Shared TCR epitope cross-reactivity could permit dyads of Foxp3+ regulatory and IL-2-producing T cell precursors to escape thymic purge

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PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27853v1 | CC BY 4.0 Open Access | rec: 11 Jul 2019, publ: 11 Jul 2019

Abstract

The thymus-derived Foxp3+ regulatory T cells (Tregs) represent a unique population of CD4+ T cells responsible for maintaining dominant tolerance to auto-antigens, beneficial microbiota and potential irritants such as allergens on the one hand and efficient but balanced defense against pathogens on the other. How Tregs with high-affinity TCRs for thymically expressed epitopes survive thymic deletion or display such broad functionality is presently unclear. We recently introduced a novel framework dubbed SPIRAL (Specific ImmunoRegulatory Algorithm) which suggests that antigen cross-reactivity of thymic Treg repertoire could provide a mechanistic basis for its broad functionality. Here we further develop this model to propose how escape of high-affinity Tregs from thymic purge could be achieved in dyads with high-affinity natural IL-2-producing T cells (IL-2p T cells) sharing TCR epitope cross-reactivity. We believe this interpretation could reconcile contradictions related to Treg ontogeny in the thymus and their role in modulating antigen-specific immune responses.

Introduction

Developing thymocytes have to carefully navigate a thorny dilemma in the thymus. Reacting with high affinity towards MHC II/epitope complexes leads to their deletion while weaker reactivity rescues them from apoptosis, allowing them to differentiate into naive T cells. However, mature Foxp3+ Tregs seem to violate this binary choice as their thymic precursors require high functional affinity interaction with specific, agonist MHC II/epitope complexes (Weissler and Caton, 2014), something that should delete them at this stage of cellular development (Lio and Hsieh, 2008). The immunological parameters influencing bifurcation between deletion and Treg fate have been long debated but remain unresolved (Lee et al., 2012).

Two competing or rather complementary models for thymic Treg development have been proposed (Hsieh et al., 2012; Klein et al., 2019). The affinity model suggested thymic precursors expressing TCRs with neither too high nor low but rather some affinity presumed to be below an ambiguous deletional threshold for agonist MHC II/epitope complexes develop into mature Tregs (Stadinski et al., 2019). However, Treg selection based solely on TCR affinity below such a deletional threshold lacks experimental support (Cozzo Picca et al., 2011; Wirnsberger et al., 2011). The avidity model attempts to rescue affinity model by suggesting that in addition to TCR affinity, interaction with low to intermediate density of agonist MHC II/epitope complexes in the thymus enable maturation of thymic precursors into Tregs (Legoux et al., 2015). Yet how Treg precursors survive such single or serial high-affinity interaction with cognate MHC II/epitope complexes to become Treg without getting deleted remains unclear (Li and Rudensky, 2016).

Foxp3+ Treg development also critically depends on signaling through IL-2Rα/IL-2Rγc chains, mainly via IL-2 (Chinen et al., 2016). Though not yet definitively identified, conventional T cells in both thymus and periphery (Liu et al., 2015; Owen et al., 2018) are thought to be the natural source of this IL-2 to Tregs though nature of its delivery also still remains a mystery. Such IL-2-producing T cells (IL-2p T cells) must presumably express high-affinity TCRs to engage MHC II/epitopes with sufficient strength to transcribe and secrete IL-2. But if IL-2p T cell precursors express high-affinity TCRs as do Treg precursors, how does either subset escape thymic deletion? (Lee et al., 2012).

We previously introduced a novel framework dubbed SPIRAL (Specific ImmunoRegulatory Algorithm) which suggests that antigen cross-reactivity of TCRs expressed by thymic Tregs could provide a mechanistic basis for their broad functionality (Usharauli and Kamala, 2018).

SPIRAL predicts that generation of thymus-derived epitope-specific Tregs and their maintenance in the periphery requires their persistent engagement with cross-reactive MHC class II/epitope complexes presented sequentially, first in the thymus to select them and later derived from endogenous microbiota in the periphery to maintain them (Delpoux et al., 2012). SPIRAL postulates that thymic Treg specificities comprising such unique cross-reactive epitopes were evolutionarily selected to prevent ineffective immune responses to self and certain nonself antigens such as microbial antigens or allergens. We further develop SPIRAL here to posit that escape of high-affinity Tregs from thymic deletion requires tandem survival with high-affinity natural IL-2p T cells with whom they share epitope cross-reactivity (Figure 1). As a

corollary, we predict such thymus-derived IL-2p T cells are precisely those that drive pathological, polarizing T helper responses to the same evolutionarily selected specific cross-reactive epitopes in absence of partner Tregs.

Dyads of Treg and IL-2p T cell precursors allow their escape from thymic deletion

Up till now research on the thymic microenvironment that guides the bifurcation choice between deletion and Treg formation has been viewed from the perspective of an individual cell's fate. This approach has however failed to explain how Tregs escape thymic deletion. We thought the bifurcation event that determines deletion or Treg (or IL-2p T cell) fate (Klein et al., 2019) could instead be readily resolved if envisaged as not a single cell but rather as a two-cell phenomenon orchestrated through epitope cross-reactivity.

Since SPIRAL mandates antigen-specific inhibition of ineffective T cell responses by Tregs (Usharauli and Kamala, 2018; Pohar et al., 2018; Akkaya et al., 2019), their sensing of IL-2 as a proxy for such ineffective T cell responses must be antigen-specific as well (Setoguchi et al., 2005; Almeida et al., 2006; Amado et al., 2013; Liu et al., 2015). In other words, Tregs cannot just sense IL-2 produced by random T cells but instead must share antigen-specificity in the form of cross-reactivity with such IL-2 producing T cells (Wolf et al., 2016). Applying this principle to the thymus suggests that IL-2p T cell and Treg precursors whose progeny recognize

similar epitopes in the periphery via shared epitope cross-reactivity must be able to do likewise in the thymus as well.

Here is how such a process could work. In a stochastic process, close proximity of thymic precursors sharing high-affinity TCR cross-reactivity at MHC II/epitope level would enable them to form temporary dyads wherein one behaved as a Treg precursor (IL-2 recipient) and another as a IL-2p T cell precursor (IL-2 donor) (Kitagawa et al., 2017). Such spatiotemporal pairing could modify their TCR signaling so as to prevent high-affinity deletion and permit their escape 'in tandem'. Such stochastic pairing process would strictly depend on such high-affinity TCRs sharing epitope cross-reactivity as well as on MHC II/epitope density.

Foxp3+ Treg development in the thymus through stochastic dyad formation provides a novel interpretation of experimental data. First, not every TCR would be able to form a pair through shared epitope cross-reactivity (Ohkura et al., 2012). Second, density of cross-reactive MHC II/epitope complexes would greatly influence such pair formation as well. Too few epitopes and potential partners would miss forming surviving dyads with each other and get deleted individually. Too many epitopes and potential partners would engage cognate epitopes too soon before being able to form surviving dyads with each other and get deleted individually (prozone-like effect). Chance of potential Treg and IL-2p T cell precursors finding each other to form surviving dyads would dramatically rise only at optimal MHC II/epitope density and precursor frequency and only if they shared high-affinity TCRs cross-reactive for a similar pool of epitopes (Figure 2).

We also believe dyad formation committing thymic precursors to a Treg fate occurs prior to Foxp3+ upregulation (Kitagawa et al., 2017). This could account for the immunological phenotype of scurfy and Foxp3+ knockout mice where proto-Foxp3+ Tregs still come out but cannot function to control IL-2p T cells (Ohkura et al., 2012; Morikawa and Sakaguchi, 2014).

In summary, this novel interpretation of experimentally observed data links cross-reactivity to saturable TCR-dependent niche to explain thymic Treg formation. Doing so could also explain the experimentally observed inverse correlation between antigen density, precursor frequency and Treg formation in the thymus (Bautista et al., 2009). It solves the 'bifurcation' dilemma by postulating dyad formation between Tregs and IL-2p T cells sharing TCRs specific for cross-reactive epitopes as a determining factor that rescues Treg precursors from thymic purge.

IL-2p T cells are also the source of pathogenic T helper cells

We previously proposed that peripheral maintenance of thymic Foxp3+ Tregs requires the presence of specific microbiota species that supply epitopes cross-reactive to those the thymus presents to select such Tregs in the first place (Pacholczyk et al., 2007; Malchow et al., 2013; Al Nabhani et al., 2019). We propose that disruption of this antigen-specific relationship between microbiota and thymic Tregs rather than lack of 'innate' training is the natural basis for specific allergies, autoimmune diseases and other inflammatory disorders under the 'Hygiene hypothesis' (Usharauli and Kamala, 2018).

We now expand this model to propose that as in the thymus, upon seeding the periphery, Treg and IL-2p T cell dyads should coexist in the periphery in equilibrium (Almeida et al., 2006; O'Gorman et al., 2009), both recognizing microbiota-derived cross-reactive epitopes, a process that enables steady-state delivery of IL-2 to Tregs in an epitope-specific manner as long as specific microbiota species supplying them cross-reactive epitopes are available.

What would happen to the immune system when such specific microbiota species are lost due to nutritional or ecological changes? First, Tregs specifically maintained by those microbiota would stop functioning properly as their TCR signaling would be disrupted (Xu et al., 2018). In turn, their partner IL-2p T cells would no longer be controlled though they would not be pathogenic at this stage since their epitopes would be lost as well. However, if an immune system with such Treg 'holes' later encountered environmental antigens such as a pathogen or allergen or a pathobiont that expanded during dysbiosis that happened to express epitope(s) recognized by such 'orphaned' IL-2p T cells due to cross-reactivity then they would become activated and pathogenic. Commonly observed TCR or antigen cross-reactivity could thus lead to pathology but only if corresponding epitope-specific Tregs become dysfunctional.

In other words, such thymus-derived IL-2p T cells are precisely those that could drive pathological, polarizing immune responses to antigens but only in the absence of partner Tregs with whom they share TCR cross-reactivity.

Unlike conventional naive T cells, we think such IL-2p T cells could be epigenetically poised through thymic imprinting for rapid pathological polarization in

159 'orphaned' state when recognizing cognate epitopes in absence of 'cross-reactive'
160 partner Tregs (Cosmi et al., 2008; Marks et al., 2009; Mazzoni et al., 2015; Bacher et
161 al., 2019). Existence of CD4+ T cell subset with rare phenotype common to multiple
162 autoimmune disorders has recently been described (Christophersen et al., 2019).

163 If conventional naive T cells were natural IL-2 producing T cells in a steady-
164 state condition then these naive T cells should express high-affinity TCRs recognizing
165 similar epitopes as Tregs in order to deliver IL-2 to them in an epitope-specific
166 manner. If so, the same naive T cells would bind similar, cross-reactive epitopes in
167 the thymus as well and couldn't wholly escape deletion (Lee et al., 2012) unless they
168 formed surviving dyads with Tregs and thus acting as thymus-derived IL-2p T cells. It
169 is thus likely that ineffective, polarizing T helper cells and thymic IL-2p T cells are
170 actually one and the same population.

171 It is noteworthy to highlight here that a process of random dyad formation in
172 the thymus between Treg and IL-2p T cell precursors described above could explain
173 another puzzle related to tolerance. If Treg precursors share TCR cross-reactivity in
174 the thymus with high-affinity polarizing T cell precursors that are driving pathology,
175 why not simply delete all of them in the thymus and be done with it? We believe TCR
176 cross reactivity sets up an evolutionary bottleneck that prevents total 'clean' deletion
177 of every undesirable TCR specificity and thereby itself serves as the reason for Treg
178 formation in the first place. In other words, dyads between Treg and IL-2p T cell
179 precursors in the thymus represents the basis for both antigen-specific tolerance as
180 well as pathology.

181

Pathological T helper polarization is initiated at the TCR/epitope level

Mechanisms responsible for T helper phenotype differentiation have been long debated. Combination of various innate signaling molecules and pathways are widely accepted to primarily contribute to T helper phenotype selection. Even though TCR/epitope interaction is necessary for T helper phenotype differentiation, requirement for such types of other signal(s) became logically mandated by the widely accepted premise that TCR/epitope affinity alone as signal 1 could not possibly provide specific signaling to initiate various T helper phenotypes such Th_1, Th_2, Th_{17}, T_{FH}, Th_{22}, etc (van Panhuys et al., 2014; Becattini et al., 2015; Trautwein-Weidner et al., 2015). T cell epitopes which are merely short amino acid sequences displayed on MHC molecules were thought to simply provide activating TCR signaling in cognate T cells to render them receptive to polarization-initiating inflammatory cytokine milieu (Yang et al., 2014; Sallusto, 2016; Sallusto et al., 2018).

In general, any T cell response to self epitopes is ineffective (non-productive) by default because the immune system cannot eliminate them without incurring evolutionary fitness cost. On the other hand, T cell response to nonself epitopes could be effective or ineffective. Effective ones would not be directly subject to evolutionary selection pressure. However, ineffective ones must be stopped as they would be cross-inhibitory to other types of T helpers by default. Were this not so, there wouldn't even be ineffective T cell responses to nonself epitopes since at least one of out of many heterogeneous T helper cell responses (Th_1, Th_2, Th_{17}, Th_{22}, Th_9, etc) would be effective. Moreover, both ineffective T helper responses to nonself and their control by thymic Tregs must be epitope-specific (Usharauli and Kamala, 2018;

DiToro et al., 2018; Kotov et al., 2019; Akkaya et al., 2019). In contrast, ineffective, polarizing T helpers must cross-inhibit other types of T helper differentiation in an epitope non-specific manner (otherwise, again, there would not be an ineffective T helper response to begin with).

We previously proposed that both self-nonself discrimination and T helper phenotype selection are one and the same process controlled by epitope-specific thymic Tregs (Usharauli and Kamala, 2018). We initially considered that specific innate signaling intrinsic to some nonself antigens in conjunction with specific TCR signaling intrinsic to epitope-specific T cells could produce such ineffective T helper polarization.

However, as discussed above, a tendency for pathological T helper polarization could be thymically imprinted for a given T cell based on its TCR affinity, specificity and cross-reactivity to epitopes shared with Tregs (Tubo et al., 2013; Persaud et al., 2014; Tubo and Jenkins, 2014). Innate signaling would then serve to merely amplify rather than initiate such pathway in 'orphaned' IL-2p T cells.

Why would such 'orphaned' IL-2p T cells be pathogenic? Again, this stems from cross-reactivity. The SPIRAL framework predicts that thymic Treg TCR epitope specificities have been evolutionarily selected to prevent ineffective (non-productive) T cell responses to certain antigens. Such antigens or rather epitopes are certain self epitopes and environmental nonself epitopes that share one common feature: capacity to engage ineffective T cell responses that dominantly cross-inhibit other effective T helper responses. Such ineffective T cell responses must be stopped but in an epitope-specific manner. SPIRAL postulates that thymic Tregs evolved to do just

this, most likely by a combination of sequestering specific MHC II/epitopes and locally made IL-2 (Akkaya et al., 2019).

Though innate signaling is currently assumed to drive pathological T helper polarization, such a system is unselectable at the Treg level. This is because innate signaling cannot initiate T helper polarization against one epitope of an antigen in isolation but has to do so for every one of its associated epitopes. However such 'total' polarization becomes non-selectable at the T cell level since effective T cell response requires Tregs to silence ineffective ones directed to some, not all, epitopes. 'Total' polarization would render all such T cell responses ineffective with all of them needing to be shut down. This would not benefit the host, which would be evolutionarily out-selected.

On the other hand, innate signaling that initiated T helper polarization to some but not all of an antigen's epitopes by physical association wouldn't be selectable at the pathogen level. Such 'partial' polarization would allow other epitopes not associated with polarization signaling to generate heterogeneous T cell response sufficient to clear the pathogen. This is because a default T helper response to any epitope is heterogeneous and balanced.

An evolutionarily selectable polarization should thus be 'partial' at the innate (PAMP/DAMP) level and 'total' at the adaptive (T cell) level. This is only possible if some epitopes drive an ineffective T cell response that can cross-inhibit other T cells specific to other epitopes (of pathogen, allergen) and thus behave as a 'total' polarization. Innate signaling simply piggybacks on already existing special category of TCR/epitope interactions that happen to drive such responses. These considerations

logically imply that such determination should instead be at TCR/MHC II/epitope level. Indeed, such epitopes and T cells (i.e. IL-2p T cells) involved in this 'pseudo-total' polarization are precisely the primary targets of Tregs (Han et al., 2014).

What is special about TCR/epitopes that drive 'pseudo-total' polarization? Such T cell specificities are not conventional, naive T cells. A mechanism that determines dominant, cross-inhibitory behavior must be T cell intrinsic. We think these T cells are thymus-derived 'memory-like' T cells poised for pathogenic polarization, exactly the same T cell population normally providing IL-2 to Tregs in steady-state via shared TCR cross-reactivity as discussed earlier. Note that such pathogenic polarization is strictly a feature of antigen-specific immune dysregulation following Treg repertoire hole that is unrelated to the normal process of heterogeneous T helper phenotype development (Th_1, Th_2, Th_{17}, Th_{22}, Th_9, etc) (Figure 3).

In summary, we outline here a novel interpretation related to generation of thymus-derived Tregs and epitope-specific mechanism that drive pathological IL-2p T cell responses to self or environmental antigens. We reasoned that high-affinity Treg precursors survive thymus negative selection in dyads with IL-2p T cell precursors that express cross-reactive TCR specificities for shared epitopes. We also hypothesize how pairing frequency that would determine total number of thymically generated Tregs would depend on MHC II/epitope density. In the periphery, these IL-2p T cells supply IL-2 to Tregs in steady-state and can in turn initiate pathological T cell polarization to antigens in 'orphaned' state in the absence of partner Tregs. It implies that Tregs and IL-2p T cells recognize epitopes derived from the same cross-reactive pool to initiate tolerance or pathological T cell responses, respectively. Thus, seemingly unrelated

mechanisms such as Treg fate determination, origin of IL-2p T cells, ontogeny of polarized T helper responses are all based on this one principle of TCR cross-reactivity, a *lingua franca* of the adaptive immune system.

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

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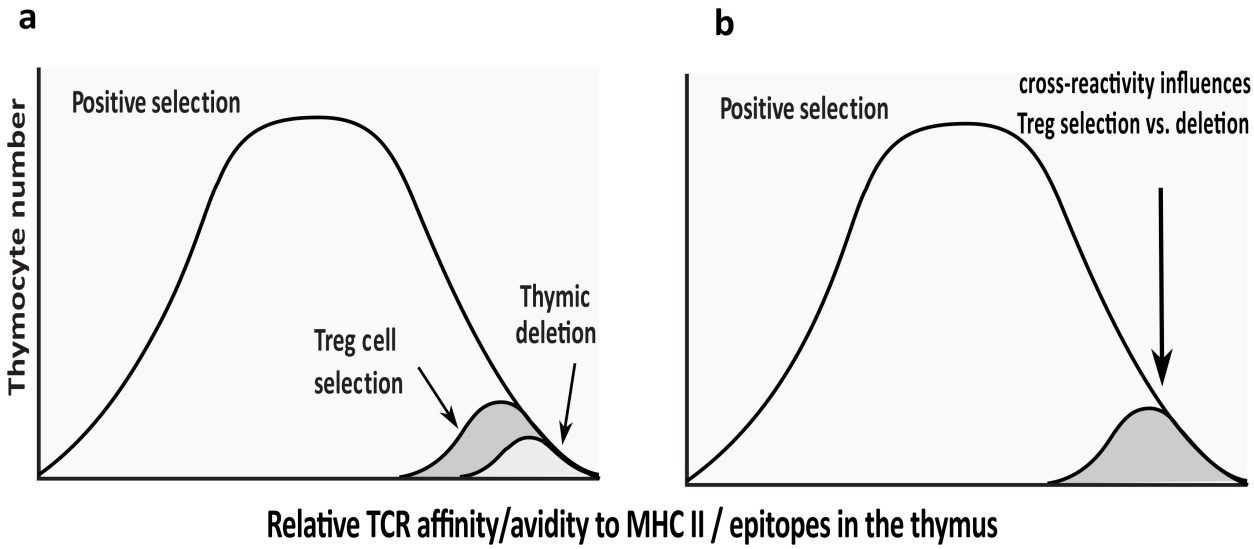

Figure 1. (a) current view for bifurcation between high-affinity thymocyte deletion versus Treg selection wherein TCR affinity below a certain ambiguous deletional threshold is supposed to facilitate thymic Foxp3+ Treg formation. (b) SPIRAL model wherein Treg precursors are rescued 'in tandem' with IL-2p T cell precursors through shared TCR epitope cross-reactivity.

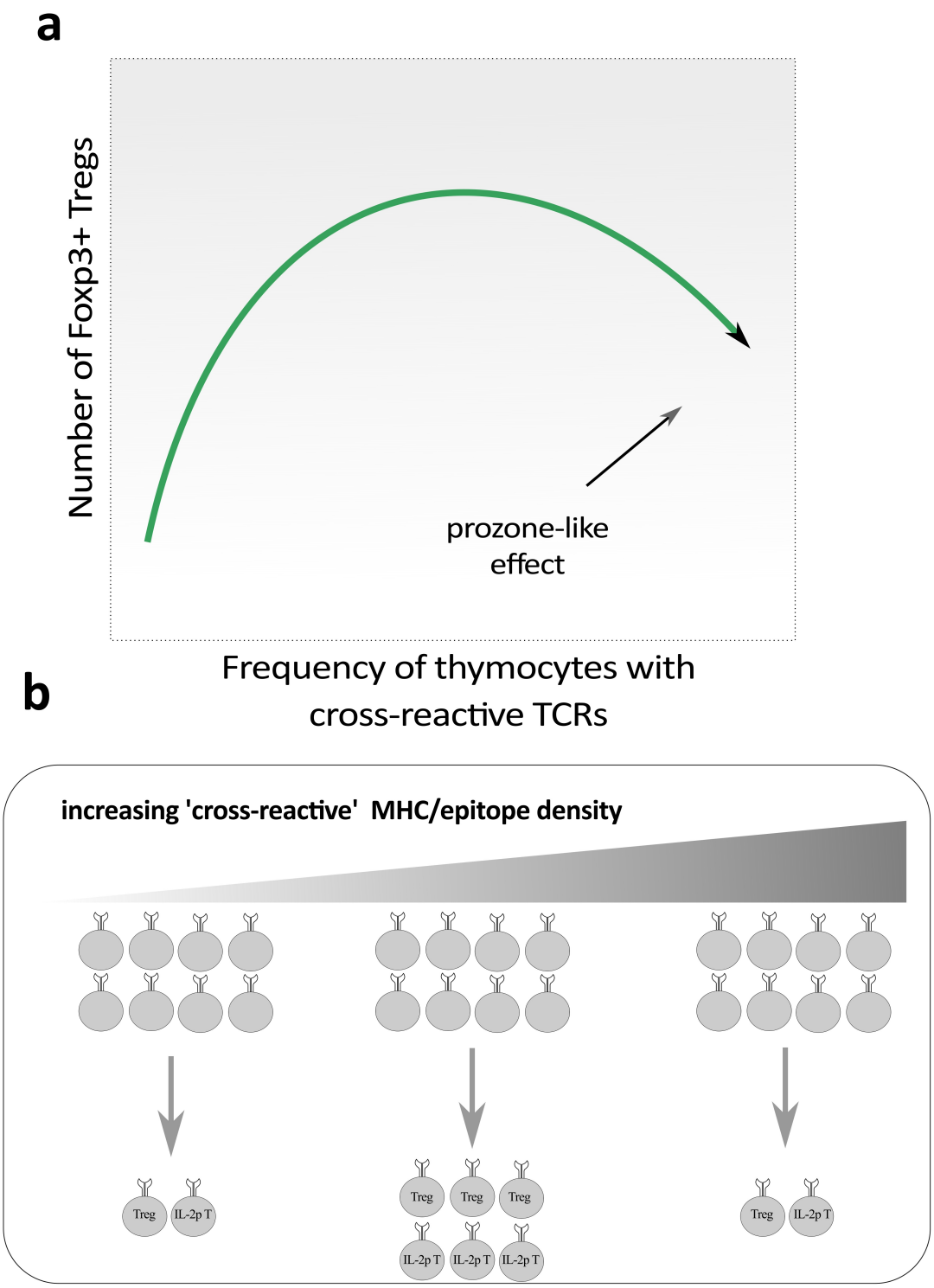

Figure 2. Both (a) the frequency of thymocytes recognizing cross-reactive MHC II / epitope complexes and (b) the density of such cross-reactive MHC II / epitope complexes would greatly influence dyad formation between Treg and IL-2p T cell precursors.

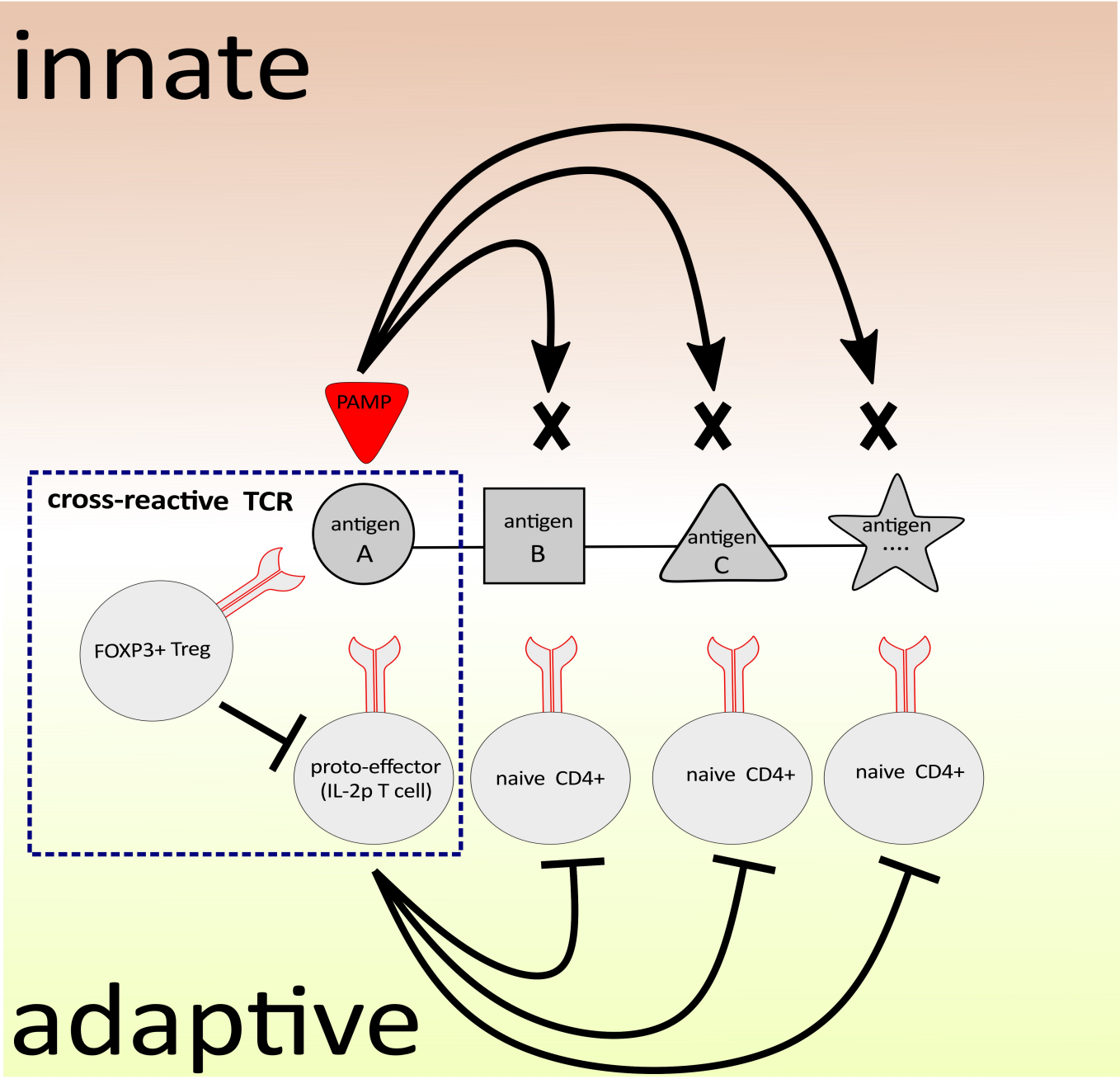

Figure 3. Top, current view that holds innate PAMP ligands control pathogen-tailored T cell responses. However, innate signaling cannot initiate T helper polarization against one epitope of a pathogen-derived antigen in isolation but has to do so for every one of its associated epitopes rendering such a process unselectable from the evolutionary point of view. Bottom, SPIRAL model wherein Tregs normally block immunodominant, pathological IL-2p T cell responses against evolutionarily selected cross-reactive epitopes in an antigen-specific manner.