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Model-driven Experimentation: A new approach to understand mechanisms of tertiary lymphoid tissue formation, function and therapeutic resolution.

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Model-driven Experimentation: A new approach to understand mechanisms of tertiary lymphoid tissue formation, function and therapeutic resolution.

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Abstract:

The molecular and cellular processes driving the formation of secondary lymphoid tissues have been extensively studied using a combination of mouse knockouts, lineage specific reporter mice, gene expression analysis, immunohistochemistry and flow cytometry. However, the mechanisms driving the formation and function of tertiary lymphoid tissue (TLT) experimental techniques have proven to be more enigmatic and controversial due to differences between experimental models and human disease pathology. Systems-based approaches including data-driven biological network analysis (Gene Interaction Network, Metabolic Pathway Network, Cell-Cell signalling & cascade networks) and mechanistic modelling afford a novel perspective from which to understand TLT formation and identify mechanisms that may lead to the resolution of tissue pathology. In this perspective, we make the case for applying model-driven experimentation using two case studies which combined simulations with experiments to identify mechanisms driving lymphoid tissue formation and function, and then discuss potential applications of this experimental paradigm to identify novel therapeutic targets for TLT pathology.

Formation and function of secondary and tertiary immune microenvironments

Lymphoid tissues are responsible for the orchestration of functional immune responses. This is achieved through the development and maintenance of niches that support the retention, activation and proliferation of adaptive immune cells in response to antigenic stimulation. Adult lymphoid tissue architecture is organised by an underlying network of stromal cells that produce extracellular matrix (e.g. collagens) and provide survival (e.g. BAFF, IL-7), migratory (CCL19/21, CXCL13) and immune activation (the storage and presentation of immune complexes by follicular dendritic cells) signals (Junt et al, 2008). Distinct stromal subsets with unique secretion profiles (chemokines, other cytokines, survival factors) develop in response to signalling from lymphocytes with a key role for TNF superfamily receptors; this stromal-lymphocyte cross-talk ensures the correct cell type is stimulated (or regulated) at the right time and place. Sustained cross-talk between mesenchymal stroma and lymphocyte subsets is a core feature of lymphoid tissue formation and maintenance, and occurs irrespective of the tissue type or anatomical location.

Formation of lymphoid tissues can occur by different cellular and molecular mechanisms. During foetal development secondary lymphoid tissues form in a process dependent on the RAR-related orphan receptor gamma, RORγ transcription factor expressing lymphoid tissue inducer cells (LTi) responding to localised chemotactic gradients leading to formation of lymph nodes (LN) and Peyer's patches (PP) in a lymphotoxin β (LTβ) dependent process (Pavert & Mebius, 2010). Localised mesenchyme, lymphoid tissue organiser (LTo) cells differentiate into adult marginal reticular cells (MRCs), fibroblastic reticular cells (FRCs) and follicular dendritic cells (FDCs) (Jarjour et al., 2014). Likewise, in the adult, innate lymphoid cells type 3 (ILC3s), the adult equivalent of LTi cells, have a key role in regulating crypto-patches that

can mature into isolated lymphoid follicles (Mowat and Agace, 2014). These specialised lymphoid structures contain predominantly B cells and often contain germinal centre (GC) reactions.

In humans, tertiary lymphoid tissues (TLT) are found in inflammatory immune responses associated with chronic pathology from hip joint replacements, keloids, tissues in autoimmune disease (e.g. the salivary gland in Sjogren's syndrome, multiple sclerosis and rheumatoid arthritis) to solid tumours and follicular lymphomas in the bone marrow (Mittal et al., 2013; Bombardieri et al., 2012; Bagabir et al., 2012; Dieu-Nosjean et al., 2016; Guilloton et al., 2012). Although the role of specific cell types has been controversial, there is an emerging paradigm of a multi-step process where localised inflammation induces stromal cell activation in a lymphocyte independent process, leading to localised microenvironments permissive for T and B cells entry (De Silva & Klein, 2015). These lymphocytes have the potential to drive the formation of organised tertiary tissue in an autocrine dependent process. This process closely resembles the capacity of naïve B cells to drive B cell follicle formation in secondary lymphoid tissues in a TNFα and LTβ dependent process, and the capacity of activated B cells to generate the germinal center (GC), a transient microenvironment that drives high affinity immune responses in a self-regulating autocrine dependent process. In both secondary immune tissues (LN, PP and spleen) and tertiary lymphoid tissues including ILFs and TLT, activated B cells prime the formation of the GC reaction. This specialised microenvironment contains both activated and proliferating B cells and different stromal compartments of CXCL12 secreting stroma (dark zone) and CXCL13 secreting FDCs (light zone). This facilitates the cyclic selection and expansion of antigen specific B cells (Zhang et al., 2016).

Non-lymphoid inflammatory immune structures, granulomas, can form in the liver, intestine, adipose tissue (crown-like structures) and lung induced by chronic infection/inflammation associated with tuberculosis, leishmaniasis, schistosomiasis, cell death and Crohn's disease (Sandor et al., 2003; Beattie and Kaye, 2016; Bolus et al., 2015). The formation of these highly dynamic microenvironments superficially resemble TLT, however their formation and organisation is driven by activated macrophages rather than by the mesenchymal-lymphocyte cross-talk observed in lymphoid tissues thus do not exhibit lymphocyte compartmentalisation. Granuloma structures are very heterogeneous in presentation within individual patients in a continuum between early macrophage centric granulomas, self-resolving granulomas to fibroblastic structures, these often being fibrotic rather than taking on a supportive stromal network phenotype. The triggers that drive granuloma formation instead of TLT formation appear not to be due to differences in the different chemotactic cues delivered by activated macrophages compared to those delivered by activated stromal fibroblasts leading to a very different cellular make up to the inflammatory foci of leukocytes (primarily myelo-monocytic (granuloma) vs. lymphocytic (TLT)).

Current approaches to studying lymphoid tissue formation: Limits, challenges ad new approaches.

Experimental studies, principally performed in gene knockout, lineage specific fluorescent protein and Cre reporter mouse lines have contributed significant insights into the roles of multiple different cell types and molecules in lymphoid tissue formation and function. This has been further validated using histology and flow cytometry analysis on human secondary lymphoid tissues. However, in contrast to secondary lymphoid tissues there are some distinct differences in human tissue pathologies to those found in mice including the cellular

composition of TLTs, granulomas and other inflammatory tissues. This arises in part from genetic and physiological differences between human and mice including the timing and duration of the immune response (chronic vs acute inflammation), the inflammatory triggers (infection, autoimmunity and cancer) and transcriptional differences in immune cells in the different species. In general, mouse models of immune mediated inflammatory disease are acute and fail to replicate the chronic human disease characterised by disease flairs followed by remission, limiting their translational capacity to human disease. Infection and tumour models in mice either rapidly resolve (too quickly for chronic pathology to establish) or lead to the mouse having to by euthanized for health and welfare prior to tertiary lymphoid pathology occurring. In comparison, humans may live the rest of their life with the disease pathology, particularly in the context of treatment with biologics and small molecules, thus pathology has the opportunity to evolve from localised inflammation to fibrotic tissue failure, systemic inflammation and autoimmunity working together to prevent disease resolution. Increasingly human 3-dimensional tissue culture models containing both stroma and lymphocytes have become increasingly common and useful in understanding underlying molecule mechanisms of TLT formation. However, it is not currently possible to represent the full complexity of chronic human pathology *in vitro*.

Experimental systems (*in vivo* and *in vitro*) to date have proven limited in their ability to explain chronic clinical pathology and resolve established Sjogren's pathology, although TNF has an important role in FDC differentiation and B cell organisation, anti-TNF fails to induce resolution disease (Sankar, 2004). To better understand the form and function of TLTs, current knowledge of stromal regulation through molecular signals and immune cell behaviour within lymphoid tissue must be consolidated and considered in a quantitative, systems-based approach. The development of systems-level stochastic computational models can bring

155 together a broad understanding across spatiotemporal scales of how genetic and molecular
156 factors relate to cellular and tissue level form and function, and give rise to the complex,
157 functional architectures observed in secondary lymphoid organs and disease specific TLT.
158 These models permit *in silico* experimentation providing a unique platform driving further
159 experimentation and assessing novel mechanistic targets and intervention strategies where *in*
160 *vivo* observed heterogeneity can be replicated.
161
162 Alan Turing (of code breaking fame) in seminal early work in mathematical biology (Turing,
163 1952) noted that gastrulation, arose from symmetry breaking, this leads to fundamental insights
164 and principles that drive modern mathematical and computational biology: the notion that
165 chaotic, non-linear behaviour of individual biological processes, including the self-
166 organisation of complex biological structures (e.g. TLT), can result in emergent properties that
167 cannot be understood from consideration of each individual component in isolation. The
168 development of models that capture the essential, emergent behaviour of specific biological
169 processes, with extraneous components excluded, enables understanding of how complex
170 molecular and cellular interactions govern complex, emergent biological processes and can
171 therefore lead to new insights and quantitative predictions (Callard and Yates, 2005). Emergent
172 properties in a TLT model would include stromal networks, lymphocyte organisation,
173 migration and interactions with antigen presenting cells, and localised cytokine/chemokine
174 production.
175
176 **Application of model-driven experimentation to understand mechanisms of lymphoid**
177 **tissue development and function.**
178

Advances in computing resources and computational modelling technology has provided the capacity to generate complex *in silico* models of lymphoid tissues that incorporate space, time and cellular heterogeneity found in immune tissues including TLT. Applying *in silico* approaches to understand secondary lymphoid tissue formation and function requires the integration of experimental data across cellular, molecular and tissue levels of organisation. Ensuring that the biological processes are appropriately described requires a fine balance between model abstraction and interpretation (quantitative and qualitative) of experimental data. A number of different modelling approaches may be utilised (summarised in **Table 1**), increasingly, integration of different mathematical/computational techniques into a hybrid model is a common strategy to address the limitations of using each technique in isolation. This approach also facilitates the consolidation of data across different levels of organisation (molecular, cellular, tissue and patient) into a single multiscale model. For example, an agent-based model can capture an individual cell, which in turn incorporates a differential equation-based model capturing a 'lower-level' aspect of that individual's behaviour, such as surface expression of a receptor. Adopting an *in silico* approach provides a platform that can provide insights and generate predictions that can be verified *in vivo:* verification that can lead to increased biological understanding and incrementally improved *in silico* models for further experimentation. This iterative approach of combining *in vivo*, *in vitro* and *in silico* approaches has been termed 'model-driven experimentation' (MDE)(Ganesan & Levchenko, 2012).

Case Study 1: Insights from MDE to secondary lymphoid tissue formation:

Peyer's patches (PP) are specialised secondary lymphoid tissues of the intestine that develop during a fixed window in foetal development and have an essential role in maintaining intestinal immunity. PP form stochastically along the mid-gut, with mice developing 8-12

patches, however, as the absence of or reduction in the number of PPs is observed in several different gene knockouts, the molecular process which triggers patch formation was unclear (Veiga-Fernandes et al., 2007). Using an MDE based approach had the potential to provide new insight into how different signalling pathways (RET, chemokine receptors, cytokine receptors, TNF superfamily, adhesion molecules) might integrate to induce PP development *in silico* and to subsequently design key experiments to test hypotheses *in vivo*. PPSim is an agent based Peyer's patch simulator that captures key processes during the 72-hour period of tissue development in prenatal mice and replicates (statistically similar) emergent cell behaviours found *in vivo,* specifically Populations of haematopoietic cells, known as Lymphoid Tissue Initiator (LTin) and Lymphoid Tissue Inducer (LTi) cells, migrate into the developing gut, with data from laboratory observations suggesting these cells follow a random motion. Both cell populations express receptors for the adhesion molecule VCAM-1, expressed by stromal Lymphoid Tissue Organizer (LTo) cells residing in the gut wall. (Alden et al., 2012; Patel et al., 2012). In this computational model LTi and LTin are captured as individual entities that migrate into the developing mid-gut serosa and undergo a random walk, interacting with their localised simulated environment through signalling pathways including GDRFs/Ret signalling pathways, adhesion molecules and chemokine receptors, as is observed *in vivo*. On ensuring PPsim adequately represented individual cell responses, statistical analysis techniques, specifically sensitivity analyses, were used to explore mechanisms driving prenatal lymphoid organ formation (Alden et al., 2013; Butler et al., 2014). This exploration of the simulated biological pathways revealed which pathways had significant impacts on simulated cell behaviour at different time points during PP development. By examining correlations in the level of activity of simulated pathways and cell behaviour, the hypothesis was derived that contact between LTin and LTo cells that leads to the localised upregulation of VCAM-1 on stromal cells was the key triggering event that determined the site of PP formation on the mid-

gut (Patel et al., 2012). Utilising this prediction, an *in vitro* assay imaging foetal mid-gut explants incubated in the presence or absence of anti-VCAM-1 antibodies was developed. Using this assay, it was verified that early upregulation of VCAM-1 was the triggering event that was essential for the initiation of LTi & LTin cell clustering. The model simulation results, supported by replicated experimentation and safety-critical systems-based fitness-for-purpose argumentation that details the knowledge integration in model composition, provide evidence that the simulation was fit for the purpose of aiding exploration of this specific research question: understanding the triggering of lymphoid tissue development which was not possible by conventional genetic approaches (Alden et al., 2015a; Alden et al., 2015b).

Case Study 2: Applying MDE to understand germinal centre dynamics and function

The GC reaction is a transient microenvironment in which affinity maturation occurs in response to immunisation and infection bearing key similarities to TLT in its evolution in the role of lymphocytes in inducing highly organised stromal networks, the essential role of TNF superfamily members in regulating its induction and the induction of chemokine gradients (De Silva and Klein, 2015; Victora and Mesin, 2014). However, in comparison to TLT, the GC is a self-resolving tertiary lymphoid microenvironment. Recent technological advances, particularly the advent of intravital multiphoton imaging including photo-activated fluorescent proteins has led to the unprecedented availability of data on the dynamics B-cell migration and selection (Allen et al., 2007; Schwickert et al., 2007; Shulman et al., 2013, 2014). However, imaging datasets provide a narrow window of insight into a process that occurs over a timescale of days and weeks. Furthermore, as imaging techniques are optimised for a given time and length scale, they are limited in their ability to link molecular, cellular and tissue level processes. This has made the interpretation of imaging datasets in the context of the wider

literature challenging. To address this issue modelling approaches have been used to test the validity of different hypotheses for mechanisms controlling B-cell migration and selection within the GC (Chan et al., 2013; Figge et al., 2008; Meyer-Hermann, 2006; Meyer-Hermann et al., 2012).

With respect to the germinal centre, model-derived insights have proved useful not only in the analysis of existing datasets but also as a driver for further experimentation. Specifically, an MDE approach to examine the effects of antibody-feedback on the process of affinity maturation (Zhang et al., 2013). Analysis of an *in silico* GC reaction yielded the prediction that GC B-cells, which require antigen on FDCs for positive selection, were competing for antigen by early low-affinity antibodies. Only higher affinity B-cells were able to outcompete for antigen to receive the necessary survival signals. To experimentally validate this prediction, the authors manipulated the GC response with monoclonal antibodies of defined affinities and were able to confirm that antibody feedback provides a dynamic selection threshold to maximise Ig affinities (Zhang et al., 2013). A similar approach was employed to investigate the role of Toll-like receptor 4 (TLR4) on the GC where an iterative cycle of *in silico* and *in vivo* experimentation dissected the importance of TLR4 signalling on the maturation of Follicular Dendritic Cells, key regulators of B-cell selection in the light zone of the GC (Garin et al., 2010). Both of these MDE examples highlight the use of *in silico* experimentation as a means of refining the use of experimental animals and available resources through the identification of key time-points and conditions to test *in vivo*. These case studies together provide example of how theoretical models can consolidate data from different sources as a platform for the development novel hypotheses and a driver for further experimentation.

Perspective on MDE as applied to tertiary lymphoid tissue formation, function and therapeutic resolution.

When computational modelling is combined with the knowledge that can be derived from next generation imaging, multi-dimensional cytometry and gene expression analysis of human TLT pathology, MDE has the potential to provide novel insights to key questions on molecular and cellular mechanisms involved in TLT formation, maintenance and function similar to its capacity to impact on our understanding of lymphoid stromal network and granuloma dynamics (**Table 2**)(Kislitsyn, A et al., 2015; Novkovic M et al., 2016; Warsinke et al., 2016; Marino et al., 2016). One of the key advantages of applying multi-scale modelling is it permits capture of a wide range of different phenomena that occur on different orders of magnitude in terms of time and length scales that are critical in the stochastic processes involved in TLT induction. These include different cell types, states and interactions, inflammatory molecules, extracellular matrix, adhesion molecules and chemotactic signals all in the context of an evolving tissue microenvironment. Developing *in silico* models permits temporal inhibition of different signalling pathways and cellular depletions during different stages of TLT pathology using statistical tools (**Figure 1**). This permits identification of key pathways that could be targeted to induce resolution of pre-existing TLT rather than inhibiting its formation as has been used to make in silico predictions for the treatment of tuberculosis (Pienarr et al., 2015). A large number of novel antibody therapies, biologics and small molecular inhibitors have been developed to target immune function for the treatment of immune mediated inflammatory diseases. These therapies are unlikely to show maximal efficacy against existing tissue pathology when used as mono-therapies, rather it is more likely that use of therapeutic combinations that is most likely to show clinical efficacy. The clinical challenge is that there are already over 20,000 possible different combinations using existing therapeutics that would

need to be trialled to find optimal targeting strategy to resolve TLT pathology. Thus MDE based approaches provide a rational approach to identify novel combination therapeutic regimes that have a best potential in clinical trials.

Although the adoption of MDE has only recently started to impact on immunology research, it is starting to have a very significant impact on other areas of biology. We propose that the increased accessibility of computational models, high-performance computing resources, the increased familiarity and understanding of simulations as tools to understand immune function and the capacity to apply *in silico* approaches to identify potential therapeutic approaches and disease biomarkers will accelerate the application of MDE as a methodology understand and target disease resolution.

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507 **Table 1:** Mathematical and Computational Techniques for Modelling Immune Processes

Technique	Description	Comments
ODE	Ordinary Differential Equations: Describe the rate of change with respect to one other variable (e.g. population change over time, *t*).	Commonly used technique that can be used to quantify changes in population size over time.
PDE	Partial Differential Equations: Describe rate of change of a function of more than one variable with respect to one of those variables (e.g. motion through space *x,y,z* as a function of time *t*).	Often used to describe changes occurring over both time and multiple spatial dimensions.
Monte Carlo	Statistical random sampling method where outcomes are determined at random from input probability distribution functions.	Stochastic technique to model deterministic processes, very frequently integrated within ABM, CPM and other stochastic modelling approaches.
Petri Nets	Graph based model describing network of events or 'transitions' that occur depending on given conditions or 'places'; a stochastic methodology.	Computationally efficient, can be effectively defined using SBML2. Capturing explicit spatial representation can be difficult.
ABMs	Agent Based Models are composed of individual entities specified as agents which exist independently in a well-defined state: a set of attributes at a specific point in (e.g.) time and space, with state-transitions governed by a rule-set, often described in terms of Finite State Machines and other diagrammatic constructs using the UML (Unified Modelling Language).	There are a number of methodologies to generate ABMs. There are tools with user interfaces for constructing simpler lattice-based ABMS, or 'unconstrained' models manually coded as software in languages such as Java and C++.
(Extended) Cellular Potts Modelling	A lattice based modelling technique for simulating the collective behaviour of cells. A cell is defined as a set of pixels within a lattice (sharing a 'spin state'), and is updated pixel-by-pixel according to a mathematical function which incorporates cell volume, and surface/adhesion energies.	Similar to an ABM, but relies on effective energy functions (the Hamiltonian) to describe cellular adhesion, signalling, motility and other physical phenomena.
Hybridised Models	Bringing together a range of different techniques generally within the context of an ABM or CPM, incorporating differential equations and a variety of other mathematical and computational techniques to effectively capture phenomena occurring over different spatiotemporal scales (e.g. intracellular activity)	Can take advantage of different modelling techniques, particularly applicable where there are multiple processes occurring in different scales of time and space.

508 **Table 2:** Key questions on TLT formation and maintenance that can be address in hybridised

509 TLT models

Formation
What are the minimum cellular requirements to initiate TLT formation? Is this driven by different types of stroma, lymphocytes, dendritic cells or tissue resident macrophage?
What is the relative importance of inflammation and antigen in TLT induction? Is autoantigen required for induction or just an outcome of the pathology?
What is the role of different cytokines and chemotactic signals on TLT formation?
Maintenance
What is the relative role of inflammatory cytokines, lymphocyte – stromal cross talk, immune cell entry, cell death, antigenic stimulation on TLT maintenance?
What are the key signalling pathways required to maintain TLT once it has formed? Can these pathways be targeted to induce TLT resolution?
Can TLT self-resolve in humans? If so what is the balance between new TLT induction and resolution of existing structures?

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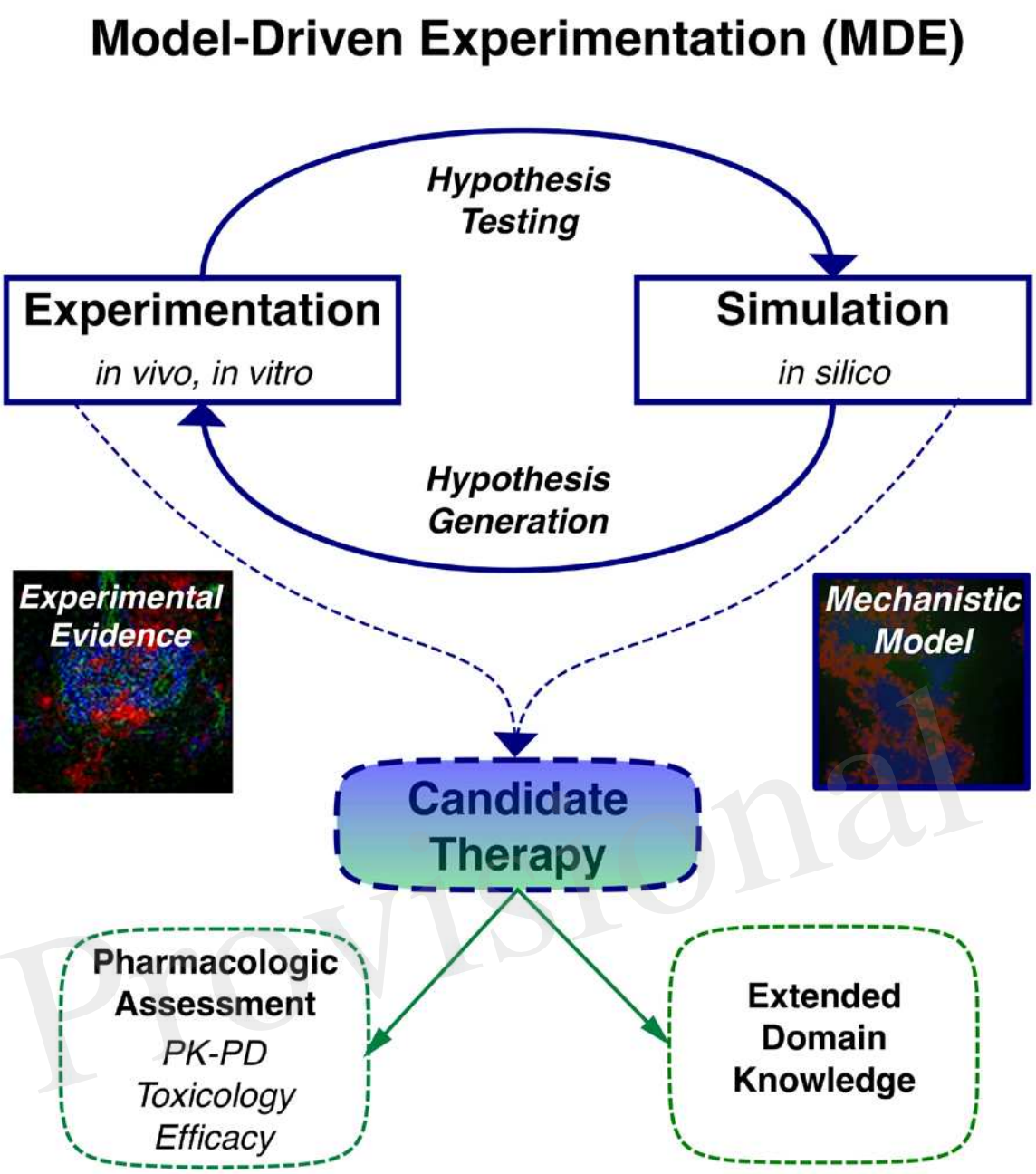

Figure 1: Application of Model-driven Experimentation to develop new mechanistic understanding of TLT formation and maintenance permitting identification of novel therapeutic approaches to resolve localised TLT pathology.

Figure 01.JPEG