature immunology* **16**, 663-673 (2015).
11. Saylor, C., Dadachova, E. & Casadevall, A. Monoclonal antibody-based therapies for microbial diseases. *Vaccine* **27 Suppl 6**, G38-46 (2009).
12. Sabbatino, F. et al. Novel tumor antigen-specific monoclonal antibody-based immunotherapy to eradicate both differentiated cancer cells and cancer-initiating cells in solid tumors. *Semin Oncol* **41**, 685-699 (2014).
13. Weiner, L.M., Murray, J.C. & Shuptrine, C.W. Antibody-based immunotherapy of cancer. *Cell* **148**, 1081-1084 (2012).
14. Caspi, R.R. Immunotherapy of autoimmunity and cancer: the penalty for success. *Nat Rev Immunol* **8**, 970-976 (2008).
15. Murphy, W.L., McDevitt, T.C. & Engler, A.J. Materials as stem cell regulators. *Nature materials* **13**, 547-557 (2014).
16. Pernet, O., Yadav, S.S. & An, D.S. Stem cell-based therapies for HIV/AIDS. *Adv Drug Deliv Rev* **103**, 187-201 (2016).
17. Lam, S. & Bollard, C. T-cell therapies for HIV. *Immunotherapy* **5**, 407-414 (2013).
18. Lunzen, J.V. et al. Transfer of Autologous Gene-modified T Cells in HIV-infected Patients with Advanced Immunodeficiency and Drug-resistant Virus. *Mol Ther* **15**, 1024-1033 (2007).

19. Krenger, W., Blazar, B.R. & Hollander, G.A. Thymic T-cell development in allogeneic stem cell transplantation. *Blood* **117**, 6768-6776 (2011).
20. Hudson, L.L., Louise Markert, M., Devlin, B.H., Haynes, B.F. & Sempowski, G.D. Human T cell reconstitution in DiGeorge syndrome and HIV-1 infection. *Semin Immunol* **19**, 297-309 (2007).
21. Davies, E.G. Immunodeficiency in DiGeorge Syndrome and Options for Treating Cases with Complete Athymia. *Front Immunol* **4**, 322 (2013).
22. Dudley, M.E. et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* **298**, 850-854 (2002).
23. Kalos, M. & June, C.H. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. *Immunity* **39**, 49-60 (2013).
24. Takahama, Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol* **6**, 127-135 (2006).
25. Gordon, J. & Manley, N.R. Mechanisms of thymus organogenesis and morphogenesis. *Development* **138**, 3865-3878 (2011).
26. Radtke, F., MacDonald, H.R. & Tacchini-Cottier, F. Regulation of innate and adaptive immunity by Notch. *Nat Rev Immunol* **13**, 427-437 (2013).
27. Awong, G., La Motte-Mohs, R.N. & Zuniga-Pflucker, J.C. In vitro human T cell development directed by notch-ligand interactions. *Methods Mol Biol* **430**, 135-142 (2008).
28. Murata, A. et al. A Notch ligand, Delta-like 1 functions as an adhesion molecule for mast cells. *J Immunol* **185**, 3905-3912 (2010).
29. Schmitt, T.M., Ciofani, M., Petrie, H.T. & Zuniga-Pflucker, J.C. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* **200**, 469-479 (2004).
30. Shah, D.K. & Zuniga-Pflucker, J.C. Notch receptor-ligand interactions during T cell development, a ligand endocytosis-driven mechanism. *Curr Top Microbiol Immunol* **360**, 19-46 (2012).
31. Taqvi, S., Dixit, L. & Roy, K. Biomaterial-based notch signaling for the differentiation of hematopoietic stem cells into T cells. *J Biomed Mater Res A* **79**, 689-697 (2006).
32. Chung, B. et al. Engineering the human thymic microenvironment to support thymopoiesis in vivo. *Stem Cells* **32**, 2386-2396 (2014).
33. Pinto, S. et al. An organotypic coculture model supporting proliferation and differentiation of medullary thymic epithelial cells and promiscuous gene expression. *J Immunol* **190**, 1085-1093 (2013).
34. Fan, Y. et al. Bioengineering Thymus Organoids to Restore Thymic Function and Induce Donor-Specific Immune Tolerance to Allografts. *Mol Ther* **23**, 1262-1277 (2015).
35. Marshall, D. et al. T cell generation including positive and negative selection ex vivo in a three-dimensional matrix. *J Hematother Stem Cell Res* **12**, 565-574 (2003).
36. Anderson, K.L., Moore, N.C., McLoughlin, D.E., Jenkinson, E.J. & Owen, J.J. Studies on thymic epithelial cells in vitro. *Dev Comp Immunol* **22**, 367-377 (1998).
37. Chaudhuri, O. et al. Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium. *Nature materials* (2014).
38. Patel, R.G. et al. Microscale Bioadhesive Hydrogel Arrays for Cell Engineering Applications. *Cell Mol Bioeng* **7**, 394-408 (2014).
39. Lee, T.T. et al. Light-triggered in vivo activation of adhesive peptides regulates cell adhesion, inflammation and vascularization of biomaterials. *Nature materials* **14**, 352-360 (2015).
40. DeForest, C.A. & Tirrell, D.A. A photoreversible protein-patterning approach for guiding stem cell fate in three-dimensional gels. *Nature materials* **14**, 523-531 (2015).
41. DeForest, C.A. & Anseth, K.S. Cytocompatible click-based hydrogels with dynamically tunable properties through orthogonal photoconjugation and photocleavage reactions. *Nat Chem* **3**, 925-931 (2011).

42. Kloxin, A.M., Tibbitt, M.W., Kasko, A.M., Fairbairn, J.A. & Anseth, K.S. Tunable hydrogels for external manipulation of cellular microenvironments through controlled photodegradation. *Advanced materials* **22**, 61-66 (2010).
43. Dallas, M.H., Varnum-Finney, B., Delaney, C., Kato, K. & Bernstein, I.D. Density of the Notch ligand Delta1 determines generation of B and T cell precursors from hematopoietic stem cells. *J Exp Med* **201**, 1361-1366 (2005).
44. Lee, J. & Kotov, N.A. Notch ligand presenting acellular 3D microenvironments for ex vivo human hematopoietic stem-cell culture made by layer-by-layer assembly. *Small* **5**, 1008-1013 (2009).
45. Irvine, D.J., Stachowiak, A.N. & Hori, Y. Lymphoid tissue engineering: invoking lymphoid tissue neogenesis in immunotherapy and models of immunity. *Seminars in immunology* **20**, 137-146 (2008).
46. Stachowiak, A.N. & Irvine, D.J. Inverse opal hydrogel-collagen composite scaffolds as a supportive microenvironment for immune cell migration. *J Biomed Mater Res A* **85**, 815-828 (2008).
47. Goldrath, A.W. & Bevan, M.J. Selecting and maintaining a diverse T-cell repertoire. *Nature* **402**, 255-262 (1999).
48. Alge, D.L., Azagarsamy, M.A., Donohue, D.F. & Anseth, K.S. Synthetically tractable click hydrogels for three-dimensional cell culture formed using tetrazine-norbornene chemistry. *Biomacromolecules* **14**, 949-953 (2013).
49. Allen, C.D., Okada, T. & Cyster, J.G. Germinal-center organization and cellular dynamics. *Immunity* **27**, 190-202 (2007).
50. Allen, C.D., Okada, T., Tang, H.L. & Cyster, J.G. Imaging of germinal center selection events during affinity maturation. *Science* **315**, 528-531 (2007).
51. Randolph, G.J., Angeli, V. & Swartz, M.A. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol* **5**, 617-628 (2005).
52. Schwickert, T.A. et al. In vivo imaging of germinal centres reveals a dynamic open structure. *Nature* **446**, 83-87 (2007).
53. Swartz, M.A. The physiology of the lymphatic system. *Advanced drug delivery reviews* **50**, 3-20 (2001).
54. Klein, U. & Dalla-Favera, R. Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol* **8**, 22-33 (2008).
55. McHeyzer-Williams, M., Okitsu, S., Wang, N. & McHeyzer-Williams, L. Molecular programming of B cell memory. *Nature reviews. Immunology* **12**, 24-34 (2012).
56. Schwickert, T.A. et al. A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *The Journal of experimental medicine* **208**, 1243-1252 (2011).
57. Purwada, A. & Singh, A. Immuno-engineered Organoids for Regulating the Kinetics of B cell Development and Antibody Production. *Nature Protocols* **12**, 168-182 (2017).
58. Purwada, A. et al. Ex vivo engineered immune organoids for controlled germinal center reactions. *Biomaterials* **63**, 24-34 (2015).
59. Wang, X., Rodda, L.B., Bannard, O. & Cyster, J.G. Integrin-mediated interactions between B cells and follicular dendritic cells influence germinal center B cell fitness. *Journal of immunology* **192**, 4601-4609 (2014).
60. Zhang, Y. & Wang, H. Integrin signalling and function in immune cells. *Immunology* **135**, 268-275 (2012).
61. Cayrol, F. et al. Integrin $\alpha_v\beta_3$ acting as membrane receptor for thyroid hormones mediates angiogenesis in malignant T cells. *Blood* **125**, 841-851 (2015).
62. Tian, Y.F. et al. Integrin-specific hydrogels as adaptable tumor organoids for malignant B and T cells. *Biomaterials* **73**, 110-119 (2015).

63. Wang, X., Rodda, L.B., Bannard, O. & Cyster, J.G. Integrin-mediated interactions between B cells and follicular dendritic cells influence germinal center B cell fitness. *The Journal of Immunology* **192**, 4601-4609 (2014).
64. Victoratos, P. et al. FDC-specific functions of p55TNFR and IKK2 in the development of FDC networks and of antibody responses. *Immunity* **24**, 65-77 (2006).
65. Goodnow, C.C., Vinuesa, C.G., Randall, K.L., Mackay, F. & Brink, R. Control systems and decision making for antibody production. *Nat Immunol* **11**, 681-688 (2010).
66. Carrasco, Y.R. & Batista, F.D. B-cell activation by membrane-bound antigens is facilitated by the interaction of VLA-4 with VCAM-1. *The EMBO journal* **25**, 889-899 (2006).
67. Allen, C.D. & Cyster, J.G. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Semin Immunol* **20**, 14-25 (2008).
68. Song, J. et al. Extracellular matrix of secondary lymphoid organs impacts on B-cell fate and survival. *Proceedings of the National Academy of Sciences* **110**, E2915-E2924 (2013).
69. Purwada, A., Shah, S.B., Beguelin, W., Melnick, A. & Singh, A. Modular Immune Organoids with Integrin Ligand Specificity Differentially Regulate Ex vivo B Cell Activation. *ACS Biomater. Sci. Eng.* **3**, 214-225 (2017).
70. Munson, J.M., Bellamkonda, R.V. & Swartz, M.A. Interstitial flow in a 3D microenvironment increases glioma invasion by a CXCR4-dependent mechanism. *Cancer research* **73**, 1536-1546 (2013).
71. Wiig, H. & Swartz, M.A. Interstitial fluid and lymph formation and transport: physiological regulation and roles in inflammation and cancer. *Physiol Rev* **92**, 1005-1060 (2012).
72. Swartz, M.A. & Lund, A.W. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. *Nat Rev Cancer* **12**, 210-219 (2012).
73. Nia H.T. & Hao Liu, G.S., Meenal Datta, Dennis Jones, Nuh Rahbari, Joao Incio, Vikash P. Chauhan, Keehoon Jung, John D. Martin, Vasileios Askoxylakis, Timothy P. Padera, Dai Fukumura, Yves Boucher, Francis J. Hornicek, Alan J. Grodzinsky, James W. Baish, Lance L. Munn, Jain R.K. in *Nature Biomedical Engineering*, Vol. 12017).
74. Tomei, A.A., Siegert, S., Britschgi, M.R., Luther, S.A. & Swartz, M.A. Fluid flow regulates stromal cell organization and CCL21 expression in a tissue-engineered lymph node microenvironment. *J Immunol* **183**, 4273-4283 (2009).
75. Apoorva, F. et al. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. *Journal of biomedical materials research. Part A* (2017 (Accepted)).
76. Buskohl, P.R., Gould, R.A. & Butcher, J.T. Quantification of embryonic atrioventricular valve biomechanics during morphogenesis. *Journal of biomechanics* **45**, 895-902 (2012).
77. Buskohl, P.R., Sun, M.J., Thompson, R.P. & Butcher, J.T. Serotonin potentiates transforming growth factor-beta3 induced biomechanical remodeling in avian embryonic atrioventricular valves. *PLoS One* **7**, e42527 (2012).
78. Gjorevski, N. et al. Designer matrices for intestinal stem cell and organoid culture. *Nature* **539**, 560-564 (2016).