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Butler, James Andrew, Cosgrove, Jason, Alden, Kieran James et al. (2016) Model-driven Experimentation: A new approach to understand mechanisms of tertiary lymphoid tissue formation, function and therapeutic resolution. *Frontiers in immunology*. pp. 1-23. ISSN: 1664-3224

<https://doi.org/10.3389/fimmu.2016.00658>

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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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Submitted to Journal:

Frontiers in Immunology

Specialty Section:

Inflammation

ISSN:

1664-3224

Article type:

Perspective Article

Received on:

26 Aug 2016

Accepted on:

16 Dec 2016

Provisional PDF published on:

16 Dec 2016

Frontiers website link:

www.frontiersin.org

Citation:

Butler JA, Cosgrove J, Alden K, Timmis J and Coles MC(2016) Model-driven Experimentation: A new approach to understand mechanisms of tertiary lymphoid tissue formation, function and therapeutic resolution.. *Front. Immunol.* 7:658. doi:10.3389/fimmu.2016.00658

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Provisional

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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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Running Title: Application of Model-driven Experimentation to TLT

Keywords: Multi-scale Modelling, Tertiary Lymphoid Tissue, Systems Immunology

37 **Abstract:**

38

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

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56 **Formation and function of secondary and tertiary immune microenvironments**

57

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

71

72 Formation of lymphoid tissues can occur by different cellular and molecular mechanisms.
73 During foetal development secondary lymphoid tissues form in a process dependent on the
74 RAR-related orphan receptor gamma, ROR γ transcription factor expressing lymphoid tissue
75 inducer cells (LTi) responding to localised chemotactic gradients leading to formation of lymph
76 nodes (LN) and Peyer's patches (PP) in a lymphotoxin β (LT β) dependent process (Pavert &
77 Mebius, 2010). Localised mesenchyme, lymphoid tissue organiser (LTo) cells differentiate into
78 adult marginal reticular cells (MRCs), fibroblastic reticular cells (FRCs) and follicular
79 dendritic cells (FDCs) (Jarjour et al., 2014). Likewise, in the adult, innate lymphoid cells type
80 3 (ILC3s), the adult equivalent of LTi cells, have a key role in regulating crypto-patches that

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

84

85 In humans, tertiary lymphoid tissues (TLT) are found in inflammatory immune responses
86 associated with chronic pathology from hip joint replacements, keloids, tissues in autoimmune
87 disease (e.g. the salivary gland in Sjogren's syndrome, multiple sclerosis and rheumatoid
88 arthritis) to solid tumours and follicular lymphomas in the bone marrow (Mittal et al., 2013;
89 Bombardieri et al., 2012; Bagabir et al., 2012; Dieu-Nosjean et al., 2016; Guilloton et al.,
90 2012). Although the role of specific cell types has been controversial, there is an emerging
91 paradigm of a multi-step process where localised inflammation induces stromal cell activation
92 in a lymphocyte independent process, leading to localised microenvironments permissive for
93 T and B cells entry (De Silva & Klein, 2015). These lymphocytes have the potential to drive
94 the formation of organised tertiary tissue in an autocrine dependent process. This process
95 closely resembles the capacity of naïve B cells to drive B cell follicle formation in secondary
96 lymphoid tissues in a $TNF\alpha$ and $LT\beta$ dependent process, and the capacity of activated B cells
97 to generate the germinal center (GC), a transient microenvironment that drives high affinity
98 immune responses in a self-regulating autocrine dependent process. In both secondary immune
99 tissues (LN, PP and spleen) and tertiary lymphoid tissues including ILFs and TLT, activated B
100 cells prime the formation of the GC reaction. This specialised microenvironment contains both
101 activated and proliferating B cells and different stromal compartments of CXCL12 secreting
102 stroma (dark zone) and CXCL13 secreting FDCs (light zone). This facilitates the cyclic
103 selection and expansion of antigen specific B cells (Zhang et al., 2016).

104

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

120

121 **Current approaches to studying lymphoid tissue formation: Limits, challenges and new**
122 **approaches.**

123

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

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

147

148 Experimental systems (*in vivo* and *in vitro*) to date have proven limited in their ability to explain
149 chronic clinical pathology and resolve established Sjogren's pathology, although TNF has an
150 important role in FDC differentiation and B cell organisation, anti-TNF fails to induce
151 resolution disease (Sankar, 2004). To better understand the form and function of TLTs, current
152 knowledge of stromal regulation through molecular signals and immune cell behaviour within
153 lymphoid tissue must be consolidated and considered in a quantitative, systems-based
154 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

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

198

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

200

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

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

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

238

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

240

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

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

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

276

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

279

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

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

305

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

Provisional

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Provisional

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

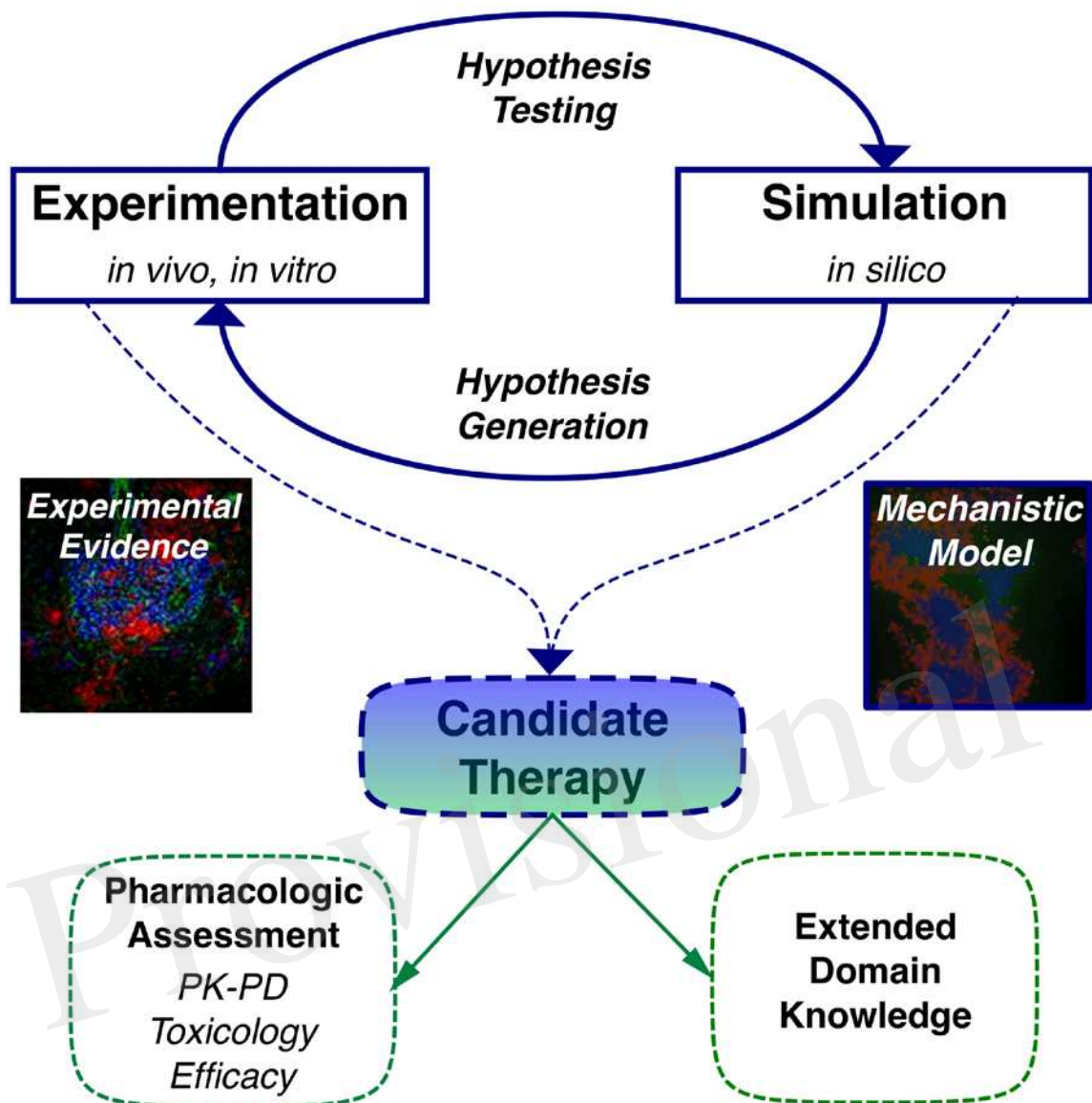
Technique	Description	Comments
ODE	Ordinary Differential Equations: Describe the rate of change with respect to one other variable (e.g. population change over time, t).	Commonly used technique that can be used to quantify changes in population size over time.
PDE	Partial Differential Equations: Describe 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
<i>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?</i>
<i>What is the relative importance of inflammation and antigen in TLT induction? Is autoantigen required for induction or just an outcome of the pathology?</i>
<i>What is the role of different cytokines and chemotactic signals on TLT formation?</i>
Maintenance
<i>What is the relative role of inflammatory cytokines, lymphocyte – stromal cross talk, immune cell entry, cell death, antigenic stimulation on TLT maintenance?</i>
<i>What are the key signalling pathways required to maintain TLT once it has formed? Can these pathways be targeted to induce TLT resolution?</i>
<i>Can TLT self-resolve in humans? If so what is the balance between new TLT induction and resolution of existing structures?</i>

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Model-Driven Experimentation (MDE)



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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.

Model-Driven Experimentation (MDE)

