# Loss of microbiota depletes cross-reactive Foxp3$^+$ Tregs leading to selective immunopathologies

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## Abstract

The 'Hygiene hypothesis', a cornerstone model to account for the role of exogenous pathogens and later of endogenous microbiota in immune disorders, is currently presumed to operate at the innate immunity and metabolite levels to properly 'educate' the immune system. Doing so however fails to satisfactorily account for the antigen-specific nature of such disorders. SPIRAL is a novel interpretive framework that resolves this dilemma. It represents the periodic table of cross-reactive Foxp3$^+$ regulatory T cell (Treg) epitopes selected from commensal microbiota over evolutionary time to mediate self-nonself discrimination and effector class regulation. Here, we utilize the SPIRAL's predictive power to provide a mechanistic antigen-specific basis for the initiation of allergies and autoimmune diseases as well as for the failure to mount effective anti-tumor and vaccine responses through selective loss of microbiota and corresponding cross-reactive Foxp3$^+$ Tregs.

## Introduction

Merit of an immunological model is proportional to its ability (a) to account for prior observations, (b) to help design and predict outcomes of experimental and clinical studies, and (c) to provide a blueprint for effective therapeutic interventions. To this end, we apply the SPIRAL model (Usharauli and Kamala, 2017) to four clinically relevant immunological



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phenomena to illustrate its interpretive power.

## Allergy

Allergy represents an excessive, highly polarized immune response, usually $Th_2$ but other effector classes as well, to seemingly innocuous environmental antigens. While antigen-specific IgE is a normal response *per se*, excessive IgE to the same antigen is an allergic response, an immunopathology.

Why do some individuals mount such exaggerated responses to antigens called allergens? Why does a given allergen with inherent propensity to promote a particular effector class, for example, highly polarized $Th_2$ response, manifest only in some, not every, individual?

The extended version of the 'Hygiene hypothesis' attributes recent rise of allergy in urbanized, 'clean' hosts to reduced exposure to environmental microbes, both commensal and pathogenic, due to modern sanitation including chlorinated water supply, childhood vaccination and antibiotic (over)usage (Kondrashova et al., 2013). Though host interaction with evolutionarily co-evolved pathogen-associated molecular patterns (PAMPs) became its chief focus, such non-specific genetic and epigenetic changes at the innate immune system level alone cannot account for such dramatic rise of selective allergies in individuals. We thus conclude that the 'Hygiene hypothesis' must be placed at the adaptive immune system level in line with $Foxp3^+$ Treg biology.

SPIRAL explains that any non-productive (ineffective) T cell response to nonself antigen is by default dominated by a highly polarized effector class that constrains other effector classes. The SPIRAL model predicts that initiation of specific allergy is a two-step process. First, non-specific genetic or epigenetic variations would predispose certain individuals to excessive, highly polarized forms of immune response to some polarizing environmental nonself antigens, though ordinarily such a propensity would remain unexpressed or silent unless and until second,



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such individuals would also display an antigen-specific defect, a 'hole' in their Foxp3$^+$ Treg repertoire that evolved to control non-productive T cell responses to such polarizing nonself antigens.

The SPIRAL model predicts allergens that promoted non-productive and highly polarizing T cell responses were subject to antigen-specific control by thymic Foxp3$^+$ Tregs. In the SPIRAL model, hosts maintain allergen-specific Foxp3$^+$ Tregs by acquiring over evolutionary time commensal microbiota that express antigens cross-reactive to allergens. The model predicts the exact overlap between allergens and microbiota that determine Foxp3$^+$ Treg specificity necessary to prevent allergies (Figure 1). Subsequent exposure to specific allergens in predisposed individuals who have, for any reason, lost specific microbiota would yield a polarizing T cell response rather than tolerance due to complementary loss of allergen-specific Tregs as well.

In brief, when exposed to allergen, specific microbiota loss and attendant 'hole' in a given host's antigen-specific, cross-reactive Foxp3$^+$ Treg repertoire reveals their underlying non-specific genetic defects that would have otherwise remained silent.

## Autoimmunity

A subset of autoimmune diseases likely has a purely genetic basis independent of failure of self-nonself discrimination, such as seen in AIRE or Foxp3 gene hypomorphisms (Bacchetta et al., 2016) and such genetic malfunctions would equally affect both self and nonself immune responses.

Nonetheless, in canonical autoimmune diseases, failure of self-nonself discrimination leads to specific and selective auto(self)-responses. Increasingly, such autoimmune responses are found to be associated with changes in host microbiota composition as well. Typically, autoimmune disease initiation has been interpreted in the light of either the 'Hygiene hypothesis'



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or cross-reactivity between microbe and host antigens (molecular mimicry).

According to a broader version of the 'Hygiene hypothesis', autoimmune diseases develop as a result of failure to properly 'train' host immune system (Kostic et al., 2015). This concept, widely adopted in the field following discovery of PAMPs, suggests that immune systems of today's urbanized, 'clean' hosts experience minimal interaction with evolutionarily co-evolved PAMPs (Vatanen et al., 2016). As a consequence, such hosts mount inappropriate immune response to even nominal antigens. As Foxp3$^+$ Tregs also express receptors for PAMPs, the argument goes they too are defective in their function in 'clean' hosts. Essentially, failure of innate 'training' of the host's adaptive immune system supposedly leads to antigen-specific inflammatory tissue damage (Bach and Chatenoud, 2012).

However, such interpretation fails to account for antigen-specificity of autoimmune diseases as defect in Foxp3$^+$ Tregs due to defect in PAMP signaling should lead to 'total body' autoimmunity rather than selective autoimmune diseases. We thus conclude that here too the 'Hygiene hypothesis' must be placed at the adaptive immune system level in line with Foxp3$^+$ Treg biology.

Separately, according to the 'cross-reactivity' hypothesis, autoimmune diseases develop as a result of infections that share antigenic similarities with susceptible hosts (Root-Bernstein and Fairweather, 2015). However, it is not entirely obvious how 'cross-reactive' infection could break tolerance to auto(self) antigens. Wouldn't a host expressing self antigen cross-reactive to commonly occurring infectious antigen lead to co-evolution of appropriate tolerance mechanisms, such as thymic deletion or thymic Foxp3$^+$ Tregs generation, to prevent autoimmunity? And if autoimmune diseases are a more recent development in urbanized 'clean' hosts due to modern sanitation, childhood vaccination and antibiotic (over)usage, how do microbiota fit in the picture?

The SPIRAL model resolves these above-mentioned contradictions by proposing that



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tolerance to self is thymus-generated and peripherally-maintained by commensal microbiota-derived cross-reactive Foxp3$^+$ Tregs with a focus on those self and nonself antigens that applied evolutionary selection pressure on the immune system by initiating non-productive T cell responses.

The SPIRAL model predicts that commensal microbiota-derived cross-reactive antigens (epitopes) rather than self antigens themselves maintain Foxp3$^+$ Tregs required for self-tolerance. Were it the latter, Foxp3$^+$ Treg loss would entail loss of self-antigen expression which would by default eliminate the possibility of autoimmunity. Such host-microbiota arrangement for self-tolerance was likely favored by peripheral tissues not expressing cryptic autoimmune target self-epitopes at levels necessary to maintain cross-reactive Foxp3$^+$ Tregs on their own since self-antigen expression sufficient to directly maintain Tregs would likewise be sufficient for thymic negative selection of auto-reactive T cells as well. Not surprisingly, depletion of commensal microbiota that express epitopes cross-reactive to cryptic self-antigens would compromise antigen-specific Foxp3$^+$ Treg repertoire leading to autoimmune response against such self epitopes but only when responding to cross-reactive pathogens. The SPIRAL model thus predicts the exact overlap between auto-antigens, microbiota and pathogens that determine Foxp3$^+$ Treg specificity necessary to prevent autoimmunity (Figure 2).

So under what conditions would cross-reactive pathogens cause autoimmune disease? We believe, as in allergy, initiation of specific autoimmune disease is a two-step process. In the first stage, the host develops a 'hole' in cross-reactive Foxp3$^+$ Treg repertoire either due to

- Selective depletion of commensal microbiota that maintained them, or
- HLA polymorphism not stably supporting particular cross-reactive Foxp3$^+$ Tregs, or
- Exposure to completely novel cross-reactive pathogens that hosts had not encountered in evolution and thus did not have chance to develop corresponding Foxp3$^+$ Tregs.



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However, Treg depletion alone is insufficient to initiate autoimmunity since target antigen remains cryptic and not 'visible' for auto-reactive T cells at this stage. Post-Treg loss, the second stage initiates autoimmunity only when cryptic self antigens are rendered visible to autoreactive T cells amplified by a pathogen expressing antigens (epitopes) cross-reactive to such self-antigens.

In brief, tolerance to specific self-antigens could be compromised by

(a) failure to generate self antigen-specific Foxp3$^+$ Tregs in the thymus, and/or

(b) failure to maintain them by failing to harbor commensal microbiota expressing cross-reactive antigen (epitopes).

The host's genetic makeup, including HLA allele polymorphism, contribute to these antigen-specific tolerance failures through destabilization of microbiota-Treg axis.

The SPIRAL model also predicts that anti-self Tregs maintained by microbiota-derived cross-reactive antigens (epitopes) could explain differences in autoimmune disease rates between male and female. Since reproductive physiology inherently entails cyclical hormonal fluctuations, the microbiota responsive to such fluctuations would also be more prone to instability, especially in today's 'hygiene' era. We believe such relatively greater instability of hormone-responsive microbiota and corresponding instability of the cross-reactive antigen (epitope)-specific Foxp3$^+$ Treg repertoire they support underlies differences in autoimmune disease propensity between genders and specifically, the much higher rates of several autoimmunities in females.

## Tumor immunology

Tumors represent a special challenge to the immune system from the standpoint of self-nonself discrimination and effector class regulation. On the one hand, tumors develop from self tissues but frequently express novel antigens called neo-antigens, reminiscent of late appearing



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150 self antigens expressed by normal tissues. On the other hand, tumors frequently promote non-
151 productive immune effector classes that in fact support tumor growth, reminiscent of microbial
152 PAMPs.

153 The SPIRAL model proposes that thymus plays a mandatory role in establishing
154 peripheral tolerance by generating and seeding the periphery with a repertoire of antigen-
155 specific Foxp3$^+$ Tregs determined by 'antigenic experience' over evolutionary time. These
156 antigen-specific Foxp3$^+$ Tregs are maintained in the periphery by microbiota that supply relevant
157 cross-reactive antigens.

158 The SPIRAL model predicts that tumors would be under selection pressure to express
159 polarizing neo-antigens:

160 (a) cross-reactive to microbiota antigens ordinarily seen by Foxp3$^+$ Tregs. If polarizing
161 neo-antigens, such as carcinoembryonic antigens, happen to be 'unplugged' in particular hosts
162 who have lost the corresponding microbiota and cognate Foxp3$^+$ Tregs, then simply by chance,
163 such a tumor would end up exploiting such Foxp3$^+$ Treg repertoire 'holes' to drive polarizing yet
164 ineffective anti-tumor immune responses. This scenario fits examples where reconstitution with
165 specific bacterial species jumpstart an effective anti-tumor immune response (Vétizou et al.,
166 2015; Sivan et al., 2015).

167 (b) not yet selected over evolutionary period to be 'plugged' by Foxp3$^+$ Tregs. In this
168 scenario tumor would grow by driving polarizing, ineffective immune response as no
169 corresponding antigen-specific Foxp3$^+$ Tregs are present to inhibit ineffective effector class.

170 Essentially, contrary to prevailing assumptions, the SPIRAL model predicts that
171 presence of tumor-associated Foxp3$^+$ Tregs are more often than not beneficial. As with microbe-
172 host interactions, different classes of immune response target different tumor proteins and if one
173 leveraged the evolutionarily selected antigen-specific Foxp3$^+$ Treg repertoire to stop such
174 dominant, nonproductive effector class directed to polarizing tumor antigens that cross-inhibits



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other effector classes to other tumor antigens, a productive immune response capable of eliminating the tumor would naturally emerge. The model predicts the exact overlap between tumor antigens and microbiota that determine Foxp3$^+$ Treg specificity capable of protecting against tumors (Figure 3).

## Vaccines

Today vaccine development has to navigate the stalemate between a rock and a hard place in trying to make regulator-friendly 'safe' minimally reactogenic subunit vaccines that are simultaneously highly immunogenic and therefore effective. The former, which entails deconstructing organisms, and then picking and choosing from the smorgasbord of resulting individual antigens, comes at the expense of the latter. To make up for this predictable deficiency, adjuvants are added to more effectively engage the innate arm of the immune system. However, knowing little about how adjuvants actually work makes them more a handicap than asset, a problem compounded by the fact that old generation vaccines already cover most naturally self-limiting acute infections while vaccines are currently lacking for chronic infections such as HIV, malaria, tuberculosis.

The SPIRAL model predicts that the same rule governs effectiveness of immune response to vaccines as it does for infections, namely, through Foxp3$^+$ Tregs specific for antigens (epitopes) cross-reactive between microbiota and natural infection or vaccines. An effective anti-pathogen immune response emerges naturally in the presence of an antigen-specific Foxp3$^+$ Treg repertoire capable of blocking response to its polarizing antigens.

Presently, it is commonly but erroneously accepted that subunit vaccines lacking Foxp3$^+$ Treg epitopes would be able to drive strong and effective immune response (Moise et al., 2014). In fact, the SPIRAL model predicts the opposite would be true. The effectiveness of a vaccine would chiefly depend on whether polarizing antigens within it are 'plugged' by evolutionarily



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selected cross-reactive Foxp3$^+$ Tregs to enable development of proper immune effector class(es). Treg-epitope depleted vaccines would not have any advantage against the actual pathogen which would after all express such polarizing antigen (epitopes) that would take over the focus of the host's immune response, resulting in dominance of non-productive–effector class.

Not surprisingly, the SPIRAL model predicts that effectiveness of any vaccine would primarily depend on host microbiota that specifically maintain cross-reactive Treg repertoire to naturally prevent non-productive immune response to polarizing antigens of a given pathogen. In short, vaccines that appear ineffective could be converted into effective ones by specific modulation of host microbiota (Valdez et al., 2014). The SPIRAL model predicts the exact overlap between vaccines and microbiota that determine Foxp3$^+$ Treg specificity necessary to drive development of effective immune response to pathogens following vaccination (Figure 4).

## Conclusion

Here we have presented a practical guide to SPIRAL, a unique interpretive framework that demonstrates the central role of the microbiota-Treg axis in self-nonself discrimination and effector class regulation. Its predictive power has the potential to transform the field of antigen-specific immunotherapy.

**Conflict of interest statement:** Tirumalai Kamala and David Usharauli are founders of Tregeutix Inc., a biotech company that focuses on developing microbiota guided antigen-specific immunotherapies.

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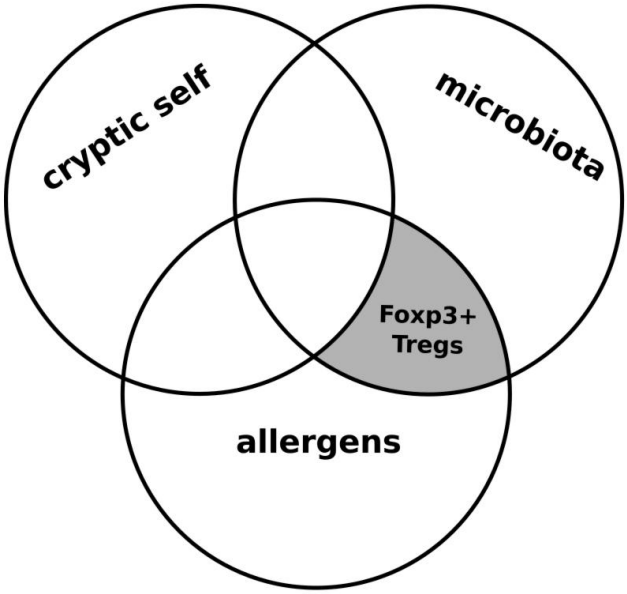


Figure 1. Map of relevant antigenic overlap between allergens and microbiota that determines antigen-specificity of thymic Foxp3+ Tregs necessary to prevent allergies, as predicted by the SPIRAL model.



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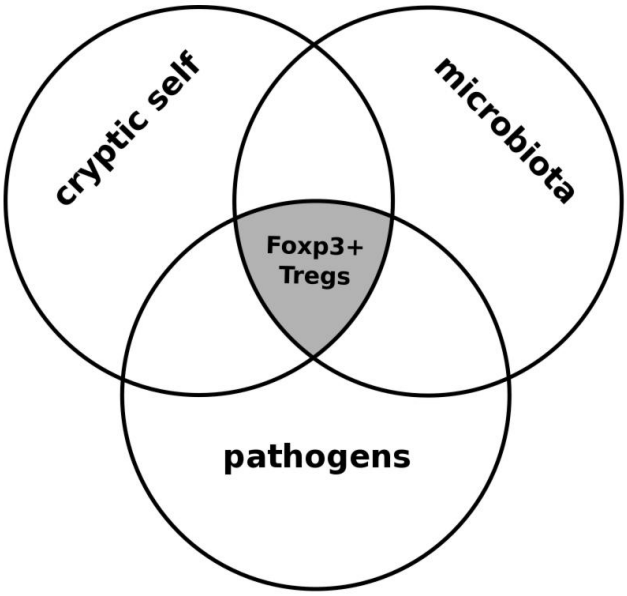


Figure 2. Map of relevant antigenic overlap between auto-antigens, microbiota and pathogens that determines antigen-specificity of thymic Foxp3+ Tregs necessary to prevent autoimmunity, as predicted by the SPIRAL model.



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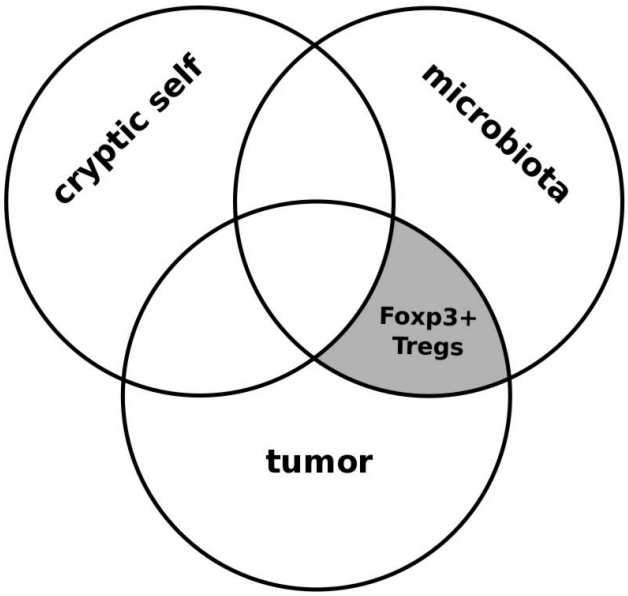


Figure 3. Map of relevant antigenic overlap between tumors and microbiota that determines antigen-specificity of thymic Foxp3+ Tregs that can protect against tumors, as predicted by the SPIRAL model.



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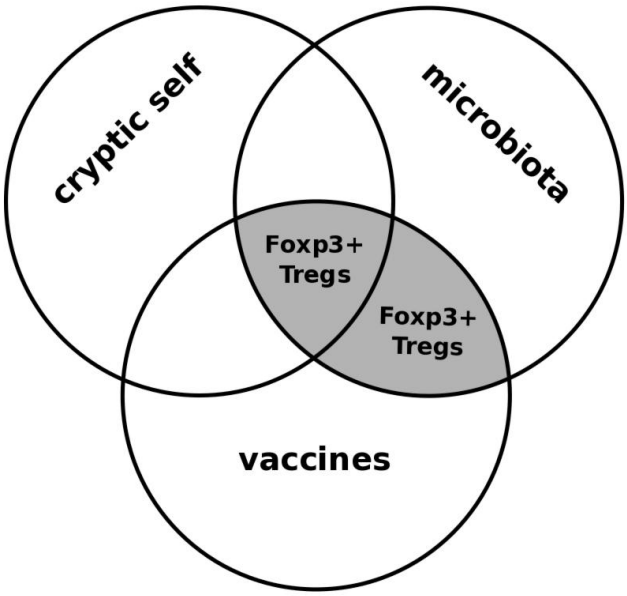


Figure 4. Map of relevant antigenic overlap between vaccines and microbiota that determines antigen-specificity of thymic Foxp3+ Tregs that can drive effective immune response to pathogens following vaccination, as predicted by the SPIRAL model.



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