Regulatory T Cell Generation During Lifetime, a Central Tolerance Difference and Later Organism Homeostasis Repercussion

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Abstract

Regulatory T cells (Tregs) permit an individual to host a large T cell repertoire while avoiding potentially pathologic autoreactivity. The population of Tregs generated in perinatal mice differs from that produced in adults, transcriptome and TCR repertoire. The function of these early Tregs remains quite enigmatic. We wondered whether thymic Treg progenitors differ in perinatal and adult mice. A time-course revealed that the ratio of the two T regulatory T cell progenitors (Foxp3+CD25- to Foxp3-CD25+) changed considerably during early life. In addition, thymic medullary epithelial cells from perinatal and adult mice showed differences in the antigen presentation machinery, as well as in their repertoires of cytokines and costimulatory molecules, according to RNA-seq analysis. Interestingly, depletion experiments using Foxp3-DTR mice revealed differential recovery of the Treg populations in different parenchymal tissues, suggesting that adult mice might not be able to generate these populations.

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"Research is seeing what everybody has seen and thinking what nobody has thought"

Albert Szent-Györgyi

Chapter one: Introduction and contextualization.

The classic definition of tolerance is the loss of reactivity towards self-antigen while retaining the immunological competence to still react against other types of antigens (foreign antigens). Presently, that definition needs to be modified with the incorporation of two new elements that have been shown to play a crucial role in maintaining homeostasis and immune system fitness. Those new elements are the commensal microbiome (including parasites, bacteria, and viruses) (1,2) and the inhaled or ingested antigens (3,4).

Going back to the main concept of immunological tolerance, it is impressive that even though it was defined more than 60 years ago, still on our days many lacunae remain in the field in terms of understanding of how the immune cells in the body maintain the equilibrium between not reacting against self-tissues but mounting an extremely efficient and strong response against pathogenic challenges. When one of these mechanisms of tolerance staggers, an autoimmunity phenomenon appears; when a mechanism fails or several start to stagger, in many cases those evolve to an autoimmune disease with sometimes irreversible tissue damage.

These two concepts, that of autoimmunity and tolerance, were coined at the end of the 19th century and the middle of the 20th century, 60 years apart. The historical evolution of these concepts arose at the end of the 19th century when the German scientist (and later Nobel Prize-recipient along with I.I. Mechnikov in 1908) Paul Ehrlich, observed that within the blood of an animal there were "magic bullets" that destroyed the erythrocytes of another animal. He performed these experiments in different species, between different individuals of the same species (iso-antibodies or allo-antibodies), and in the same individual. He found that hemolysis occurred in all cases except when using the blood of the same animal on itself. This led Ehrlich to generate the term *horror autotoxicus* as an explanation for the body not attacking itself by the formation of autotoxins (5,6):

"The organism has certain means, by which the immunity reaction is prevented from acting against the organism's own elements and thus giving rise to self-toxins ... so that it could be justified by speaking of an" autotoxicus horror "of the organism. These artifices are naturally of the highest importance for the existence of the individual. "-Paul Ehrlich. (5)

Approximately fifty years later (1953) Medawar et al. (7) proposed, before they were able to characterize the basic elements of the immune response (Major HistocompatibIlity Complex -MHC-, T cell receptor - TCR-, the stages of thymic selection or the different subtypes of T cells), that the immune system had discrimination filters to determine what is yours and what is not. In addition, these filters mature, becoming more restrictive over time. He demonstrated his hypothesis by proving that if an immature immune system, during development, was exposed to new antigens including allo-antigens, a specific immunological tolerance was induced to those that in an adult were rejected immediately.

Though Ehrlich enunciated his theory and the medical community rejected it for being too transgressive, demonstrations of autoimmunity increased with time (6). In 1957, Sir Frank McFarlane Burnet proposed his theory of clonal selection (which earned him a Nobel Prize) (8), introducing with it the possibility that autoimmune diseases could arise from a somatic mutation in the antigenic receptor of the lymphocyte (without differentiating between B or T lymphocytes) and provide the system with "forbidden" clones that by mistake were not eliminated during lymphocyte development and exerted an autolytic action. Currently the concept has evolved to that of a tolerance system, which has become much more complex, specific and dependent on the lymphocyte type to which we refer (T lymphocyte or B lymphocyte).

The human being has primary lymphoid organs, such as the thymus and bone marrow, and secondary, referring to the spleen and lymph nodes. Both lymphocyte subpopulations, B and T lymphocytes, are found in the secondary lymphoid organs (SLO). Instead the primary lymphoid

organs (PLO) are specialized for a specific subtype: the thymus harbors the maturation and selection of naive T lymphocytes and their main subtypes (cytotoxic T lymphocyte (CTL) characterized by the expression of the co-receptor CD8 and helper lymphocytes (TH) that are differentiated by CD4 co-receptor expression). The passage through the thymus of the lymphoid progenitors (Common T cell progenitor, CTP), which comes from the bone marrow, generates a repertoire capable of generating a specific effector response against the antigen that the TCR is able to recognize.

In contrast, the bone marrow is responsible for developing and partially maturing the B lymphocytes due to the fact that in the SLO there will be a hyper specification by the B cell receptor (BCR) towards a certain antigen presented in a specific environment (germinal center, GC) by specific cells (TFH), and with a unique enzyme possessed by these cells (AID).

There is a hierarchy within immunological tolerance where PLO grant a central tolerance to the outgoing repertoire and in the SLO the peripheral tolerance is generated. The peripheral tolerance is of capital importance. This is due to the fact that central tolerance is leaky and isn't able to generate a tolerant repertoire towards all the body's own antigens with 100% efficiency in order to preserve a faster generation of naïve T cells with a broader repertoire. The necessity for generating a suppressive cell with specificity toward certain antigens appears, the T regulatory cell (Treg).

At this point it can be understood that failure in any of the regulatory layers of the immune system causes the loss of tolerance towards a specific organ or, even at a systemic level. With this simplified definition of autoimmunity, we can see that there is a compendium of immuno-mediated pathologies of a very heterogeneous nature (9), both between different diseases and within the same disease. This suggests that there may be certain common mechanisms that develop autoimmunity and are common in all, but depending in which tissue it occurs could create more characteristic pathologies that, depending on where the main autoimmune response develops, receive different denominations. Therefore, autoimmune diseases are categorized as complex pathophysiological

diseases that result from a combination of environmental factors, age, sex, microbiota (10), and genetic predisposition (for example PTPN22 or HLA). To try to answer why autoimmunity occurs and how we can reverse it we need first to understand how tolerance and the immunoregulation mechanisms are generated, established and maintained. In my case we focus on the connection between central and peripheral tolerance: Tregs. By connection I mean they are purposefully generated in the same place of central tolerance but develops their function in the periphery. The importance of Tregs is seen when there is a Treg defect or, in the most extreme phenotype, a Treg absence that generates autoimmunity and death, respectively. So, we can say that Tregs are the guardians of life.

1) Thymus biology: the epithelial compartment.

The thymus is a primary lymphoid organ located on the superior mediastinum. The thymus is a considerable size until we reach puberty where sexual hormones, androgens, induce its involution (11) leaving a residual organ by the age of 25 years in humans. As the thymic mass decreases so does the production of lymphocytes, although we never lose the capacity to generate new lymphocytes during life (12). Despite the relatively short life of this organ, it plays a transcendental role for generating a proper immune system. It attracts progenitor lymphoid cells and provides an ideal environment for their proliferation and differentiation through a series of stages into T naive lymphocytes, which will be able after a licensing process to execute an effector response if they are required. Although only 5% of the thymocytes are able to leave the thymus to mature in the secondary lymphoid organs, that is enough to generate a vast and diverse repertoire able to keep us alive. If you are curious to see how they affect infections, age, hormones or the different factors that influence their function as an organ, I recommend going to ref. 11.

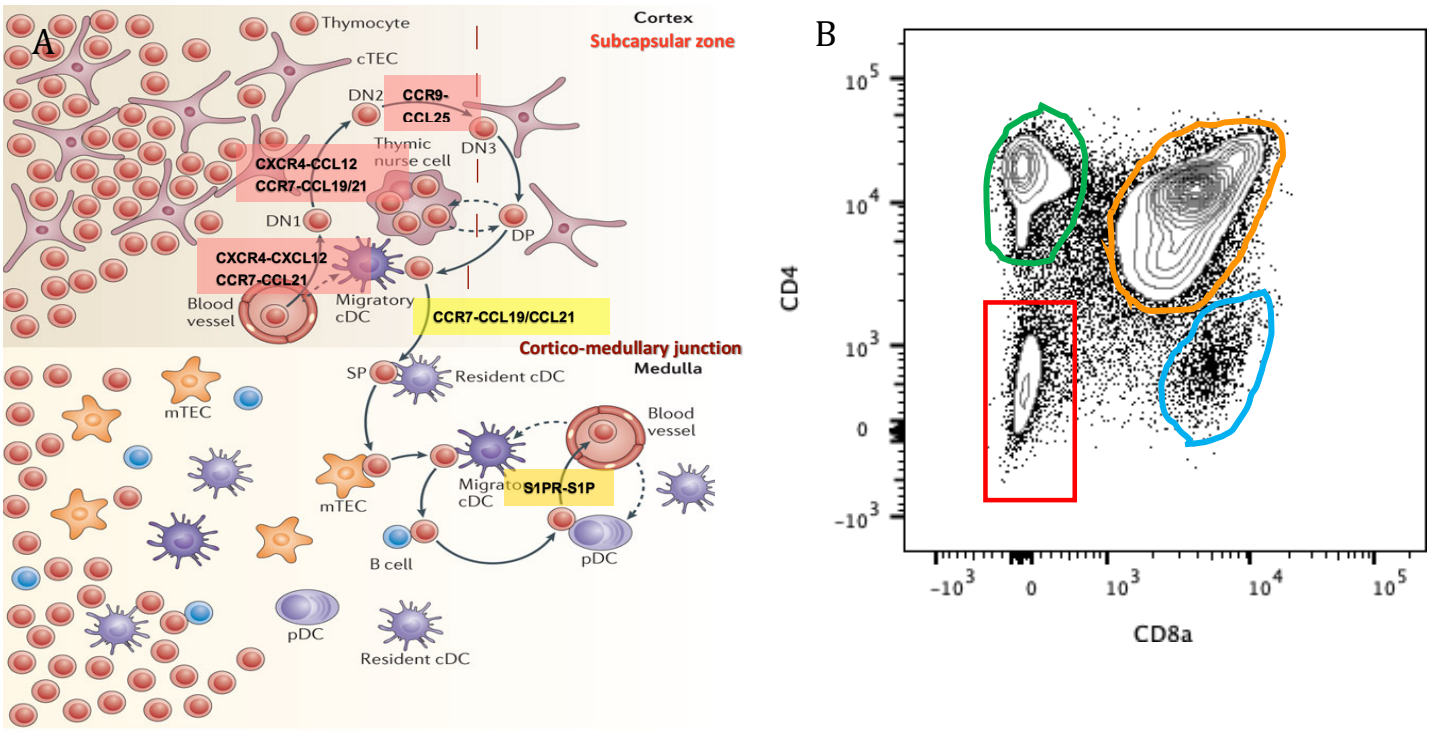

Figure 1. **Migratory steps of the lymphocyte progenitors in the thymus and the main chemokine clues**. **(A)** Image adapted from Klein L., et al Nat Rev Immunol 2014; (14) 377-391. It shows the steps that a thymocyte needs to pass to reach the SLO. In boxes are highlighted the main chemokines responsible of the guidance of the thymocytes around the thymic architecture.**(B)** Representative plot of the thymocytes population in a 3-week-old male mouse. Red square gates the double negative population (which can be subdivided into four stages by CD44 and CD25 expression markers), orange square represents the double positive, blue circle the CD8+ CD4- single positive thymocytes, and the green circle the single positive CD4 thymocyte population.

When examined under a microscope, the thymus can be divided into lobes that subdivide into follicles (1-2mm). The follicles are composed of two main areas, the cortex and the medulla. Each of these areas has a specific function and play an important role in the thymocyte maturation and selection process.

The lymphocyte precursor migrates from the bone marrow to the thymus guided by a chemokine gradient generated by the cortical thymic epithelial cells (cTEC). cTEC express CXCL12 and CCL21 that bind to their receptors (CXCR4 and CCR7) on the circulating lymphoid progenitors (Figure 1). The binding of the chemokine receptor promotes the expression of PSGL1 on the progenitors that bind to P selectin, expressed exclusively on the thymic endothelium by the cytokine activation and modulation induced by cTEC. This leads to the rolling and extravasation of lymphoid progenitors and eventual entry into the thymus. Once inside the thymus the lymphoid progenitors get in contact with

Delta like Canonical notch ligand 4 (DLL4), inducing their differentiation into the T lymphocyte lineage (12). Also, the high concentration of Interleukin 7 (IL7) and stem cell factor (SCF) found at this level induce the proliferation of the progenitors and the differentiation into the first of the four double negative stages. They are called double negative because at this point they don't express either of the TCR coreceptors: CD4 or CD8. In this DN stage, which is subdivided into 4 phases, thymocytes start reorganizing their TCR beta chain, if they succeed they will start reorganizing the alfa chain. They do so induce by Dll4 and IL7 that induce Rag expression among other genes (13) . If the stochastic reorganization of both chains is successful they become αßTCR+ and co-express both CD4 and CD8. All this process is followed by a migration of the DN thymocyte to the cortex and subcapsular zone via another gradient, this time mediated by CCL25, produced by cTEC, and CCR9 expression on the DN. Once they have the TCR rearranged they start doing a random walk in the cortex, contacting the cTECs present in the area to test them for the ability to recognize a peptide MHC complex (pMHC). Depending on what type of pMHC (class I or II) they recognize they will lose the opposite coreceptor and, via induction of a specific transcription factor (Runx3 for the SP CD8+ lineage and ThPok for the SP CD4+ lineage (14,17)), become single positive cells (SP). Also, here there is a game of affinities as a high affinity binding will lead to the death of that thymocyte and a total inability to bind the TCR won't promote its survival signaling and the thymocyte will die by neglect. For more details on the development of CD4 and CD8 in the thymus please refer to (14). This process is known as positive selection and is mediated by cTECs. Nurse cells, a subtype of cTECs, have an interesting function in helping to rearrange the alpha chain of certain thymocytes (15).

If the thymocyte survives positive selection, it starts expressing CCR7. Medullary epithelial cells produce CCL19 and generate a gradient that leads to migration of the SP thymocytes to the medulla to continue their maturation (16,17,18). All this is fine-tuned by the expression of the decoy receptor for CCL19, CCL21 and CCL21, CCRL1 who makes a gradient of this chemokines to avoid overlapping patterns and guidance throughout the thymus structure(19). There is also a negative

feedback on the maturation of DP thymocytes, which is mediated by TGBß produced by cTEC. This will lead to the arrest on the immediate stage before DP, the $CD4^{-}CD8^{lo}$ (20). At the same time, an increase in the levels of TGBß will decrease the tissue restricted antigens (TRA) expression from mTEC and speed up the SP maturation in the thymus, helping them to leave the thymus earlier (21).

1.1 Positive selection: cortical thymic epithelial cells (cTEC)

The cTEC has unique mechanisms for the presentation of antigens either for MHC I or for MHCII. This leads to the generation of a unique peptide repertoire (12). This unique machinery they possess has the objective of generating the largest possible TCR repertoire that is able to recognize the pMHC. The major responsible for the majority of cTEC functions is the transcription factor Foxn1 (22). It promotes the expression of the chemokines that attract and guide the thymic precursors and DN thymocytes in the thymus (CCL25, CXCL12), also of the lymphoid induction in those progenitors (Dll4), of the peptide presentation pathway (Tap, etc) and the thymic selection (tß5-Cd8 thymocytes and CD83 for CD). So, in summary, Foxn1 is responsible for the biology of the cTEC and also the maintenance of the epithelial thymic progenitors throughout the induction of TP63 (22). In reference to the antigen processing for MHC class I (MHCI), cTECs use a unique proteosomic unit, tß5, that generates a different proteasome, the thymo-proteasome. There are 3 types of proteasomes: the constitutive proteasome (cß5**)**, expressed by all cells, the immuno-proteosome (iß5), exclusive for APCs, and the thymoproteosome (tß5), only expressed by cTECs. The difference between the three types is the ß5 subunit at the catalytic site. The particularity of tß5 is the preference for different a cutting residue. cß5 and iß5 cut the amino acid after a hydrophobic residue, as MHCI molecules have a preference towards small peptides with a hydrophobic residue at the C terminal end. tß5 does that really inefficiently, generating a pool of peptides that are inefficient at binding MHC I. This leads to a faster TCR-off rate between MHCI-TCR, as prolonged contacts will increase intracellular calcium levels in the thymocyte, stop their movement and their apoptosis (17). At this stage what matters is

if the TCR is able to recognize the MHC molecule rather than the peptide (that will take place in the medulla during the negative selection by mTECs), so shorter, lower intensity, and faster contacts leading to small increases of intracellular calcium levels and brief stops in their movement give the intrinsic signals to be selected to survive and keep progressing in their maturation. The intensity of the bindings is reflected in the level of CD5 and NR4A1 (12) as a fingerprint of the thymocyte. If they are able to go through the negative selection this lifelong fingerprint will imprint also some of their abilities and dynamics of the response (12,23)

The importance of CD8 biology is apparent in the study Nitta et al (24). The KO of tß5 causes a massive loss in the CD8 compartment, without affecting the CD4 numbers, and even the substitution for the cß5 or Iß5 are able to recover the decrease in numbers (12). This had a huge repercussion for the mice as a normal challenge with a virus caused the death in the KO group. So, the thymoproteosome regulates the generation of a diverse CD8 T cell repertoire more than generating a tolerant repertoire.

In terms of peptide presentation on MHCII, cTEC are provided with two key mechanisms: a substantially high basal level of autophagy (all cTEC have greater than 5 autophagosomes, with the mature cells containing more than 35, compared with mTECs where only 10% of cells at the mature stage ($CD80^{hi}$ $MHCII^{hi}$) have them) and a unique set of proteases (Cathepsin L (instead of Cathepsin S that is expressed by APC and mTEC) and thymus specific serine protease, TSSP). The impact of each of these is revealed when working with their KO models, but we can say that autophagy shapes the CD4 repertoire and the proteases control the generation of peptides leading to the actual TCRs selection (25,26). In the Atg5 KO mouse (27,28) the authors of the study observed a decay of the positive selection of certain clones of CD4, without affecting CD8, albeit the peripheral repertoire of T naïve and Treg was still polyclonal. But this lack of autophagy generated a qualitative change on the pMHCII repertoire leading to gross autoimmunity and the death of the mice around 8-16 weeks

due to colitis, adenopathy, and multi organic inflammation. This showed that autophagy is involved in the induction of tolerance in the CD4 population. Instead the proteases diversify the CD4 TCR repertoire as shown by the KO of Cathepsin L where the CD4 SP repertoire is reduced by 60 to 80% in the thymus; whereas the TSP KO results in the loss of 15% of the TCR repertoire. In reference with TSSP, in a study (29) they show that it controls the repertoire and function of the CD4 in inflammation. They observed that TSSP KO the mice have an increased incidence of tumors, with higher expression of proinflammatory Th17 cytokines (Il17 and Il4), and reduce the number of Tregs and Il10 expression.

In general, cTECs aim is to generate the most variable repertoire of TCR that can recognize an MHC so in the negative selection there is a lower chance to skew or undersize the lymphocyte repertoire, and they do so by diversifying the peptides that they present on the pMHC.

1.2 Negative selection: medullary thymic epithelial cells (mTEC) & immune antigen presenting cells.

The single positive thymocytes arrive in the medulla guided by CCL19 gradient produced by the mTECs. In the medulla thymocytes who recognize with high affinity the pMHC will be induced to apoptosis and resident macrophages will clear them. There are 3 types of negative selection based on the antigens:

I. Self-antigens with ubiquitous expression throughout the body (30).
II. Self-antigens from blood and peripheral tissues brought to the thymus by different populations of dendritic cells (30).
III. Tissue restricted antigens (TRA), which are classical antigens from specific tissues that are ectopically expressed in mTECs thanks to the role of Aire (31).

For each of the above-mentioned categories, we have a pool of different cells that are in charge of mediating the negative selection process. It is important to mention, that although we have described a clear-cut division in terms of roles in the thymus where cortex is positive selection, medulla negative selection; we have done this in order to simplify the process. However, i*n vivo* this difference is more diffuse as negative selection towards ubiquitous antigens starts in the cortex as KO of CCR7 (that induces migration of DCs to the thymus and the DPs toward the medulla) or its ligands leads to autoimmunity against group 2 and 3 but not towards group 1.

The level of expression of a self-antigen and its distribution affects CD4 negative selection (32). It was shown that:

1. If the peptide is distributed homogeneously by the thymic DCs and/or mTECs, this will generate an intrathymic depletion of any clone that recognizes this peptide, eliminating its appearance at the peripheral level.

2. Proteins (both cytosolic and nuclear) expressed only outside the thymus are ignored by the immune system.

3. Peptides with limited expression in the thymus induce a partial elimination of those clones and alteration of effector functions (high CD5 that counteracts, shorter life, higher requirement of survival signals) while also inducing the formation of specific Tregs.

So as the DP/SP thymocytes start descending they face a first wave of negative selection on the corticomedullary region by dendritic cells (33), and a second wave of negative selection carried out by B cells, pDC, cDC, and mTECs(12,17).

- Dendritic cells (12,34,48): Represent 0.5% of the cellularity in the thymus and stablish their niche and localization through the expression of Jag 1 in the outer medulla (35). They include migratory subset, which constitute 2/3 of the major DC subsets at steady state (36): pDC (1/3

of the thymic DC) and conventional DC (2/3). They migrate to the thymus via CCR7 (37), CCR2 (38,126), and CCR9 (39) only in homeostatic conditions. If an infection occurs and they get activated through TLRs, the migration of pDC and Sirpα+ DC is blocked, preventing the import of bacterial antigen to the thymus and the development of tolerance towards them (12). There are 2 populations of conventional DC (cDC). One is the CD8a+ Sirpα- DC. Their progenitor arrives to the thymus via CCR7, and it is in the thymus where they differentiate. CD8a+DC tend to localize near mTECs in the medulla due to a gradient that mTECs generate by the expression of XCL1. This is important as CD8a DC have to capture TRA from mTEC in a highly efficient way and present them on their membrane. By doing this cross presentation they are able to expand the area of negative selection toward the mTEC antigens reaching a higher number of thymocytes (12,40). In addition, they do have their own antigen processing machinery, providing a bigger repertoire to discriminate which TCRs are autoreactive and which are not. The other cDC population is the migratory Sirpa+ CD8a- DC. They come from the periphery to the thymus and localize around the corticomedular junction or perivascular areas. They tolerize against blood antigens and peripheral antigens. They are able to crosspresent some antigens from mTECs but in a less efficient way than CD8a DCs (30). Finally, the pDCs, which cannot crosspresent, but do present blood borne antigens and some antigens from food or microflora (still debatable). They express IFN type 1, and this has been shown to induce a transitory stop on thymopoiesis and induce apoptosis of mature mTECs. This can be a mechanism to avoid generating tolerance toward foreign antigens that may arrive to the thymus in the case of an infection. The have been shown to be able to generate Treg (39, 41, 42).

- B cells: They represent around 0.3% of the thymic population. Their origin can be thymic or from the periphery but either way once in the thymus their phenotype changes. This is due to the contact with DP thymocytes and interaction via CD40-CD40L. This induces the

maturation and increased expression of CD80, MHCII, and expression of AIRE (50% of thymic B cells express Aire). Once are educated by the environment in the thymus, B cells acquire an APC role inducing tolerance towards the specific B cell components (the BCR) via Aire, and the antigens that autoreactive B cells capture on their BCR. The expression of Aire by B cells is interesting; Yamano T., et al (43) showed that although the transcripts induced by Aire in B cells are not enriched (24% of transcripts are induced by Aire similar to any other random gene) they qualitatively generate different peptide repertoire that mTECs cannot express, specific for B cell tolerance.

- mTEC: mTECs come from the same progenitor of cTEC (44,45). These cells localize in the center of the medulla and as they divide and differentiate they go to the outer parts. Once they reach maturation, they will divide one or two rounds more and form small clusters of around 2-4 mTEC. On those miniclusters the expression of TRA is the same but a single TRA is expressed at the same time in a minority of clusters (1-3%) at any time point (12,46). The purpose of those miniclusters is believed to increase the local concentration of antigens in every mini spot making all lymphocytes that go through them encounter them are become and tolerized (47). Then the expression of the TRA in that cluster, which is inherited by the daughter cells, is established as a bookmark. Once they express the first cluster of genes, the minicluster of mTECs start changing or sliding through the different antigen clusters (total of around 36 genes clusters (12)) in a model called sliding model or colinear expression of TRA due to the transitory nature of the promiscuity gene expression induced by Aire, generating a mosaic model of TRA expression. This increases efficiency so that a SP thymocyte can scan most of the self-antigen repertoire in the same area of the medulla, decreasing the time or the chances of missing a cluster. If they move around they could randomly go from the same cluster to the same and never get checked for their autoreactivity at others. This helps to make the stay of the lymphocytes around 4 days of a total of 5-6 days

they spend in the thymus. It has been shown that if thymocytes prolong their stay in the medulla 2 days more, they will virtually see all possible antigens and no need for Treg and peripheral tolerance is required (52).

In terms of antigen presentation, mTEC can present and do negative selection by themselves or have their TRA cross presented by CD8a DC(which also induce negative selection), generating a mosaic of self when you add all in clusters (12).

Recent reports (49,50) have described in more detail the immune cell compartment and the possible roll of other minority populations such as ILC, NKT, or Tuft cells in the thymus.

1.3 mTEC and Aire: a unique role to shape the TCR repertoire

MTEC closely depend on thymocytes to mature and execute their functions. As thymocytes mature they start expressing ligands of the TNF super family as RANKL, LTa, LTb, TNFa, FasL, Ox40L, and CD30L; while mTECs express a big portion of those receptors and ligands (RANKL, LTRb,Fas, CD40, CD30). We will focus on the three most important for mTEC maturation: RANK-RANKL, CD40-CD40L and LTbR. All these ligand receptors signal in the mTEC via NFkB, a capital importance factor in mTEC biology. For a more in depth read please refer to reference 16 or specially, reference 44.

The thymocytes that reach the medulla contact the mTECs present there via RANK to RANKL and CD40-CD40L promoting NFkB activation and mTEC maturation. In the case of RANK-RANKL (51), which plays the main role in mTEC survival, followed in order of importance by CD40-CD40L, it's signaling and association with NIK promotes the activation of RelB and ultimately the activation of NFkB. Among NFkB target genes is Spi-B (52). Spi-B (which has a prominent role in the development of pDC and BCR signaling of the lymphocyte among other functions) in mTEC promotes the induction of maturation by inducing the expression of costimulatory molecules such as CD80 and CD86, induces the expression of certain TRA independently of AIRE, and promotes the expression of osteoprogesterin (OPG). OPG is the decoy receptor of RANKL who blocks it by inhibiting its action.

The function of OPG is, therefore, to perform a negative feedback to control the mTEC population and avoid an increase in mTEC cellularity (as it happens in its KO or SpiB - / -). This would lead to a greater number of Tregs (they have more niches to be produced) and greater peripheral tolerance to a greater number of antigenic variants but reduce the T naïve repertoire compromising the defensive response, exemplified in Spib KO mice (52). It is important to note that OPG does not have a negative role in the formation of Tregs, its function is on mTEC, finely regulating their amount to ensure an optimal balance of the immune response, but it does not affect the efficacy of mTEC to induce Treg (53). The other negative regulator of mTEC is TGFβ (21). TGFβ is expressed both by cTECs and by thymocytes that do not pass the screening of the negative selection and die (via Fas / FasL). The binding to its receptor (TGFßRII) limits the growth, differentiation and function of mTEC by blocking NFkB (it is responsible for survival and maturation) and c-MYC (responsible for proliferation). Its control over mTECs is so important that by performing a conditional KO to the mTEC lineage of its receptor (TGFßRII), the IPEX phenotype (Foxp3ko which prevents Treg development) is rescued. It recovers the phenotype because it lengthens the stay of thymocytes and greatly increases the mTEC cellularity, which leads to greater control of cells that can escape the surveillance of negative selection. The speed that prevents the SP thymocytes from remaining in the cortex is in part due to LTßR, which is expressed in TEC and DC (45). LTßR, through the binding of its ligands (LTα1ß2 or LIGHT), induces the production of RANK in the mTEC .

Regarding the impact of Aire on the polyclonality of the TCR, Aire usually affects low frequency TCRs and since the APC perform most of the elimination of CD4 SP (they eliminate 30% of the total SPs while the mTECs 10% of the population) it is not surprising that APCs capture the Aire-induced antigens in mTEC through the autophagy-dependent pathway and perform 50% of the Aire-dependent SP CD4 and Treg selection (30). Specifically, the population of APC that capture the antigens of the mTEC and select the Treg are the cDC CD8a+ as we mentioned before, by surrounding

the mTEC via XCL1-XCR1. Therefore, Aire mediates its effects through the presentation of antigens by cDC and mTEC.

If we focus a bit more on the function Aire, we observe that Aire is not a traditional transcription factor whose function is to bind DNA and induce the expression of genes. Aire performs its function by interacting with transcriptional protein complexes instead of DNA directly, and within that associated machinery it has been seen that it tends to bind inactive regions of chromatin with the H3K4 mark (54). Likewise, it has been observed that to express TRA Aire can induce double chain breaks in DNA and hence is associated with a proposed proapoptotic role (55). The binding of Aire to inactive regions of the genome is accompanied by the ability to induce the expression of transcripts that are usually expressed in low frequency, increasing it, and grouping them into small expression groups. These miniclusters are expressed at the same levels in all mTECs, but their content varies among them, which indicates that there is an initially random expression, but then an order follows (47). In addition, Aire does not have a particular preference for certain TRAs, so that the genes that are co-expressed in the clusters have no relation neither in position in the genome, nor biological function, nor mechanisms of transcriptional regulation. Although Aire may have more preference for certain regions of the chromosomes (greater number of epigenetic marks such as H3K4 or greater density of TRA) its function is not specific, since it opens a region of DNA facilitating the entry of transcription factors that induce the expression of the TRA that are in that area. We must think of the nucleus as a structure in 3D and Aire, together with its complex of associated proteins, detect an auspicious region and open it as if a speck posed on a point of a string opening and letting see only that on which it has perched (it is just that mottled pattern seen in the mTEC core when Aire is activated). This can affect several completely different and far away chromosomes and also to regions surrounding the TRA sequences. The authors of the study (47) propose that once a pattern is induced, it is maintained and transferred with the divisions of the mTEC to the daughter cells, ensuring a more efficient expression of the TRA and promoting that they express themselves at high concentrations

on the mTEC. In this way, the SP thymocytes are prevented from scanning by said microclusters due to poor expression.

Aside from this, the study of Meredith et al (47) showed that the clusters of genes induced by Aire vary between individuals. This generates small differences in the autoantigens that occur leading to small variations in the chosen TCR repertoires. This has an important advantage at the population level because as a species small variation do not have common weak points in the repertoire maintaining global health and protecting it against pathogens. The repercussions of this are felt at the individual level, given that autoimmunity can be promoted in certain individuals. In addition, this is well suited to the existing problem of restriction of presentation by MHC, where one MHC cannot present the same peptide as another MHC from a different individual, which generates a specific tolerance and autoreactivity repertoire for each individual (hence the need to have an MHC concordance very high in transplants, so that the immune system continues to recognize the same peptides and will not see them as foreign) implying that not all individuals will respond to the same autoantigens (56).

On the other hand, a group from the University of Heidelberg (57) demonstrated two things, very important in my opinion, within the biology of Aire and the promiscuous expression of genes (pGE). First of all, they demonstrated that pGE differs in many aspects from the specific tissue regulation of these TRA (different patterns of splicing, use of cryptic promoters, mixing of expression between monoallelic with biallelic and the possible differences of SNPs that may have been inherited from the mother and father) which can lead to differences in the peptide that occurs in the thymus and the one that is actually in the tissue. But this is solved by transferring TRA induced by Aire to cDC CD8 +, since its processing machinery is more similar to that in the periphery and can normalize the differences in expression. Regarding the Aire expression pattern, these researchers propose a model where, in the beginning, different TRA expression groups are formed with shared genes and others

specific to each group (maintaining a common background with small variations between them) generating mosaic groups. These groups modify the expression of their TRA as they mature the mTEC (sliding coexpression groups). In this way if each mTEC makes this model of continuity of expression of the TRA (resembling a range continuous chromatics, where each mTEC expresses a group of TRA that vary during its life, and this evolution is coordinated) would cause the thymocytes would be enough to be patrolling in a small microdomain to have recognized all the TRA. Given that each mTEC group makes this progressive antigen slide, this allows to the thymocyte to remain in a relatively small area because in the end with a small group of mTEC (you estimate there would be 36 groups) you would be presenting all TRA.

Apart from these functions related to pGE and the expression of TRA, Aire has a transcendent capital in all spheres of the mTEC because it induces the production of chemokines. These cytokines, CCL17, CCL19, CCL22 and CCL25, favor the trafficking of thymocytes to the marrow. XCL1, which by binding to its receptor XCR1 (which is only expressed in cDC CD8α +), causes this subpopulation of cDC to surround the mTEC and be more efficient in the transfer of antigens on a population of APC specially designed for it. It induces the chemokines that cause the Sirpα + DC and pDC to migrate to the thymus (CCL2 and CCL25 respectively). In addition, Aire, which is expressed once the mTEC cell has matured, may also be responsible for its apoptosis (as for the expression of TRA the mTEC alter and suffer cuts in their DNA) limiting their survival and promoting the periodic replacement of these thus preventing them from accumulating damage at the DNA level and/or losing efficiency (34), as a mTEC lifespan is around 14 days.

1.4 Self-recognition as a survival mechanism in the periphery

The human body cannot predict what it will face, which is why it selects its lymphocytes based on what they will have to recognize each day. Through a process of positive and negative selection the body ensures that the TCRs of the thymocytes that are generated are able to recognize their own

MHC. Even though the generation of the TCR is stochastic, there is still a consensus domain on the CDR 1 and CDR2 that make them recognize MHC molecule (58). These two regions of the TCR, located in the part that contacts the two alpha helices and the groove of the MHC molecule, have more or less conserved sequences that always bind to the MHC alpha 1 and 2. The part that makes contact with the peptide, and therefore has to have the greatest variability of the TCR molecule, is the CDR3 region (63), and this variability is greatly done by the addition of N additions via the enzyme TdT (130). CDR3 gives the TCR a spectrum of affinities of which the range of low or intermediate affinities include possible self-peptides. It is important that they have some self-recognition because in general all T lymphocytes are programmed to die unless they are actively given signals to survive, as demonstrated by Raff M.C. back in 1992 (59). The positive survival signal is the contact that T naive cells constantly have with different pMHC expressed by the APC of the secondary lymphoid organs (60,58). A molecular level semi-activation state is described as a partial phosphorylation of the TCR / CD3 complex (pp21), with recruiting the activating kinase, ZAP70, but not Lck (58,61), maintaining a signal tonicity that activates pathways of survival (Bcl-2 partially) but does not activate the lymphocyte.

1.5 TCR reactivity

The TCR does not recognize a single antigen, indeed there is considerable cross-reactivity of the TCR, allowing it to recognize different peptides to a different degree and affinity, this is known as T cell receptor promiscuity. This cross-reactivity is essential for two reasons: it reduces the number of lymphocytes needed to cope with any event (62) and also assures that there will be a large proportion of lymphocytes able to respond to any exogenous peptide presentable. This affinity spectrum ensures there will always be a set of lymphocytes that can recognize and mount a specific response to a pathogen, preventing it from proliferating and invading, in case its specific TCR lymphocyte is at the other end of the body (a lymphocyte takes an average a day in crossing the whole body) (62). In addition, it has been shown that self-recognition favors a greater sensitivity of these lymphocytes to

respond to an antigen, as it spatially redistributes the TCRs and kinases associated with the receptor, favoring the antigenic recognition (TCR-pMHC) of a foreign antigen expressed in low density by an APC in sufficient quantity for lymphocyte activation to develop (63). Therefore, self-recognition increases the sensitivity by spatially redistributing the TCR complexes and signaling molecules, increasing their focal density and, in addition, the rest of the TCRs, even though they do not bind to the foreign peptide (in low concentrations there is not enough to occupy all the TCRs) serve as co-agonists (64), facilitating the activation of T lymphocytes in conditions of low amount of agonist peptide (64).

So, a single TCR of a mature T cell can initiate different responses depending on the quality of the interaction with which it binds to the peptide-MHC complex (pMHC). Also, the maturational state of the lymphocyte will drive a different response from the same or similar interaction (mature versus naïve for example). An example of this is a low affinity interaction is required in the thymocyte negative selection, but in the periphery, it has an antagonistic effect avoiding the activation of the mature T lymphocytes.

Likewise, there is the concept of polyspecificity of the TCR. This refers to the ability of a TCR to recognize different peptides, even with great affinity for several of them, within the same MHC (62, 65). Add to this that the TCR must respond differently depending on whether affinity with pMHC is high or low, which is crucial to generate an effective effector response and stay alive, respectively. In addition, it is interesting to note that the recognition by the TCR of a proprietary peptide presented by MHC has the same specificity as that of another agonist but what marks the difference in response is the affinity that in the case of an antigen itself is of several orders of lower magnitude. This is achieved thanks to the fact that this autoantigen activates different signaling mechanisms, second messengers, and accessory proteins that mediate lymphocyte activation. If these signaling mechanisms are divided into proximal signaling, such as CD3e phosphorylation and signaling

molecules that are involved in the initiation of the TCR-coreceptors response, calcium-mediated signaling, and signaling via GTPase Ras-MAPK, the differentials of response between a self-antigen and a true agonist are detected already from the proximal signaling, because with higher affinity you generate conformational changes that favor a powerful signaling (65). This occurs in the same immunological synapse, since the formation of this one is already related to the interaction force TCR-pMHC. Therefore, the immunological synapse (a binding between lymphocyte and APC in the form of a target with the TCR-pMHC molecules in the center surrounded by a circle of co-stimulatory / inhibitory molecules (CD28, PD-1, etc.), and this at the same time surrounded by other molecules with adhesion) modulates TCR signaling through several mechanisms that occur at the same time:

1. The TCRs are concentrated in microclusters that are located in the center of the target, increasing the sensitivity for activation, since the dimerization of the TCR is important to overcome the minimum limit for activation.

2. There is a co-agonism between TCRs that bind to the foreign antigen and others that bind to their own peptide expressed by MHC of the APC in question. This increases the total signal strength by facilitating activation of the lymphocyte in the presence of a small amount of antigen (64).

3. The affinity of the TCR towards the presented peptide has a determining impact on the influence of the co-receptors (CD4 or CD8) recruited to the synapse. If TCR-pMHC affinity exists, the activation and transmission of signals is less dependent on the co-receptors carrying the Lck kinase with them (in the case of CD8, inverse correlation between CD8 recruitment at the synapse and TCR-pMHC affinity).

4. The arrangement of the synapse modulates TCR signaling because it attracts and concentrates proximal signaling molecules (Zap70, Lck, CD28) and excludes phosphatases. It also happens that the recruitment of all these molecules takes on more weight when the interaction is weak.

5. The immunological synapse is directly responsible, through active internalization of the TCR and its ubiquitination, to decrease and dampen the signal in case of a TCR-pMHC interaction.

In conclusion, there is a continuous adjustment of the activation limits of the T lymphocyte in the periphery, which is known as tuning co-receptor, through the TCR and cytokines. In situations of homeostasis, contact with pMHC and Il-7 in the periphery controls the total rate and composition of the naive T-lymphocyte pool. Within this pool there are different affinities for self-peptides by the TCR and different availability of these own ligands. Both variables determine the amount of signaling by the TCR and this regulates the degree of need for Il-7 to prevent them from apoptosis due to ignorance or death by neglect (66).

Finally, another advantage that the slow intensity self-recognition provide is the homogeneous distribution of the lymphocytes throughout all the secondary lymphoid organs (SLO) in the body. This occurs because, according to their higher TCR affinity self-antigen, lymphocytes will migrate to those sites where they receive more survival signals where they will be retained or spend more time. This has been already been proven with Treg (67) and is known as regional tolerance. But it could also be applied to T naïve cells, although the signals are not as strong and allow them to patrol the different lymph nodes. In conclusion, having a certain affinity of its own maintains a peripheral pool of naive T lymphocytes that survives and will be distributed homogeneously throughout the body based on the antigens it recognizes.

2 Lymphocyte development: focus on Treg.

The environment in the perinatal thymus is much more protolerogenic than the adult thymus. This is due in part by the increased concentration of glucocorticoids (GC)(68) that reduce the TCR signaling. This broadens the possible repertoire of TCR because without GC an increase of negative selection occurs. It also generates an increase in percentage and function of Tregs in the pediatric age versus adult (69).

The regulatory T lymphocytes account for 10% of the peripheral CD4 lymphocytes (which make up 60-80% of the total T cells). In spite of their small number they are the "guardians of life", as the Rudensky group, Sakaguchi group, and others showed years ago that the loss of Tregs at any moment of life generates a lethal autoimmunity. But despite its capital importance we still know little about its biology. Its main transcription factor, Foxp3, was discovered by the Sakaguchi group in Japan (70). The Rudensky laboratory described the mechanisms that induce its expression (70) and demonstrated its epigenetic regulation and stability of expression, since Conventional T-lymphocytes (Tconv) transiently elevate Foxp3 expression when activated (70).

Treg lymphocytes arise according to the affinity model of TCR (12, 71) in the high affinity spectrum, although a large number of affinities, not only high, can generate Tregs. As affinity increases the propensity to generate Tregs is greater, but the TCR repertoires expressed by naive CD4 and Tregs coincide by some degree (71).

The formation of natural or thymic Treg (nTreg) is based on a two-step process (72,73,74). The first phase is dependent on the TCR recognizing with moderate or moderate-high affinity an antigen expressed thanks to Aire (75) on an APC, and a co-stimulation by CD28. The second phase occurs when IL-2 induces the final differentiation to Treg by stabilizing its transcription factor, Foxp3. Interleukin 2 (IL-2) is only found in the medulla in limited quantities and originates from the thymocytes themselves, upon activation they secrete it in a paracrine way, and secrete it in a very localize manner by migratory DC (76).

Focusing a bit on the induction cascade (72,73,74,77, 78): All thymocytes in the DP and SP phases express Satb1, an epigenetic modulator that recruits chromatin regulators or histone tag modifiers and is required for the generation of a functional repertoire of SP CD8 and SP CD4. Sat1b also makes the CNS0 region of the Foxp3 promoter accessible. CNS0 is essential to start the expression of Foxp3 (79) and CNS3 is required later to maintain expression (80). If during these

phases the thymocytes have a TCR with moderate affinity for some antigen (a little less or equal to the limit of negative selection) or are in a niche with longer contact, cytokines available, and receive the co-stimulation by CD28, the expression of CD25 (alpha subunit of the IL2 receptor), IL2RB, and NFkB are triggered. NFkB activates cREC that binds to the CNS3 region of the Foxp3 promoter, inducing its expression. Foxp3 induction generates a proapoptotic signature (PUMA +, pBim +, pJNK +, DUSP6- and Bcl-2low) (78) that can only be over ridded if the IL-2 receptor, which it already has in its membrane, binds to IL-2 (+ / - IL15) and signals via phosphorylated STAT5 to maintain Foxp3 and activate PKB, which inhibits the Bcl-2 inhibitor, Bad. Given that IL2 is limited and the antigens capable of generating this induction are specific, the induction of Treg in the thymus is performed in very small niches, where there is a brutal intraclonal competition for IL-2 and the antigen. Those thymocytes with greater affinity for pMHCII will remain, since CD25 and Il2rβ will be expressed earlier and in greater quantity propitiating their survival. Here it is inferred that most of the lymphocytes that express Foxp3 die (the lethality of Foxp3 is prevented by the signaling of cytokines within the common gamma subunit family). In the sequence of events, we observe this two-step model which is first dependent on the binding with moderate affinity by the TCR and co-stimulation of CD28, which gives way to a phase exclusively dependent on cytokines (common gamma chain cytokines). This process has a specific and strict timing, because if the process occurs outside the window where Satb1 is expressed and opens the CNS0 region, we would never have Tregs. That is, Satb1 opens a window period for the induction of Tregs. In addition, one can explain why the CD4 lineage commonly has T regulators while CD8 do not (although there are subpopulations with regulatory capacity) because CD25 can only be expressed in thymocytes already destined to be CD4, thanks to ThPok.

Regarding the location of induction of Tregs, Cowan J.E. et al (81) showed that the thymic medulla is required for the multistage process of Treg induction but dispensable for conventional CD4 thymocyte development. This highlights the requirement of this structure for the development

of regulatory T cells (81,82, 83) and with the special relevance for the epithelial cells of Hassall's corpuscles in humans (83) and mTEC (82) and those responsible for their induction by these antigens, generating small independent niches. In ref.82 the Ludger Klein group proposes a model where mTEC are responsible for expressing and presenting the antigens to the Treg while passing their antigens to the cDCs that surround them to perform the negative selection on conventional SP CD4 +. Other groups have shown that Aire is critical for the development of Treg with different specificities (84) and Aire has a reliable impact on the polyclonal repertoire of regulatory T lymphocytes (30). TRA produced by AIRE are required for the elimination of around 6-7% of the unique TCR repertoire of Tconv, but 30% of the TCR repertoire of Treg is dependent on AIRE (34) Aire also promotes the conversion of autoreactive CD4 thymocytes into Treg (75). MTEC and DC generate Tregs with different TCR repertoires, because Aire transcripts are transferred to the DCs and through different antigen processing machinery and autophagy generate a different repertoire of self-peptides (30).

A critical population of DCs have a major role in Treg induction compared to other populations. This is the migratory Sirpα+ thymic DC (37, 42 ,85). They are more suited for this role due to their maturity (in the thymus they express more MHCII, CD86, CD80, CD40, and CD69 compared with Sirpα- DC), chemokine production (CCL17 and CCL22 facilitate their interaction with CCR4+ SP CD4), and peripheric origin; in addition of a more efficient in Ag presentation on MHC II (85).

Also, it is important to mention that DCs can produce TGF-β (86) and IL-2 in low amounts which are critical for Treg induction (76). DCs also express CD70, that when bound to its ligand cD27 on newly generated Tregs maintain their survival by blocking the mitochondrial apoptosis pathway via Bcl2 (87). A seminal paper from Perri J. S.A. et al (30) compared the repertoire that mTEC and DC induce and came to 4 interesting conclusions:

mTECs and BM APCs make non-redundant contributions to clonal deletion and Treg selection. This could suggest that each different APC has their own repertoire of self-antigens they display. Also, that DCs are the major subset of the BM derived cells that generate Tregs.

Aire expression in mTECs affect negative selection and Treg selection. Aire has an especially large impact on lower frequency TCRs.

Aire induced antigens are presented on CD8a + DCs and mTECs

CD8a DCs and Sirpa DCs play non-redundant roles in Treg selection, whereas pDC are probably less important and select lower frequency Treg clones.

Is interesting that Sirpa DCs are the ones more specialized in Treg generation (85) as Sirpa binds to CD47 in apoptotic particles, triggering phagocytosis and presentation of the particle. This could be a mechanism of how Sirpa DCs capture their peptide repertoire for inducing tolerance in the thymus. Especially interesting is this role in neonates where proliferation and apoptosis are on a nearly exponential scale and Sirpa DC are the most abundant subtype of APC in the thymus during the first days.

On the other hand, A. Rudensky (88) demonstrated that the cTEC are sufficient to generate Treg, proposing that there is no specific Treg generation location and both the cortex and medulla have an environment capable of generating Treg, although the majority of Tregs are generated on the thymic medulla. This could make the dendritic cells (also responsible for collaborating in the generation of Treg in certain models (34)) and other auxiliary cells capable of inducing Treg and each one having a different spectrum. For example, in the cortex the DCs are probably inducing Tregs against ubiquitous antigens or self-peptides produced by the immunoproteosome or blood antigens of the microbiome, while the mTEC would induce Treg specific to a particular tissue thanks to its promiscuous expression of restricted antigens due to Aire (46).

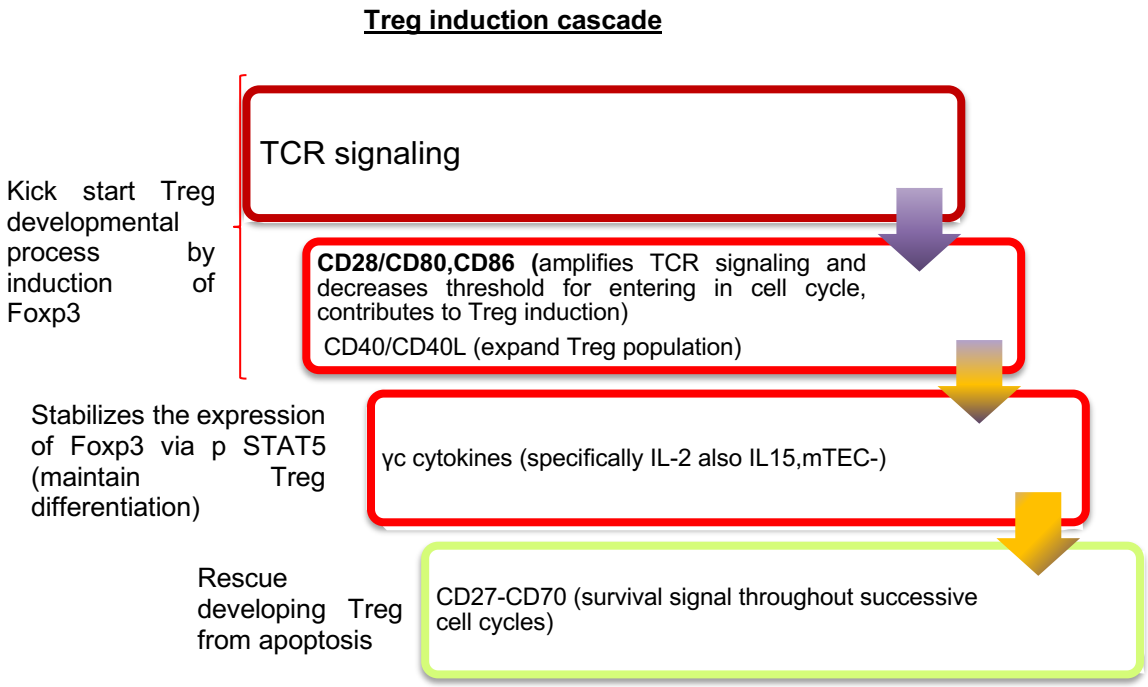

Table 1. **Thymocyte steps and checkpoints that have to succeed in order to become a Treg in the thymus.**

Up to this point I have provided an intrinsic molecular mechanism (intracellular steps and transcription factors) for the generation of Tregs. However, receptor-ligand interactions on the membrane have not been mentioned (table 1). Single positive CD4+ thymocytes must archive certain checkpoints that promotes their survival and conversion into a Treg. As mentioned before, the first event is the TCR signaling strength. We need to remember that different affinities can drive Treg generation, but they do so with different efficiencies of conversion (the highest the affinity has the greater the chance to become a Treg) (89). The group of L. Ignatowicz (90) showed that the diversity of the Treg TCR is greater than that of the non Treg CD4 (Tconv), having only around 20-30% of sequences shared. Also, in that study, they show that the clonal distribution of Tregs was similar in the thymus compared to the periphery independent of the pMHCII complexity, meaning that there is not a specific enrichment for certain epitopes or TCR. A few years later the group of Hsieh (89) showed that the TCR of Tregs have a boarder range of affinities, varying up to 1000 folds between them. Also, they established that Treg selection (via strong TCR interaction with the pMHCII) was 100-fold less than the threshold for negative selection. The same group pointed out that the higher

the affinity of the TCR, which means the higher the strength of the signal, the higher the efficiency of Treg generation, and this was correlated with a larger niche. The strength of the TCR signal directly correlated with the level of expression of CD25 on the membrane.

At the same time as the TCR -pMHC is signaling, CD28 from the SP CD4+ has to contact B7.1 or B7.2. This will amplify the TCR signaling, decrease the threshold for entering into the cell cycle, and induce expression of molecules that will be required, such as CD25 or CD122. At the same time, the CD40-CD40L interaction occurs, letting the future Treg expand. After the TCR dependent steps, or the immune synapsis dependent steps, cytokines take control. The progenitor for the Treg must come in contact with a common gamma chain cytokine (specially IL2 and IL15 (91) so they can stabilize the expression of Foxp3 directly through phosphorylated STAT5 binding to the promoter that previously has been chromatin remodeled by c-Rel to make it accessible. c-Rel is induced at the CD28-B7 interaction phase (92, 93).

A controversy has been generated with which yc cytokine is required for each progenitor to rescue them and become a Treg. The group of M. Farrar published a first study (94) showing that from all the common gamma cytokines only IL2, followed by IL15, then IL7, and lastly TSLP were able to induce *in vitro* and *in vivo*, the conversion of CD25+ Foxp3- progenitor into Treg. The group of Hsieh did a similar experiment (72), sorting CD25 hi SP CD4 and adding every common g cytokine individually. They observed the biggest conversion also with IL2, followed by IL15, and the rest could only generate a marginal 4-8% conversion to Treg. With those results IL2 seems to be the bottle neck of conversion to Treg by the CD25 + Foxp3- progenitor, leading to only the ones with more CD25 outcompeting the others to be selected. A few years later the Farrar group also showed (93) that the TCR signal strength, in addition to correlating with CD25 levels, also did correlated with some TNFSF members such as GITR, OX40 and TNFR2. They showed that these coreceptors decrease the threshold and need of IL2 for conversion of Tregs.

At this point the Treg progenitor needs a boost, as Foxp3 is proapoptotic. The common gamma chain rescues this progenitor from dying but the CD27-CD70 interaction is needed to finally rescue the developing Treg from apoptosis, and does so throughout a successive cell cycle program and blocking the mitochondrial apoptosis pathway via Bcl2 -Coquet, JM et al JEM 2013). This process we can see as SPCD4+ CD25- Foxp3-→ CD25+Foxp3- → CD25+Foxp3+ Treg, although a more complex sequence occurs as the expression of CD25 needs to be coordinated with the expression of CD122 and the downregulation of the IL7R, CD127 (72). An interesting phenomenon that has been reported several times is the correlation in expression between CD25 and GITR (72,73,93)

But there is another progenitor described, the Foxp3+ CD25- population. A possibility for the induction of this progenitor is due to the contact after the TCR signaling and CD28 with TGBß. TGBß is released with thymocyte apoptosis (and also by F4/80 medullary macrophages and DC), which have a sudden increase at day two (95). Curiously, the first record of Tregs in the thymus is just one day after (96). TGBß signaling (Smad2/3) in the context of specific TCR engagement is key for the induction of Foxp3 in Foxp3- thymocytes. The induction of Foxp3 prompts thymocytes towards the Treg lineage and once the SP CD4+ Foxp3+ thymocyte progenitor is generated the limited amount of a common gamma chain drives its stabilization and survival. Ouyang, W et al (97) showed that TGBß is required for Treg induction in the thymus, with special relevance during the first 4 days after birth (72). This is because TGFß1 maintains the suppressor function and induces Foxp3 but doesn't prevent apoptosis (72,98), therefore one needs the common gamma cytokines. Finally, there exists a correlation: the more TGBß, the more Treg generation (99) and vice versa (86), establishing an axis: apoptosis of thymocytes. TGBß release- Foxp3 induction.

An article from the Farrar group published this year (100) pointed out the differential characteristics of these progenitors in the adult mouse. Based on their results, CD25+ Foxp3- progenitors have a more mature phenotype, with faster kinetics and higher rates of proliferation but

also higher rates of apoptosis. They showed that they tend to interact with the pMHC with higher intensity (Nur77-GFP reporter mice) and rely on IL2, IL15 and the coreceptor 4-1BB.

The other progenitors (Foxp3+ CD25-) instead have a broader, non-self TCR affinities, with lower TCR signaling intensity. This makes them more dependent on CD28 and costimulatory ligands (RANK, GITR, IFNg), needing longer contacts with the APC to reach the level of signaling required to turn on Foxp3 (LFA1 KO reduces only this progenitors' levels and total Treg numbers). They tend to rely more on Il2 and IL4. IL4, in the mouse, is secreted by iNKT cells that are stimulated by tuft cells through IL25. In humans theoretically, Hassall corpuscles produce IL4 themselves and have been associated with Treg induction. In addition to this, when they did imaging they found around 20% the Foxp3+ CD25- in the cortex. This could be related to the report of the Rudensky group about a population of Tregs induced in the cortex (). In addition, a divergence in function could occur depending on the progenitor or the period of life (104) in which these progenitors are induced being more protective to the tissues and repair process (105) or controlling the immune responses (106).

An article published in 2015 (101) drew attention due to a new role of mature Treg, as they are able to migrate to the thymus and block the formation of new Treg when competing for IL-2 (they block Treg induction in the terminal phase, IL-2 dependent, but not the previous one). The authors saw that the proportion of Tregs with an active phenotype that migrated to the thymus increased with age, and according to other publications it would be the mTEC that induce this peripheral recirculation of the Treg (53). This could indicate a mechanism by which the body seeks to maintain an active immune system and for this purpose it seeks to block the continuous formation of Treg, in a feedback loop, so when the quantity increases in periphery more Treg recirculate and stop the generation of new Tregs against antigens that are not known whether or not they will be in the periphery, and enhance the Treg that have survived and expanded. This will protect the organism of an excess of immunosuppression as a single TCR from a Treg is able to recognize several targets and

suppress an immune response (102). This Treg recirculation has been confirmed by other publications, each of them showing a different maker to differentiate recirculating Treg vs recently generated thymic Treg (CD31 by Paola Romagnoli group (101), CCR6 by Graham Anderson group (103) and CD73 Michal Farrar. Group (100)).

Therefore, the selection of Treg vs conventional T cells is very different since in the conventional T cells their interactions are of low affinity and have different possible TCR-pMHC combinations that can originate with little avidity and be selected. In the case of the Treg, the ligands are much more defined and in addition the ligands that induce the expression of FOXP3 in a lymphocyte with a determined TCR cannot then induce another Treg. On top of this, the intense polyclonal competition for the ligands that induce FOXP3 result in small niches that give few clones of each.

Recently, the group of Diane Mathis (104) has discovered that the regulatory T lymphocytes that are generated in the perinatal period by the expression of Aire in the mTEC, are necessary and sufficient to prevent multi-organ autoimmunity. In addition, these perinatal Tregs persist in the adult and have a different transcriptomic and activation profile than the adult nTreg. This is because the repertoire of peptides presented by perinatal and adult ECTs is different. This means that the TCR repertoire of perinatal Treg is different from that of adults.

Going into the repertoire of Tregs that are generated in the periphery (iTreg), which represent a 5-10% Treg, it was discovered that during the specialization of CD4 into the different subtypes of Th is not only determined by the cytokine that surrounds that naive lymphocyte, but also by intrinsic factors take part as the affinity of its receptor for its own antigens. In addition, these lymphocytes that have a greater affinity and are more likely to generate iTreg are characterized by not expressing the marker Ly-6C (since the self-recognition with MHCII leads to a greater number of contacts

inhibits Ly-6C expression), which is induced in all naïve T cell in the periphery in a fast and gradual way (139).

3 The perinatal window of development: a critical time for tolerance induction.

The perinatal period of life is a stage where many changes occur simultaneously and at a high pace. It is the period, aside from the fetal stage, where the cells are multiplying -and dying- at the highest rate as tissues grows. In this period the different tissues start to establish connections between themselves, the circulatory system becomes independent from the mother's and microchimerism from the mom helps to tolerize and protect the newborn (108,109). Also, the hematopoietic progenitors change and so do the cells derived from them, including T cells (110). Extremely remarkable is that changes in this period have lifelong consequences on the immune fitness (104,107)

The perinatal immune system is phenotypically more immature than the adult one from the recent thymic emigrants (111), to the myeloid and B cell lineage (112,113 ,114). B cells are very inefficient at presenting antigens, generating antibodies in both T cell-dependent and independent. The T cell dependent response in the neonate is delayed on the onset, lower peak levels of antibodies, shorter duration of the response, and different distribution of the IgG isotype, also they tend to be lower affinity antibodies. This is in part due to the reduced number of Tfh (108) and follicular dendritic cells (FDC) in the germinal centers (113). But also, the T cell independent responses are decreased as C3 and the receptor (CR2) are decreased in plasma and in B cells, making a less intense tag on polysaccharides. There are fewer marginal B cells, although more b1 subtype. However, even when they generate plasma cells they are inefficient at migrating and staying at the bone marrow (113).

DCs are more immature in function and numbers compared to adults, although an enrichment for the first days of pDC is observed (112,113). DCs are less efficient at presenting and stimulating T

cell responses (114), expressing less costimulatory receptor (B7.1 and B7.2), less MHCII and integrins (CD11c), and increase activity of arginase 2 that suppresses immune responses (115). Also, higher levels of glucocorticoids (68) inhibit, even more, the DC maturation upon stimulation.

The thymic output does not change much when comparing adult to neonate (69, 111). What does change in the thymus is the progenitors, which is regulated by the type of lymphocyte precursors found in the thymus during development that led to the generation of a reduced ratio CD4: CD8 (1.1-1.6 at birth to 2-3 in the adult). Interestingly the production of gamma delta T cells does not vary during at these ages (116). Also remarkable is the reduced dependence on TCR signals that T cells have during the perinatal period. Although they tend to be less proliferative, express lower levels of CD40 (licensing), and IL2 among other cytokines (108,112,113).

But once they are strongly stimulated they enter faster and in greater percentage into the cell cycle, especially CD8. This is followed by an earlier cessation on the proliferation. This is inherited by the thymic precursors and can be a compensatory mechanism for the lower memory pool and capacity at this stage of life (117).

Perinatal T cells responses are skewed toward a Th2 response, where Th1 and Th17 are defective or dampened. This doesn't mean that at certain thresholds a neonate can't generate those responses, but they intrinsically tend to develop a Th2 response. This makes them more susceptible to infectious agents, have a higher propensity to develop allergies, but it is a keystone event for inducing tolerance towards intrinsic (69, 104) and extrinsic antigens (69, 118,120), as some reports have been proposed that Tregs are easier to be induced on the periphery at this stage (119).

An important feature of the T cell compartment in neonates is an early life compartmentalization of the T cells within the tissues. Whereas in adults' tissues are populated by memory cells, in neonates naïve T cells colonize those tissues, except lung, small intestine (69,112),

and colon where memory cells start appearing. However, these are also placing with a higher concentration of microbiota or air bone antigens.

During the young ages of the organism a high percentage of Tregs dominate the scene (69,110,120) although apparently, they appear in the scene later than T naïve cells (96).

Treg are not only important for generating de novo tolerance in tissues populated by the microbiome such as skin, gut, or lung against new antigens (69,118,120), but also for seeding the tissues and establishing a niche (69,104,120). Tregs are found in greater proportions than adults (30-40% vs 1-10%) (69) and they do have different functions, being capable of generating tissue tolerance that remains lifelong (104).

Specifically, interesting are the really exquisite experiments done by Mireia Guereau-de Arellano et al. (129), by removing the thymus at different time points and showing that the perinatal period is key for protecting from Aire disease. In a follow up paper, S yang and Nori (104) found that the cell type responsible for that phenotype was Tregs. Those Tregs have a different TCR and different stoma (mTEC) that influence their generation, seed the tissues, remain there lifelong to suppress autoimmunity. They are sufficient and necessary for maintaining tolerance and suppressing Aire autoimmunity. They also describe them with a specific Treg signature that was characterized by a high and unique expression of ST2. ST2 expression was maintained lifelong. ST2 expression on Tregs is important as the Th2 skewed responses in the perinatal period have shown to be reliant on the ST2-IL33 axis. This axis has a key role in this period, favoring vital functions and tissue homeostasis and remodeling, and is a crucial switch on thermogenesis after birth, is to maintain the body temperature (121,122). Also, in the neonatal lung, IL33 expression restricts DC functions to favor a Th2 response over Th1 (123).

Chapter two: DATA

MATERIALS AND METHODS

Mice

C57BL/6 (B6) , Foxp3-IRES-Gfp, Foxp3-DTR mice. In all cases, mouse lines have been backcrossed to the B6 genetic background for at least eight generations, and

experiments were conducted with littermate controls. All animals were housed at the

specific-pathogen-free facilities at Harvard Medical School (HMS). Experiments were

performed under protocols approved by HMS's Institutional Animal Care and Use Committee (protocol #IS00001257). Mice were feeded with normal diet.

Cell isolation and flow cytometry

Mice were asphyxiated with CO2 and perfused with 15ml of PBS. Epididymal VAT, liver, Parotid gland, lacrimal gland, eyes, kidney, ear skin, bone marrow, thymus , lung, and colon from male mice were excised, minced and digested in a 37°C water bath with shaking as follows: adipose tissues were digested for 20 min with 1.5 mg/ml collagenase type II (Sigma) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% fetal calf serum (FCS); liver, Kidney and lung was digested in RPMI containing 0.5mg/ml collagenase IV (GIBCO) and 0.1mg/ml DNase I (Sigma) for 45 min; the entire colon was incubated in RPMI containing 1mM DTT, 20mM EDTA, and 2% FCS, at 37ºC for 15 min to remove epithelial cells. The colon was then minced and dissociated in RPMI containing 1.5mg/ml collagenase II (GIBCO), 0.5mg/ml Dispase (GIBCO), and 1% FCS, at 37° C for 45 min with constant stirring. For ear skin epidermis and dermis were manually separated under the microscope and incubated in a mix of RPMI with 4%FCS HEPES, 0.5 mg/mL hyaluronidase , 3 mg/mL Collagenase IV (GIBCO) and 0.1mg mL DNase I(Sigma) for 30 minutes in constant stirring. For the bone marrow,

one femur was dissected. On the lab we cut the top and bottom part and flush it with RPMI 2% FCS. After spin it down 1500rp during 6 minutes we use ACK lysis buffer (Lonza) for 3 minutes on ice. Then we add 20 mL of RPMI 2% FCS and re spin it down at 1500 rpmi for 6 minutes. For the thymus we smash it throw a 100-um nylon filter strainer. After spin it down we refilter with a 70um nylon strainer. For adipose tissues, the SVF was collected after centrifugation at 450 g for 10 min. For liver, parotid gland and kidney, cells were further enriched for a leukocyte fraction by 30% Percoll (GE Healthcare), underlain with 75% Percoll, and spin for 25 min at 1126 g without brakes. For all tissues, the digested materials were filtered through a 100um nylon cell strainer and after by a 40-um nylon strainer. For T lymphocyte analysis, cells were stained with anti- -CD45.2 (104), -CD4 (GK1.5), TCRb (53-6.7), -CD25 (PC61), -KLRG1 (2F1/KLRG1), -ST2 (H1.2F3), mAbs (all from Biolegend); anti-ST2 (RMST2-2) (eBioscience); for ~25 minutes at 4°C, followed by LIVE/DEAD Fixable yellow zombie or UV zombie viability dye (Thermo Fisher) incubation for 10 minutes at room temperature (RT). Following standard surface staining cells were fixed, permeabilized and intracellularly stained for Foxp3 (FJK-16 s) (eBioscience),according to the manufacturer's (eBioscience) instructions. Cells were acquired with a Symphony flow cytometer (BD Biosciences), and data were analyzed using FlowJo software. Graph and statistical analysis re done with the software Prism version 8. Data represented show SEM and significance was archive by the stadistical analysis t Test. Significance values * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Mouse manipulations

For the IL-33 administration experiments, 13-week-old B6 male mice were intraperitoneally (ip) injected either with PBS or recombinant mouse IL-33 (BioLegend) at 2 μg/dose on days 0 and 3 and were analyzed on day 6.

For the Diphtheria toxin (DT) injection. 13-week-old B6 male mice were injected intraperitoneally with DT (Sigma) with 3 consecutive day doses concentration of 20ng/gr. For the controls we used

PBS or littermate controls DTR negative and administered DT also. Then we wait 7-8 weeks and analyze them.

Experiments

My master's thesis project stems from two questions; The first asks how Tregs in general, and perinatal Tregs in particular, are induced in the thymus. The second asks about the functions of these perinatal Tregs in the tissues they seed, and how they are maintained in those tissues so they can perform their actions throughout the lifetime of the animal. The role of perinatal Tregs, and the whole spectrum of Tregs, in tissues is far from being understood, but myself as well as many brilliant postdocs and PhD students are working to investigating the role of tissue Tregs in specific tissues/organs. The following data is going to be presented in order, starting from neonate and progressing to adult stages of life. The first section is related to the efforts we are making to understand Treg induction and generation in the thymus, comparing adults and neonates and determining if Treg generation changes during development. The second section focuses on experiments to look at Treg stability in tissues and their tissue-specific phenotype. Lastly, I will conclude with our new experiments aiming to modulate perinatal tissue Tregs in adults with the goal to exploit their phenotype to solve clinical problems.

4. Treg generation in the thymus, progenitors and dynamics.

As previously mentioned, two populations of Treg progenitors have been identified at present: Foxp3+ CD25- and CD25+ Foxp3- (Fig 2). Both populations have the potential to give rise to Tregs but with some differences in their function (100). As perinatal Tregs have different properties than adult Tregs (104) we questioned if that was due to a difference in the proportion of the progenitors during ontogeny with a special focus on the first two weeks of life, the perinatal and immediate post perinatal period, where the changes on the Treg properties occur (around day 7-8 after birth). We observed (Fig. 3a) opposing dynamics of the two progenitor populations, with the CD25+ Foxp3- cells dominating for the first days of life and decreasing with time while their counterparts, Foxp3+ CD25- cells, tend to increase with time. Both progenitors decrease in percentage (Figure 3a) and numbers around 32 weeks of age, the period in which the majority of the thymus has degenerated and been replaced by adipose tissue (11). As expected, the percentage and number of Tregs increase with time, with Tregs being detectable at day 1 in the thymus and spleen, notably different than what was found in the field (96). This difference could be due to the improvement of flow cytometry technology, with machines with increased sensitivity.

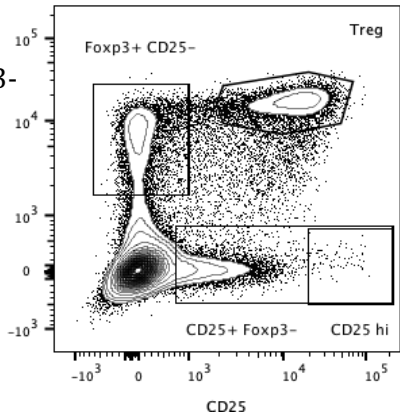

Figure 2. **Treg progenitors in the thymus**. Representative plot of an adult thymus of a B6 male. Gated on singlets, live CD4+ CD8- TCRb hi.

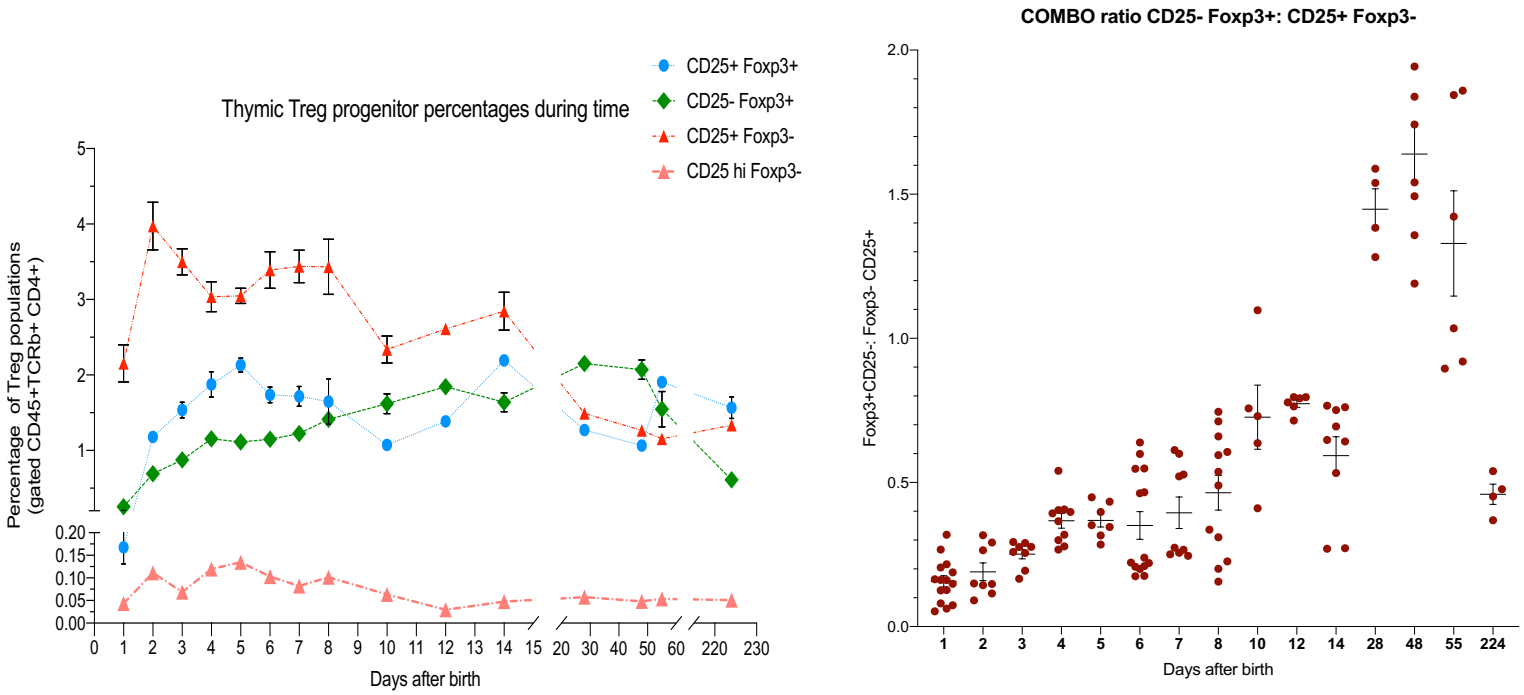

Figure 3. **Treg progenitors' dynamics in the thymus during ontogeny**. **(A)** Representation of the percentages of Treg progenitors and thymic Treg during time. **(B)** Ratio of the Treg progenitors during time. Combination of 2 independent experiments. Mean +/- SEM.

Plotting the ratio between both of the progenitors confirms our Treg progenitor switch and clearly shows this tendency of progenitor swap during time (Fig.3b).

As mentioned previously, there are two different populations of dendritic cells in the thymus playing different roles in negative selection and Treg induction (30, 48). We performed a preliminary experiment to explore the changes in the stromal compartment, with a special focus on the stromal immune compartment. We observed a mild dominance of the Sipra+ DC subset in the perinatal period, with CD8a+ DCs becoming the dominant subset after 2 weeks of life. This experiment must be repeated before any conclusions are drawn. Also, Yang S. et al (104) observed that CD8a+ DCs in the thymus are rare in the beginning of life, but increase with time. This fits perfectly with the notion that these DCs are derived from a bone marrow progenitor that seeds the thymus via CCR7 (37), and this first wave of bone marrow derived cells occurs late in the first week after birth.

Future experiments for this section will be looking at the dynamics of DC subsets during ontogeny and determining stromal compartment dynamics (cTEC and mTEC). Ongoing experiments

are trying to observe the effect of the depletion of both APCs (DC and mTEC) on the Treg precursors over time. To interrogate the mTEC contribution, I will make use of Aire KO mice. Lacking Aire alters the key step in mTEC maturation, which leads to a reduction, but not full elimination Tregs in these animals (104). Based on preliminary data, we observe an alteration in the percentages and numbers of one of the Treg progenitors. To modulate the myeloid compartment, I will use CD11c DTR GFP mice, allowing me to ablate the DC compartment systemically at a certain timepoint. Although not a perfect system, this animal model will let us interrogate if the depletion of DCs (and some macrophages (127)) during the perinatal window has an effect on the Treg progenitors. If so, will generate a more specific mouse model to solve this question and specifically target DCs.

In terms of gene expression, two experiments have been performed. We have submitted bulk RNA sequencing of both Treg progenitors and thymic Tregs (gated as CD31+CD25+Foxp3+ (101)) at day 5, day 12, and 6 weeks after birth. With this data we are aiming to answer these two major questions:

How different are Treg progenitors from each transcriptionally?

Do the gene expression profiles of progenitor populations remain unchanged overtime, or do they differ in young verses old mice?

For the first question, the group of M. Farrar recently published a study where they performed single cell RNA sequencing (scRNAseq) on these populations in adult mice. In this study the authors barely compare both progenitors aside from a heatmap that demonstrates that these Treg progenitors are transcriptionally different from each other, and also from double positive stage thymocytes and fully committed thymic Tregs, without ruling out recirculating Tregs (100). Our bulk RNA sequencing approach will give us a more in depth look at the transcriptomics of these cells and allow us to find targets that help clarify their origins and their associations with specific APC populations.

The second question, to our knowledge, hasn't been answered before and could explain why perinatal thymic Tregs are different from adult thymic Tregs. Do the progenitors change over time, leading to the difference observed in thymic Tregs? Or do the progenitors stay the same, and the difference between adult and perinatal Treg, are a result of the environment or is TCR mediated.

The TCR repertoire of perinatal Tregs is clearly different from that of adult Tregs (104); It is less clonally expanded, has shorter CDR3a stretches and fewer TCR-N- additions. These results highlight a big difference between these populations and lead us to an important question: why is the perinatal TCR repertoire critical to come out early in life and not in late time points or consistently through life? To answer this question, and specifically focus on the unique properties of perinatal Tregs, I will start by determining if perinatal Treg function is due to the specific TCR structure.

At a first glance the description of the perinatal TCR seems like a less refined TCR, with a broader surface of contact and less specificity for their targets. During the first days of life, the immune system starts seeding the different tissues for the first time and creating a niche in those tissues that will be lifelong. A broader and not so specific TCR, with the capacity of recognizing broader, and not in such fine detail, structures this is just what a cell that need to be in a tissue and get retain life long and develop their function independently of a specific cell subtype want. They need a specific TCR, to avoid being activated against every new antigen, but not such a specific TCR where they can't be kept alive and retained in the tissue to establish a broader crosstalk with several cells or cell stages in their niche. The decrease in N additions to the TCR is interesting, as this is one of the key mechanisms to increase TCR diversity, especially because N additions are made on the DJ (b chain) joining or VJ joining (a chain), where the CRD3 is generated. CDR3 is an especially important part of the TCR a and b, as the loops that it creates are positioned over the center of the

MHC/peptide complex, connecting the most surface area (and the center) of the TCR with the peptide loaded on MHCII (125).

TdT is responsible of the N– addition at the CDR3 region. Interestingly, TdT is not expressed on thymocytes (during the double negative stage) until day 3-5 after birth (104). Even then, an additional 1 to 2 days is needed until there is significant levels of N-region diversity. Then another 1 to 2 days until those thymocytes become single positive CD4 and commit to Treg and are able to leave the thymus (130). Therefore, we don't observe N additions in the TCR on peripheral T cells until 7-8 days after birth. This is the exact time that perinatal Tregs are generated. This led us to hypothesize that the cell that induces these Tregs may not be important, as they could just act as an APC that produces a cytokine for survival and differentiation of the different Treg progenitors but does not induce the special characteristics of perinatal Tregs by themselves. Maybe the TCR is the differential factor that determinates the characteristics of the future Treg independently of the Treg progenitor and/or cytokines needed for their survival. Some indirect reports on this possibility have been published already. For example, TdT deficiency decreases the incidence of diabetes, SLE, (131) , and

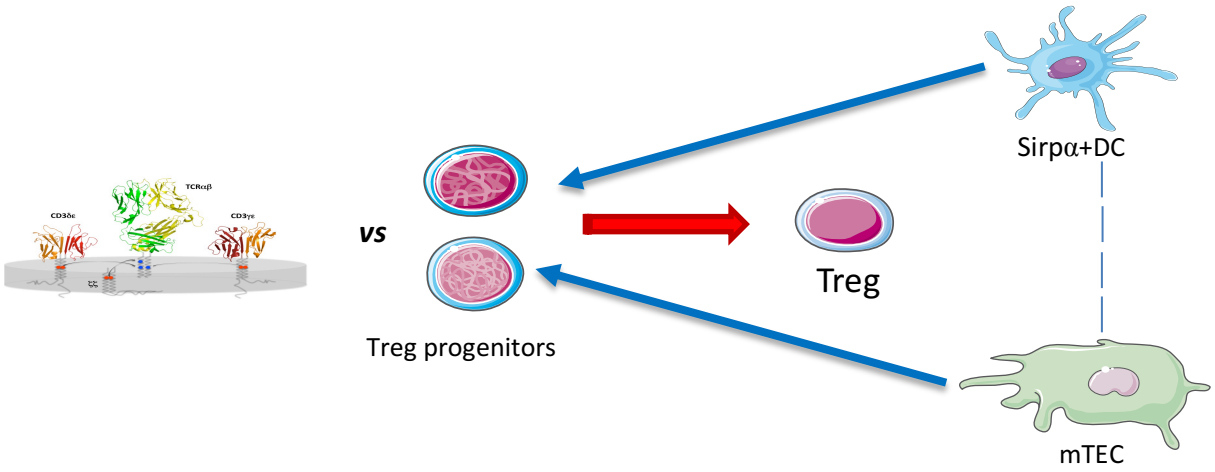

Figure 4. **Possible hypothesis that could explain the different cellular properties that neonatal Treg has compared with adult Treg**. Based on Yang S., et al paper there is a tissue niche difference but is excluded out of the equation as ACT of perinatal treg auitoimmunity models are able to revert the pathology so, the niche in the tissue is not determinant on the function of those Treg. In the thymus several factors can be the cause to give raise to this different Treg. The first one could be the increase presence of Sirpα DC, and a possible change on their phenotype, that could expand more one progenitor than the other one. The second option, differneces in mTEC transcriptomics or density of Aire+ mTEC that wlll promote the presentation in a different way of tissue restricted antigens (TRA). Finally a change on the type of precursors, due to different origin of the progenitor (fetal vs bone marrow) or different thymic mllieau. In addition of the cell intrinsic-transcriptomic difference the unalike TCR from both periods of ontogeny.

autoimmune nephritis (132) in mouse models. To address this question, we plan to do single cell TCR sequencing on the Treg progenitors at a perinatal and adult timepoint in both wildtype and Tdt KO mice to compare the TCR sequences.

In parallel, we plan to do adoptive cell therapy of TdT ko adult Tregs in AIRE autoimmunity and or a model of autoimmunity to see if the TCR only confers the rescue of the phenotype as it happened on Yang, S. et al (104).

All the previous experiments and data is exploring the Treg compartment the factors that lead to the differences between adult Tregs and perinatal Tregs (Fig.4). We have started exploring

the transcriptomic phenotypes of perinatal mTECs verses adult mTECs. Taking profit of a previous bulk RNAseq generated on perinatal versus adult mTEC we do a re-analysis of previously generated bulk RNAseq data revealed interesting differences (Figure 5 and table 2).

The bulk RNA seq was performed at two time points: 4 days of life and 4 weeks of life. Populations studied were mature mTECs (MHCII hi Aire+ and MHCII hi, Aire – and MHCII hi Aire KO). A postdoc in the lab had previously analyzed this experiment and found no

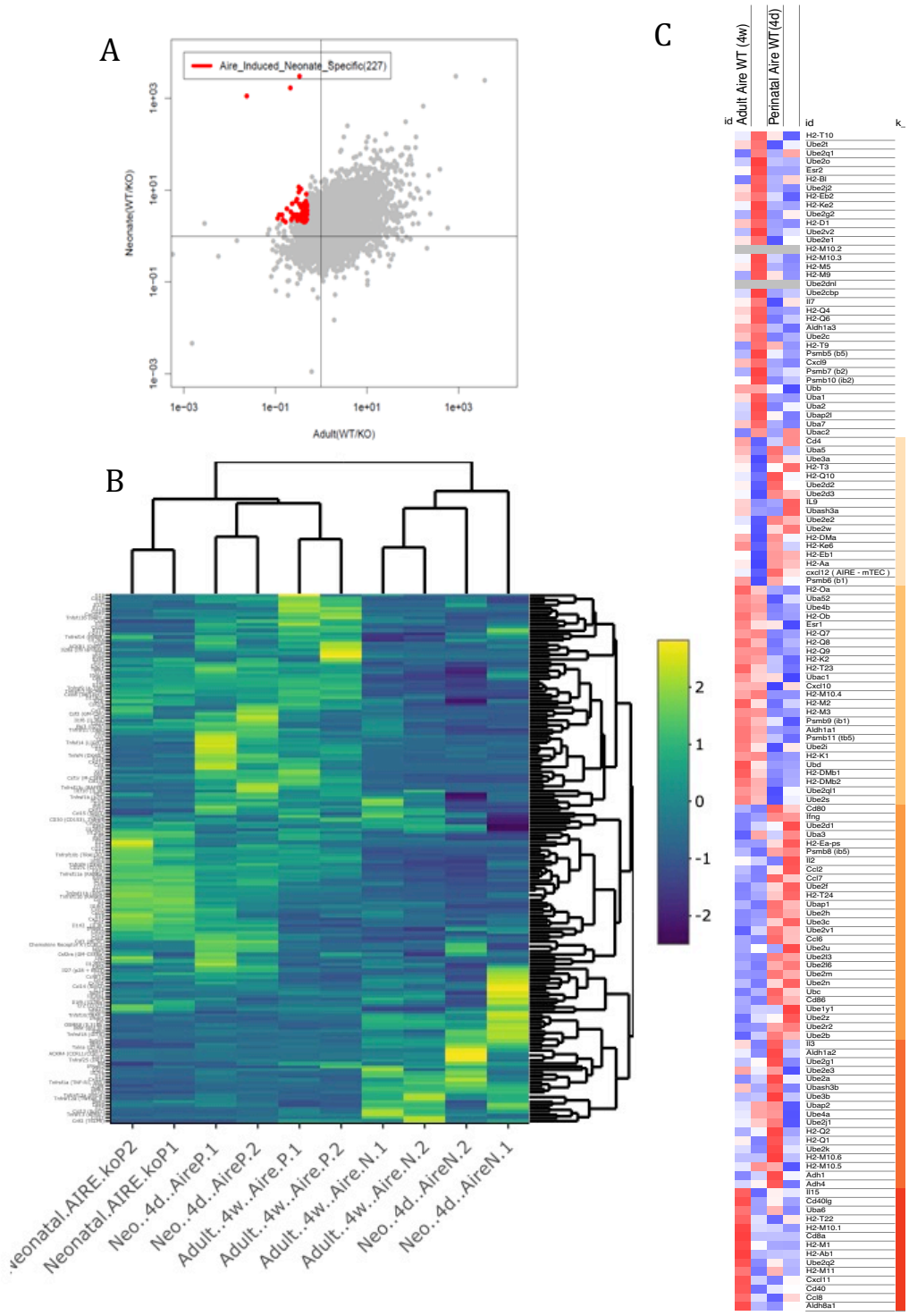

Figure 5.**mTEC differential expression perinatal period versus adult. (A)** TRA differentially expressed between neonatal vs adult mTEC. Data gently provided by K. Bansal. **(B)** Unbiased cluster analysis heatmap with all cytokines and chemokines expression in the different conditions. 2 replicates per condition. **(C)** Heatmap with all antigen presentation machinery genes included, adult versus perinatal MHCII hi Aire+ mTEC.

differentially expressed TRAs, with only 227 genes differentially expressed (Fig. 5A). Although this may seem like a modest difference, it is enough to generate a different TCR repertoire, or at least shape it.

In addition, I performed an unbiased cluster analysis based on all cytokines and chemokines known to date (Fig 5b) and determined that different stages and adult vs neonates cluster differently. I also performed an analysis on the diffential gene expression of all the molecules involved in antigen presentation (Fig 5C). This is important because eventhough the TRAs do not differ, if the antigen processing machinery is different, then a new repertoire of peptides will be presented. We also observed differences in the costimulatory molecule as CD86, cytokines as IL-7 and IL-3, chemokines as Ccl3, different MHC subtypes and ubiquitin subunits but not proteosome subunits among others (Table 2). So in general, we observe a different expression of certain important costimulatory, cytokines and antigen presenting machinery that may led to the generation of a different TCR Treg repertoire or/and education of progenitors in a different way promoting the selection of one of the progenitors over the other. This last possibility may explain the change of Treg progenitors that we hve observed on Figure 3.

mTEC Perinatal vs Adult	Pathways/targets	Difference	What is different?
Kushagra Bansal (unpublished results)	TRA	NO**	227 genes
Reanalysis data from Kushagra Bansal Rnaseq perinatal vs adul Aire ko vs WT	Ag presenting machinery	YES	
S. Yang ,et al. Science (2015)	MHC II	MILD*	H2-DO, H2-M2, H2Q6,H2-Q7, H2-Ke2 & more
Reanalysis data from Kushagra Bansal Rnaseq perinatal vs adul Aire ko vs WT	MHCI	Some	*
Reanalysis data from Kushagra Bansal Rnaseq perinatal vs adul Aire ko vs WT	Costimulatory receptors	Some	CD80, CD86, Esr2, Aldh1a1, Aldh1a2, Aldh1a3 and more
Reanalysis data from Kushagra Bansal Rnaseq perinatal vs adul Aire ko vs WT	Cytokines	Some	Several (Il3, TNFa, TSLP, Lta, TGFB2, IL9R, TSLPR…)
Reanalysis data from Kushagra Bansal Rnaseq perinatal vs adul Aire ko vs WT	Chemokines	Some	Ccl2, Ccl6
Reanalysis data from Kushagra Bansal Rnaseq perinatal vs adul Aire ko vs WT	Ubiquitin pathway	Some	Ubc, Ubd, Uba1, Uba7, Ubec2c, Ube2d3,Ube2iUbe2l3, Ube2l6, Ube 2v1, Ube4b & more
Reanalysis data from Kushagra Bansal Rnaseq perinatal vs adul Aire ko vs WT	Proteosome	Few*	Psmb11*

Table 2. **Summary of the findings in gene expression compared mTEC from a mice 4d old and mice 4 weeks old**.

5. Treg migration and colocalization within the tissue: neighbors in the niche.

In Yang, S. et al (104) , they show that perinatal Tregs stay lifelong in the spleen without changing in numbers, at least until 8 weeks old. To expand that notion, we performed parabiosis and gated on tissue Tregs (ST2+ KLRG1+). We observed similar results as Kolodin, D et al (Fig 6) in visceral adipose tissue (VAT), but due to technical problem we decided not to continue with a replicate of this experiment at least for now.

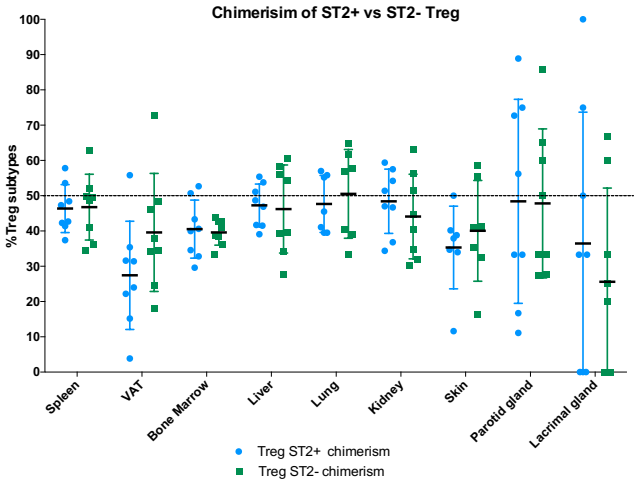

Figure 6. **Chimerism percentage of ST2+ compared to ST2- Tregs in several tissues.** In. collaboration with M. Pittet lab. Mice we SAC 5 weeks post parabiosis.

Another way to address this question is by using the Foxp3 GFP Cre ERT2 Rosa26 YFP. All Tregs in these mice express GFP and once administered tamoxifen the Tregs also express YFP, becoming double positive GFP and YFP. The plan is to administer tamoxifen during the perinatal window and then analyze adult timepoints. Three experiments will be performed on these mice. Firstly, by flow cytometry I will look for the presence of GFP+YFP+ cells in several tissues to determine which tissues get seeded by perinatal Treg and in which magnitude.

Secondly, after getting a map of tissues where perinatal tag Treg migrate and stay, I will analyze, using confocal microscopy, their localization within the tissue, and who their neighbors are. By doing this I will see if there is a special association with any other cell type across tissues.

Finally, a parabiosis experiment between perinatally tagged Treg mice and WT mice to address the possible migration of those tissue Treg could be an important option if we could improve our previous results with parabiosis.

6. Perinatal tissue Tregs: IL33 as a tissue Treg expansion model.

Perinatal Tregs express ST2 as a differential marker from other Treg populations (104). As an indirect way to determine the presence of perinatal Tregs across tissues we decided to inject its ligand, interleukin 33 (IL33), and analyze an array of tissues in order to see if we could expand the perinatal Tregs. Before continuing, I must clarify that ST2 may be expressed by other tissue Tregs that are not perinatally generated, so maybe some of the Treg expansion is due to tissue Tregs that express ST2 and seed the tissue later in life. For these experiments I applied the same protocol that colleagues have been using for Treg expansion in VAT. Briefly, I injected 2 doses of IL33 intraperitoneally (ip) spaced apart by 48 hours and harvesting organs 72 hours after the final dose.

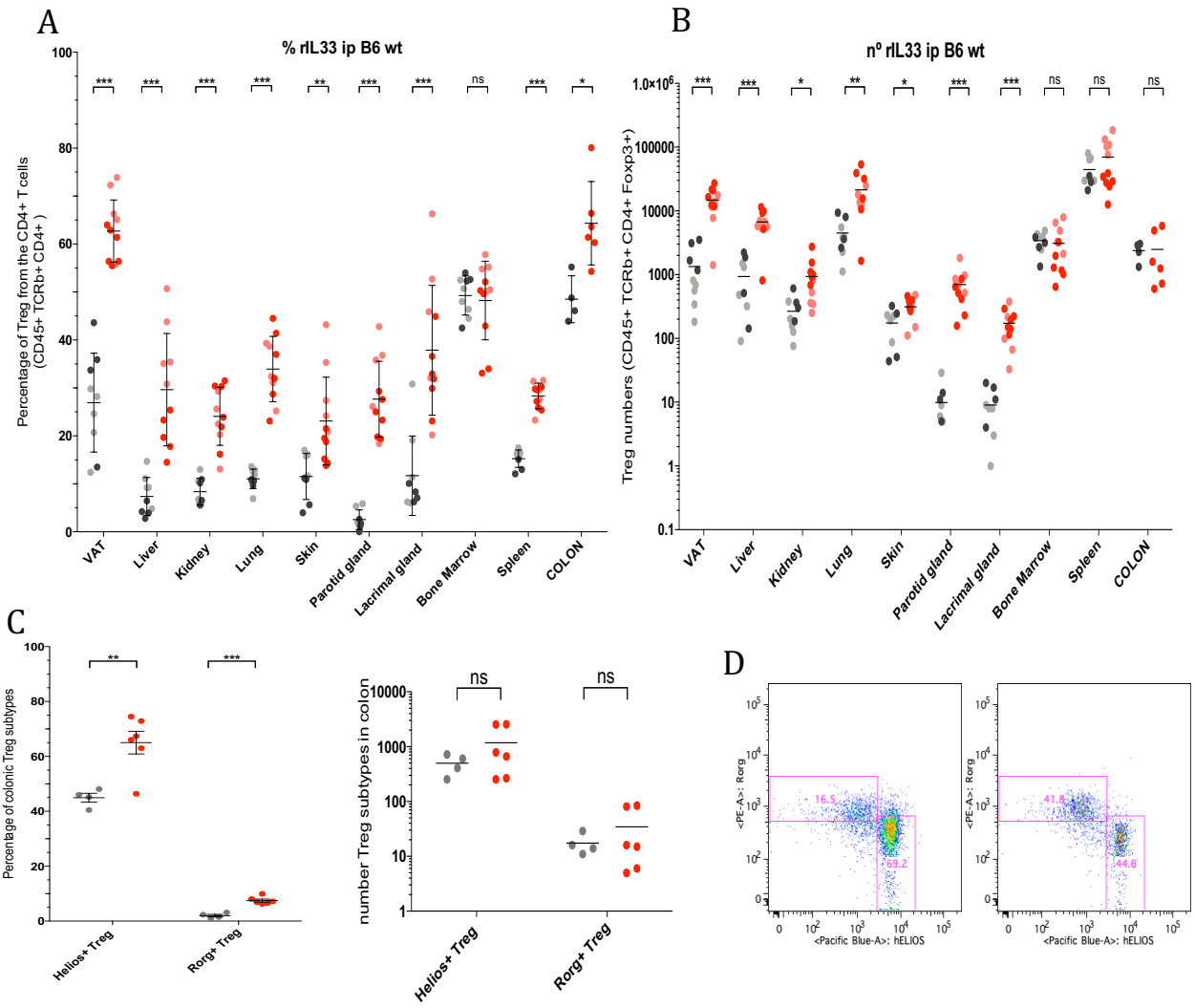

Figure 7. **IL33 injection expands tissue Treg (a)** Percentage of Treg on the CD4 T cell population. **(B)** number of Tregs after IL33 administration. **(C)** Colonic Treg subtypes percentages and numbers. **(D)** Representative flow plot of the colonic Tregs with IL33 ip injection or PBS (control)ip injection. Grey dots are the control group injected ip with PBS, red dot IL33 ip injected group. The intensity of the dots represents 2 independent experiments.

We observed that Tregs expand in all tissues, with the exception of bone marrow (BM), spleen, and colon. Bone marrow and spleen served as our negative controls, as circulatory Treg shouldn't respond to IL33 since they lack expression of ST2. For colon, we didn't observe an increase in total number but we observed an expansion of Helios + Treg compared with Rorg+ Treg, although not statistically significant. (Fig 3 C, D). Among the Treg populations across every organ , the major population that expands is the ST2+ population (data not shown) and does so independently of ILC2,

which also have ST2 and also expand in a lower magnitude to IL33 injection. T conventional cells do not increase in percentages and show only a mild increase in numbers.

In conclusion IL33 has highlighted the presence of tissue Tregs that expand in response to their ligand and opens the door to opportunities to use this in autoimmunity models to promote the expansion of tissue Tregs. By doing this, we will try to tip the balance in favor of Tregs and not of autoreactive effector cells and suppress the auto aggression. We plan to use two different mouse models of autoimmunity. The first one is the AIRE KO model, where Tregs lack a TCR repertoire (and potentially those thymocytes becoming T naïve). The autoimmunity that derives from this KO model is what I call "lacking specific Treg TCR". The other model will be an inducible autoimmunity model (arthritis or multiple sclerosis) where the tolerogenic balance is broken due to an immunization with an autoantigen. In this case we do have TCR specific Treg, but they are overrun by effector T cells that establish the autoreactivity feedback loop with epitope spreading and niche modification. By administering IL33 in these models using several different regimens we want to address if : 1) Is IL33 a promising new drug to treat or prevent autoimmunity?, and 2) Does Treg suppression need to be TCR specific or can it be mediated through bystander suppression with cytokines? These experiments are currently ongoing.

7 . Treg depletion in adult mice: Foxp3 DTR model for studying Treg recolonization of the niche.

After the positive results from the IL33 injection experiments and under the premise that perinatal Tregs can only be generated during a restricted time during life (from day 3-7), we decided to deplete Tregs in an adult mouse (13 weeks old) and wait 7-8 weeks to look at the recovery of Tregs in the same tissues (Fig 8). To our surprise, only two out of the initial array of tissues didn't show Treg recolonization after depletion. We hypothesized that this phenotype may be related to the embryological origin of those tissues, as skin and adipose tissue both derive from the mesenchymal

layer. To test this, we are performing an additional experiment that includes multiple mesenchymal derived tissues (meninges, peritoneum, muscle) to confirm or deny this hypothesis. In parallel we will be performing bulk RNA-seq on the Tregs that remain in the tissues after diphtheria toxin depletion, and those that repopulate the tissue. This will help us understand: 1) why is there still a Treg population remaining in VAT and skin after diphtheria depletion? 2) do the Tregs that repopulate the tissues have the same phenotype as the ones who don't?

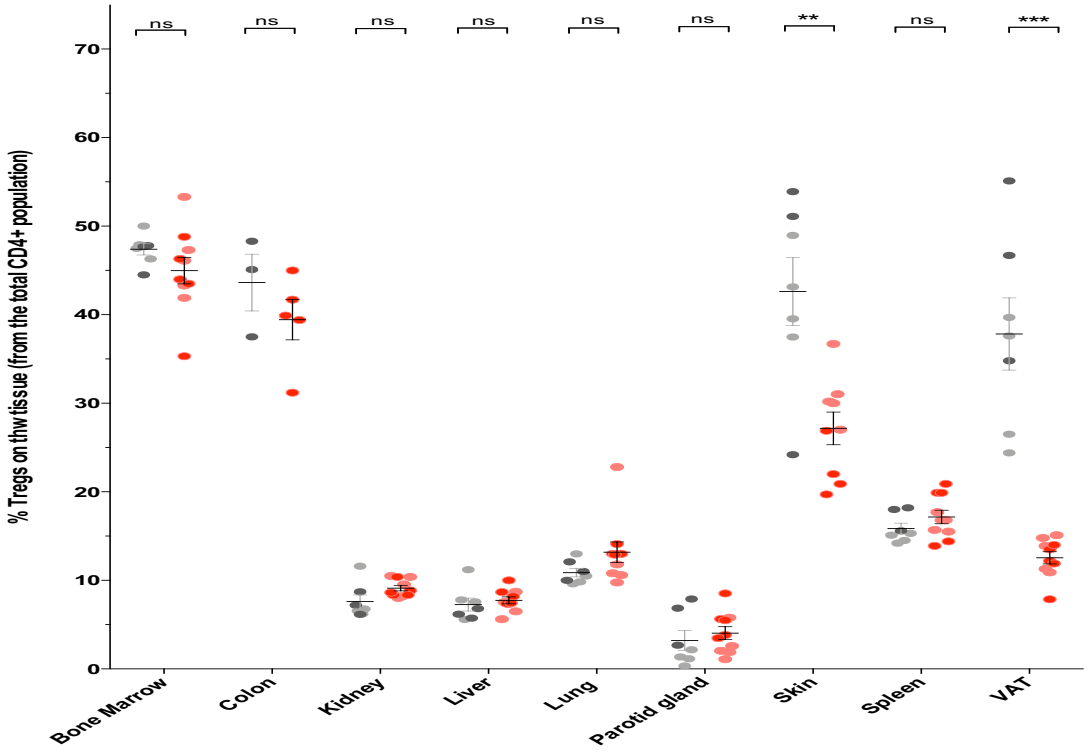

Figure 8 **Diphtheria toxin depletion of Foxp3 DTR mice generates differential recovery rate on different tissues**. Mice intraperitoneally (ip) injected at 13 weeks of age with 3 consecutive doses of diphtheria toxin (red dots) or ip injected with PBS used as a control. Then we wait 7-8 weeks post injection (20-21 weeks old) and analyze the Treg numbers. I am repeating the last experiment with littermates DTR+ DTR- all treated with DT. The intensity of the dots represents 2 independent experiments

Two additional experiments are planned to try to recapitulate the phenotype with two different approaches. The first experiment (which is currently being analyzed) was to irradiate mice (1100rads) and reconstitute their bone marrow with a littermate's. By doing this, we are depleting all the immune cells in the body via irradiation and addressing if Tregs can come back to the tissues

and if not, if VAT and skin are again the ones lacking Tregs. This experiment has the additional appeal of allowing us to make a connection with patients who receive irradiation (local or whole body) for tumors or bone marrow transplantation. Indeed, we are examining fibrosis scores in those tissues to determine if Treg loss can led to increased fibroblast activation, as this is responsible for the fibrosis that patients usually experience (124).

Last but not least, the second experiment that is ongoing to see the potential of Treg recovery is focused on obesity. Obesity depletes adipose Tregs and they never replenish. We are addressing if mice that were lean, become obese, and then go back to lean are able to recover their Treg and normalize their metabolic indexes. This is mimicking the problem that occidental society is facing as people that gain a substantial amount of weight (obesity epidemics), then try to lose it with the help of diets but gain weight again even faster. We want to know if Tregs can be responsible or at least implicated in such a process.

Chapter three: Discussion and perspectives.

Taking all this into account, this project tries to envision the mechanism of how T regulatory cells are generated. Previously, several reports have found two progenitor populations that give rise to Tregs (100), and several groups have been focused only on the CD25+ Foxp3- progenitor, leaving the other population largely unstudied. This is probably due to the clear target and straight explanation that this progenitor has, the alpha chain of the IL2 receptor. Authors have modulated that marker to reveal the importance of IL2 on this progenitor (100). Our study tries to establish the requirements for both progenitors. At the same time, we intend to find their partnering antigen presenting cell, who induces the conversion towards Treg and, if possible, understand why only some of those progenitors become Tregs. To answer these questions, we will use confocal microscopy to see with which cells Tregs are communicating with and if they are in a specific location in the thymic medulla. A delimitated space of induction of Treg has already been proved s it was shown to be

nearly exclusively in the thymic medulla (103) although some Treg can be found on the cortex, probably converted there (88). We think that the transcriptomics generated by RNAseq will be extremely helpful to find possible ligand-receptor partners that will help us to clarify how Tregs make contacts and with who. To explain the cytokine requirements for progenitor conversion into Treg, we will use the model of thymic slices (76). This model is the closest look *in vitro* to an *in vivo* system as the architecture, cells, and matrix are maintained. We can say that thymic slices are a *quasi in vivo* organ easy to manipulate to see the final output. In addition to all this, I will like to remark that we are the first group to analyze this process and generate transcriptomics data considering the recirculation of Tregs into the thymus (101) by only sorting CD31+ cells, a marker for recent thymic emigrant.

The adult side of the project is based on the lifelong permanence of these perinatal Tregs in tissues. What they do there is a hot topic in immunology, and is especially interesting for the lab I am part of, with a huge amount of resources and people trying to understand it. In my case, I just want to show in which other tissues these perinatal Tregs seed and the capacities of retention in those. Luckily, single cell RNAseq coupled with TCR sequencing of these Tregs will help to discriminate possible novel functions and help some lab mates to push forward their project or make it more complete.

The other side of the adult period of these perinatal Treg is to try to exploit their abilities to restore homeostasis in tissues that have been broken due to autoimmunity. Autoimmunity, in my personal opinion, can be classified as TCR determined or non TCR mediated. Non TCR mediated effect will include mutations, allelic variances, SNPs, metabolic alterations, or other cell intrinsic defects in immune cells or in epithelial cells. Examples of possible triggers could be an incomplete elimination of autoreactive cells in the thymus due to a defect in B cells, DC or epithelial cells, or a mis presentation of peptides at epithelial layers leading to the start of an autoreactive immune reaction, or a dysbiosis with an antigen mimicking of self-antigens or certain strains of commensal microbes/virus/parasites that will push a too high TH17-Th1 response in the context of a sterile inflammation, generating reactive cells reach and expand in the tissue and depleting or preventing Tregs to intervene. All these causes or others may elicit a small push in an individual who is already closer to the breaking point, leading to a cascade of events manifesting in autoimmunity. But that small increase in a non-predisposed individual won't cause pathogenesis, so the noxa isn't pathogenic per *se* neither the sole cause. This is what I am trying to show on figure 9 with the green bar, a non-predisposed individual during his life time independently of noxas he faces, and the orange bar, a predisposed individual without encounter a noxa that will ultimately become the red bar. The increase in autoreactivity at the beginning is unrelated with chronological time (perinatal, adult, or elderly), indeed all this probably accumulates as an increase of autoreactivity without having pathology has already been proven and that may be related with the lack of Treg

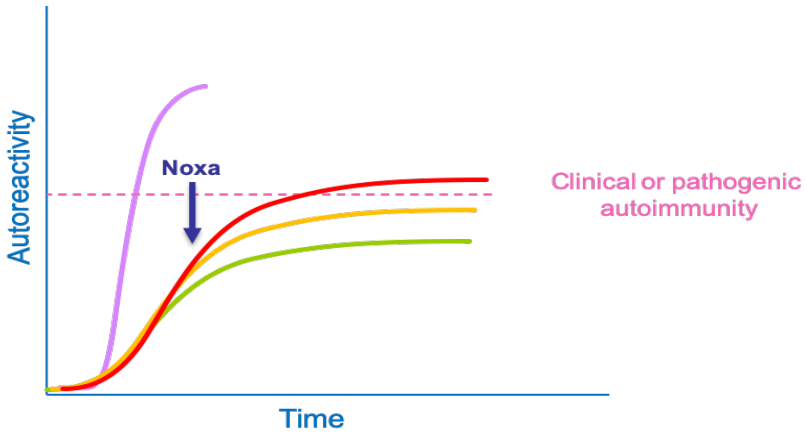

Figure **9 Different autoimmunity models for its generation**. In green, individual who never gets autoimmunity, in orange individual with a non TCR mediated autoimmunity without any noxa. In Red individual with a non TCR mediated autoimmunity that has been expose to a noxa. Lavender, TCR mediated autoimmunity.

generation and accumulation of memory cells or the death of the perinatal Treg over time. This last idea could be interesting for my project as maybe the amount of perinatal Tregs an individual can generate during his or her lifetime determines the probability of getting an autoimmune disease. It is possible that the number of perinatal Tregs in the tissues decreases gradually with time, so if you start with a low number you may not maintain enough to control a possible autoimmune reaction, thus having a breach on the peripheral tolerance mechanisms. To attempt to overcome this, we are trying to use IL33 as a way to expand Tregs and shift the balance towards tolerance. By expanding tissue Tregs we: 1) protect tissues in which tolerance is starting to fade 2) increase the local concentration of Tregs in a damaged organ that will start the repair process 3) increase Tregs within the tissue that will suppress the vicious cycle of autoimmune reaction against self. Hopefully this can be a solution that could be translated to the clinics at one point, helping to add another weapon in the arsenal of immunomodulatory drugs that rheumatologists have but hasn't advanced much more in the last 20 years aside for multiple autoimmunity treatment neither second or thrids lines of treatment since rituximab in 2006.

In conclusion, my final conceptual picture (please be aware that this is still unproven) is that perinatal Tregs seed the tissue in the first stages and have a homeostatic-organizational function within the tissue, interacting with other tissue resident immune cells (ILC2, macrophages…), neural endings, and epithelial cells to orchestrate the most exquisite system in nature: a coordinated pluricellular organ with strict functions in the body. In terms of the adult Tregs, they will act as firefighters. I think they are needed to generate tolerance towards food, commensal microbiota, and airway antigens (special remark for Rorg+ Treg). Also, they are essential in controlling the inflammation reaction, dampening the "fire" in the area and modulating the immune response to start repair. The repair and return to normal task are taken primarily, but not solely, by perinatal Tregs coordinating the other immune cells to execute a reparative role. To support this, we know that tissue Treg and not circulatory nor lymphoid Treg expand upon the expression of the alarmin IL33. We also

know that the dynamic of Treg repopulation in adult mice varies depending on the tissue you look at, showing a different repopulation ratio depending on the tissue. In addition to that, we have some data pointing to a difference on APC antigen presenting machinery and Treg progenitors skew during neonatal changes. This led to really exciting questions to be answer in the following months to try to unravel how Tregs mediate their suppressive function in the tissues.

Bibliography

1. Schirmer, M., et al. Linking the human gut microbiome to inflammatory cytokine production capacity. Cell.167(4):1125-1136 (2016).
2. Kuhn, K.A., Stappenbeck T.S. Peripheral education of the immune system by the colonic microbiota. Semin Immunol.; 25 (5): (2013).
3. Yu, W., et al. Food allergy: immune mechanisms, diagnosis and immunotherapy. Nat. Rev. Immunol. 16: 751–765 (2016).
4. Stein, M.M., et al. Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children. N Engl J Med. 375:411-21 (2016).
5. The Collected Papers of Paul Ehrlich. Yale J Biol Med.; 29(6): 628 (1957). Silverstein, A.M. Autoimmunity versus horror autotoxicus: The struggle for recognition. Nat. Immunol. 2 (4): 279-281 (2001).
6. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature 172:603–606 (1953).
7. Burnet, F. M. The Clonal Selection Theory of Antibody Formation (Cambridge University Press, London, 1959).
8. Cho, J.H., Feldman, M. Heterogeneity of autoimmune diseases: pathophysiologic insights from genetics and implications for new therapies. Nat. Medicine 21: 730–738 (2015).

9. Kau, A.L., Ahern, P.P., Griffin, N.W., Goodman, A.L. & Gordon, J.I. Human nutrition, the gut microbiome and the immune system. Nature 474, 327–336 (2011).
10. Chaudhry MS, Velardi E., Dudakov JA, van den Brink MR. Thymus: the next (re)generation. Immunol Rev. 271(1):56-71 (2016).
11. Hale, J.S., Bourslian, T.E., Turk, G. L. and Finl, P.J. Thymic output in aged mice. PNAS 103 (22), 8447-8452 (2006).
12. Klein, L., Kyewski, B., Allen, P.M., HogquistK.A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat. Rev. Immunol 14: 377–391 (2014).
13. Schatz D.G. and Swanson P.C. Annu. Rev. Genet.. 45:167–202 (2011).
14. Taniuchi, I. CD4 Helper and CD8 Cytotoxic T Cell Differentiation. Annu. Rev. Immunol. 36:579–601 (2018).
15. Takahama, Y. et al Generation of diversity in thymic epithelial cells. Nat rev. Immunol. 17: 295-305 (2017).
16. Takahama, Y. Journey through the thymus: stromal guides for T-cell development and selection. Nat. Rev. Immunology 6: 127-135 (2006).
17. Kurd N, Robey EA. T-cell selection in the thymus: a spatial and temporal perspective.Immunol Rev. 271(1):114-26 (2016).
18. Kimura MY, Thomas J, Tai X, Guinter TI, Shinzawa M, Etzensperger R, et al. Timing and duration of MHC I positive selection signals are adjusted in the thymus to prevent lineage errors. Nat Immunol 17(12):1415–23 (2016).
19. Ohigashi I, Kozai M, Takahama Y. Development and developmental potential of cortical thymic epithelial cells. Immunol Rev. 271:10-22 (2016).
20. Takahama, Y., Letterio, J. J., Suzuki, H., Farr, A. G. & Singer, A. Early progression of thymocytes along the CD4/CD8 developmental pathway is regulated by a subset of thymic epithelial cells expressing transforming growth factor beta. *J. Exp. Med.* **179,** 1495 LP-1506 (1994).

21. Hauri-Hohl M, et al.A regulatory role for TGF-β signaling in the establishment and function of the thymic medulla. Nat Immunol. 15(6):554-61 (2014).
22. Žuklys S, Handel A, Zhanybekova S, Govani F, Keller M, Maio S, et al. Foxn1 regulates key target genes essential for T cell development in postnatal thymic epithelial cells. Nat Immunol. 17(10):1206–15 (2016).
23. Mandl JN, Monteiro JP, Vrisekoop N, Germain RN. T Cell-Positive Selection Uses Self-Ligand Binding Strength to Optimize Repertoire Recognition of Foreign Antigens. Immunity 38(2):263–74 (2017).
24. Nitta T, Murata S, Sasaki K, Fujii H, Ripen AM, et al. Thymoproteasome shapes immunocompetent repertoire of CD8+ T cells. Immunity 2: 29–40 (2010).
25. Klein L, Hinterberger M, Wirnsberger G, Kyewski B. Antigen presentation in the thymus for positive selection and central tolerance induction. Nat Rev Immunol. 9(12):833-44 (2009).
26. Viret, C., et al. Thymus-specic serine protease contributes to the diversi cation of the functional endogenous CD4 T cell receptor repertoire. J. Exp. Med. 208 (1): 3-11 (2010).
27. Nedjic, J., et al.Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. Nature 455: 396-400 (Nedjic, J., et al.Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. Nature 455: 396-400 (2008).
28. Nedjic, J., et al. Macroautophagy, endogenous MHC II loading and T cell selection: the benefits of breaking the rules. Current Opinion in Immunology 21 (1): 92-97 (2009).
29. Brisson L, Pouyet L, N'guessan P, et al. The Thymus-Specific Serine Protease TSSP/PRSS16 Is Crucial for the Antitumoral Role of CD4+ T Cells. Cell Rep. 10(1):39-46 (2017).
30. Perry, J.S., Lio, C.W., Kau, A.L., Nutsch, K., Yang, Z., Gordon, J.I., Murphy, K.M., and Hsieh, C.S.. Distinct contributions of Aire and antigen- presenting-cell subsets to the generation of self-tolerance in the thymus. Immunity 41, 414–426 (2014).

31. Anderson, M. S. *et al.* Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein. *Science (80-. .* **298,** 1395 LP-1401 (2002).
32. Malhotra D, Linehan JL, Dileepan T, Lee YJ, Purtha WE, Lu J V, et al. Tolerance is established in polyclonal CD4+ T cells by distinct mechanisms, according to self-peptide expression patterns. Nat Immunol 17(2):187–95 (2016).
33. McCaughtry TM, Baldwin TA, Wilken MS, Hogquist KA. Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. J Exp Med. 205(11):2575 LP-2584 (2008).
34. Perry, J. S. A. and Hsieh, C.-S. Development of T-cell tolerance utilizes both cell-autonomous and cooperative presentation of self-antigen. Immunol Rev, 271: 141–155 (2016).
35. Martín-Gayo, E. *et al.* Spatially restricted JAG1-Notch signaling in human thymus provides suitable DC developmental niches. *J. Exp. Med.* **214,** 3361 LP-3379 (2017).
36. Li, J., Park, J., Foss, D. & Goldschneider, I. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. *J. Exp. Med.* **206,** 607 LP-622 (2009).
37. Hu et al., CCR7 Modulates the Generation of Thymic Regulatory T Cells by Altering the Composition of the Thymic Dendritic Cell Compartment. Cell Reports 21, 168–180 (2017).
38. Bonasio, R. *et al.* Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. *Nat. Immunol.* **7,** 1092 (2006).
39. Hadeiba, H. et al. Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. Immunity 36, 438–450 (2012).
40. KleinL, et al. Sampling of complementing self antigen pools by thymic stromal cells maximizes the scope of central tolerance Eur J Immunol. 31: 2476-2486 (2001).

41. Martín-Gayo, E., Sierra-Filardi, E., Corbí, A. L. & Toribio, M. L. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* **115,** 5366 LP-5375 (2010).
42. Guerri, L. *et al.* Analysis of APC Types Involved in CD4 Tolerance and Regulatory T Cell Generation Using Reaggregated Thymic Organ Cultures. *J. Immunol.* **190,** 2102 LP-2110 (2013).
43. Yamano T, Nedjic J, Hinterberger M, Steinert M, Koser S, Pinto S, et al. Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction. Immunity 42(6):1048–6 (2017).
44. Abramson, J. & Anderson, G. Thymic Epithelial Cells. *Annu. Rev. Immunol.* **35,** 85–118 (2017).
45. Lucas, B., McCarthy, N. I., Baik, S., Cosway, E., James, K. D., Parnell, S. M., White, A. J., Jenkinson, W. E. and Anderson, G. Control of the thymic medulla and its influence on αβT-cell development. Immunol Rev. 271: 23–37 (2016).
46. Malchow s, et al. Aire-dependent thymic development of tumor-associated regulatory T cells. Science.339(6124):1219-24 (2013).
47. Meredith, M. et al.Aire controls gene expression in the thymic epithelium with ordered stochasticity (2015).
48. Lopes, N., Sergé, A., Ferrier, P. & Irla, M. Thymic Crosstalk Coordinates Medulla Organization and T-Cell Tolerance Induction . *Frontiers in Immunology* **6,** 365 (2015).
49. Bornstein, C. *et al.* Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells. *Nature* **559,** 622–626 (2018).
50. Miller, C. N. *et al.* Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development. *Nature* **559,** 627–631 (2018).

51. Hikosaka Y, et al.The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. Immunity. 19;29(3):438-50 (2008).
52. Akiyama N, Shinzawa M, Miyauchi M, Yanai H, Tateishi R, Shimo Y, et al. Limitation of immune tolerance–inducing thymic epithelial cell development by Spi-B–mediated negative feedback regulation. J Exp Med. 211(12):2425 LP-2438 (2014).
53. McCarthy NI, Cowan JE, Nakamura K, Bacon A, Baik S, White AJ, et al. Osteoprotegerin-Mediated Homeostasis of Rank+ Thymic Epithelial Cells Does Not Limit Foxp3+ Regulatory T Cell Development. J Immunol 195(6):2675 LP-2682 (2015).
54. Taniguchi, R.T., Anderson, M.S. The role of Aire in clonal selection. Immunology and cell biology 89:40-44 (2011).
55. Metzger, T.C., Anderson, M.S. Control of central and peripheral tolerance by Aire. Immunol. Reviews 241:89-103 (2011).
56. Mathis D, Benoist C. A decade of AIRE. Nat Rev Immunol 7(8):645–50 (2007).
57. Rattay, K., et al. Evolutionary conserved gene co-expression drives generation of self-antigen diversity in medullary thymic epithelial cells. Journal of Autoimmunity 67: 65e75 (2016).
58. Goldrath AW, Bevan MJ. Selecting and maintaining a diverse T-cell repertoire. Nature 402(18) (1999).
59. Raff, M.C. Social controls on cell survival and cell death. Nature 365:397-400 (1992).
60. Ernst B, Lee DS, Chang JM, Sprent J, Surh CD.The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. Immunity. 11(2):173-81 (1999).
61. Theofilopoulos, A.N., et al.T cell homeostasis and systemic autoimmunity. J Clin Invest. 108(3): 335–340 (2001).

62. Manson, D. A very high level of crossreactivity is an essential feature of the T- cell receptor. Immunology toda 19 (9):395-404 (1998).
63. Stefanova I, Dorfman JR, Germain RN. Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. Nature 420(6914):429-434 (2002).
64. Lo WL, Felix NJ, Walters JJ, Rohrs H, Gross ML, Allen PM. An endogenous peptide positively selects and augments the activation and survival of peripheral CD4+ T cells. Nat Immunol 10: 1155–1161 (2009).
65. Morris GP, Allen PM. How the TCR balances sensitivity and specificity for the recognition of self and pathogens. Nat Immunol. 13(2):121-8 (2012).
66. Kieper WC, Burghardt JT, Surh CD. A Role for TCR Affinity in Regulating Naive T Cell Homeostasis. J Immunol. 172(1):40 LP-44 (2003).
67. Karen M. Wheeler, Eileen T. Samy and Kenneth S. K. Tung. Normal Regional Lymph Node Enrichment of Antigen-Specific Regulatory T Cells with Autoimmune Disease-Suppressive Capacity. J Immunol. 183:7635-7638 (2009).
68. Mittelstadt, P. R., Taves, M. D. & Ashwell, J. D. Cutting Edge: De Novo Glucocorticoid Synthesis by Thymic Epithelial Cells Regulates Antigen-Specific Thymocyte Selection. *J. Immunol.* **200,** 1988 LP-1994 (2018).
69. Thome, J. J. C. *et al.* Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. *Nat. Med.* **22,** 72 (2015).
70. Josefowicz SZ, Lu L-F, Rudensky AY. Regulatory T Cells: Mechanisms of Differentiation and Function. Annu Rev Immunol. 30(1):531 (2012).
71. Klein, L., Robey, E. A. & Hsieh, C.-S. Central CD4+ T cell tolerance: deletion versus regulatory T cell differentiation. *Nat. Rev. Immunol.* **19,** 7–18 (2019).
72. Lio CWJ, Hsieh CS. A Two-Step Process for Thymic Regulatory T Cell Development. Immunity28(1):100–11 (2008).

73. Burchill, M.A., et al. Linked T Cell Receptor and Cytokine Signaling Govern the Development of the Regulatory T cell Repertoire. Immunity 28(1): 112–121 (2008).
74. Kitagawa, Y., et al. Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. Nature Immunology 18: 173–183 (2017).
75. Pinto, S., Michel, C., et al. Overlapping gene coexpression patterns in human medullary thymic epithelial cells generate self-antigen diversity.PNAS 110 (37): E3497-E3505 (2013).
76. Weist, B. M., Kurd, N., Boussier, J., Chan, S. W. & Robey, E. A. Thymic regulatory T cell niche size is dictated by limiting IL-2 from antigen-bearing dendritic cells and feedback competition. *Nat. Immunol.* **16,** 635 (2015).
77. Beyer M, Huehn J. Epigenetic orchestration of thymic Treg cell development. Nat Immunol. 136 (2017).
78. Tai, X., et al Foxp3 Transcription Factor Is Proapoptotic and Lethal to Developing Regulatory T Cells unless Counterbalanced by Cytokine Survival Signals. Immunity 38 (6): 1116-1128 (2013).
79. Kitagawa, Y. *et al.* Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat. Immunol.* **18,** 173 (2016).
80. Feng, Y. *et al.* A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance. *Nature* **528,** 132 (2015).
81. Cowan, J.E., et al. The thymic medulla is required for Foxp3+regulatory but not conventional CD4+thymocyte development. J Exp Med. 210(4): 675–681 (2013).
82. Aschenbrenner , K. , L.M. D ' Cruz , E.H. Vollmann , M. Hinterberger , J. Emmerich , L.K. Swee , A. Rolink , and L. Klein . Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. Nat. Immunol. 8 : 351 – 358 (2007).

83. WatanabeN,WangYH,LeeHK,ItoT,WangYH,CaoW,etal. Hassall's corpuscles instruct dendritic cells to induce CD4 CD25 regulatory T cells in human thymus. Nature 436: 1181-5 (2005).

84. Malchow, S., Leventhal, D.S., Nishi, S., Fischer, B.I., Shen, L., Paner, G.P., Amit, A.S., Kang, C., Geddes, J.E., Allison, J.P., et al. Aire-dependent thymic development of tumor-associated regulatory T cells. Science 339, 1219–1224 (2013).

85. Proietto, A. I. *et al.* Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc. Natl. Acad. Sci.* **105,** 19869 LP-19874 (2008).

86. Chen, W. and Konkel, J. E. Development of thymic Foxp3+ regulatory T cells: TGF-β matters. Eur. J. Immunol., 45: 958-965 (2015),

87. Coquet, J. M. *et al.* Epithelial and dendritic cells in the thymic medulla promote CD4⁺Foxp3⁺ regulatory T cell development via the CD27–CD70 pathway. *J. Exp. Med.* **210,** 715 LP-728 (2013).

88. Liston, A. et al. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. Proc. Natl Acad. Sci. USA 105, 11903–11908 (2008).

89. Lee, H. M., Bautista, J. L., Scott-Browne, J., Mohan, J. F. & Hsieh, C. S. A Broad Range of Self-Reactivity Drives Thymic Regulatory T Cell Selection to Limit Responses to Self. *Immunity* **37,** 475–486 (2012).

90. Pacholczyk, R., Ignatowicz, H., Kraj, P. & Ignatowicz, L. Origin and T Cell Receptor Diversity of Foxp3+CD4+CD25+ T Cells. *Immunity* **25,** 249–259 (2006).

91. Burchill, M. A., Yang, J., Vogtenhuber, C., Blazar, B. R. & Farrar, M. A. IL-2 Receptor β-Dependent STAT5 Activation Is Required for the Development of Foxp3+ Regulatory T Cells. *J. Immunol.* **178,** 280 LP-290 (2007).

92. Burchill, M. A. *et al.* Linked T Cell Receptor and Cytokine Signaling Govern the Development of the Regulatory T Cell Repertoire. *Immunity* **28,** 112–121 (2008).

93. Mahmud, S. A. *et al.* Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nat. Immunol.* **15,** 473–481 (2014).

94. Vang, K. B. *et al.* Interleukin-2, -7 and -15, but not TSLP, Redundantly Govern CD4+ Foxp3+ Regulatory T Cell Development. *J. Immunol.* **181,** 3285–3290 (2008).

95. Surh, C. D. & Sprent, J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* **372,** 100–103 (1994).

96. Fontenot, J. D., Dooley, J. L., Farr, A. G. & Rudensky, A. Y. Developmental regulation of Foxp3 expression during ontogeny. *J. Exp. Med.* **202,** 901 LP-906 (2005).

97. Ouyang, W., Beckett, O., Ma, Q. & Li, M. O. Transforming growth factor-β signaling curbs thymic negative selection promoting regulatory T cell development. *Immunity* **32,** 642–653 (2010).

98. Marie, J. C., Letterio, J. J., Gavin, M. & Rudensky, A. Y. TGF-β1 maintains suppressor function and Foxp3 expression in CD4⁺CD25⁺ regulatory T cells. *J. Exp. Med.* **201,** 1061 LP-1067 (2005).

99. Konkel, J. E., Jin, W., Abbatiello, B., Grainger, J. R. & Chen, W. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. *Proc. Natl. Acad. Sci.* **111,** E465 LP-E473 (2014).

100. Owen, D. L. *et al.* Thymic regulatory T cells arise via two distinct developmental programs. *Nat. Immunol.* **20,** 195–205 (2019).

101. Thiault, N. *et al.* Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors. *Nat Immunol* **16,** 628–634 (2015).

102. Levine, A. G. *et al.* Suppression of lethal autoimmunity by regulatory T cells with a single TCR specificity. *J. Exp. Med.* **214,** 609 LP-622 (2017).

103. Cowan, J. E., McCarthy, N. I. & Anderson, G. CCR7 Controls Thymus Recirculation, but Not Production and Emigration, of Foxp3+ T Cells. *Cell Rep.* **14,** 1041–1048 (2016).

104. Yang, S., Fujikado, N., Kolodin, D., Benoist, C. & Mathis, D. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance. *Science (80-.).* **348,** 589 LP-594 (2015).

105. Arpaia, N. *et al.* A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* **162,** 1078–1089 (2015).

106. Chinen, T. *et al.* An essential role for the IL-2 receptor in Treg cell function. *Nat. Immunol.* **17,** 1322 (2016).

107. Smith, N. L. *et al.* Developmental Origin Governs CD8 + T Cell Fate Decisions during Infection. *Cell* **174,** 117–130.e14 (2018).

108. Debock, I. & Flamand, V. Unbalanced Neonatal CD4+ T-Cell Immunity . *Frontiers in Immunology* **5,** 393 (2014).

109. Mold, J. E. *et al.* Maternal Alloantigens Promote the Development of Tolerogenic Fetal Regulatory T Cells in Utero. *Science (80-.).* **322,** 1562 LP-1565 (2008).

110. Mold, J. E. *et al.* Fetal and Adult Hematopoietic Stem Cells Give Rise to Distinct T Cell Lineages in Humans. *Science (80-.).* **330,** 1695 LP-1699 (2010).

111. Opiela, S. J., Koru-Sengul, T. & Adkins, B. Murine neonatal recent thymic emigrants are phenotypically and functionally distinct from adult recent thymic emigrants. *Blood* **113,** 5635 LP-5643 (2009).

112. Zhang, X., Zhivaki, D. & Lo-Man, R. Unique aspects of the perinatal immune system. *Nat. Rev. Immunol.* **17,** 495 (2017).

113. Adkins, B., Leclerc, C. & Marshall-Clarke, S. Neonatal adaptive immunity comes of age. *Nat. Rev. Immunol.* **4,** 553 (2004).

114. Muthukkumar, S., Goldstein, J. & Stein, K. E. The Ability of B Cells and Dendritic Cells to Present Antigen Increases During Ontogeny. *J. Immunol.* **165,** 4803 LP-4813 (2000).

115. McGovern, N. *et al.* Human fetal dendritic cells promote prenatal T-cell immune suppression through arginase-2. *Nature* **546,** 662 (2017).

116. Adkins, B. Developmental regulation of the intrathymic T cell precursor population. *J. Immunol.* **146,** 1387 LP-1393 (1991).

117. Adkins, B., Williamson, T., Guevara, P. & Bu, Y. Murine Neonatal Lymphocytes Show Rapid Early Cell Cycle Entry and Cell Division. *J. Immunol.* **170,** 4548 LP-4556 (2003).

118. Gollwitzer, E. S. *et al.* Lung microbiota promotes tolerance to allergens in neonates via PD-L1. *Nat. Med.* **20,** 642 (2014).

119. Wang, G. *et al.* 'Default' Generation of Neonatal Regulatory T Cells. *J. Immunol.* **185,** 71 LP-78 (2010).

120. Scharschmidt, T. C. *et al.* A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes. *Immunity* **43,** 1011–1021 (2015).

121. Odegaard, J. I. *et al.* Perinatal Licensing of Thermogenesis by IL-33 and ST2. *Cell* **166,** 841–854 (2016).

122. Mathis, D. IL-33, Imprimatur of Adipocyte Thermogenesis. *Cell* **166,** 794–795 (2016).

123. de Kleer, I. M. *et al.* Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung. *Immunity* **45,** 1285–1298 (2016).

124. Straub, J.M., New, J., Hamilton, C.D. et al. J Cancer Res Clin Oncol 141: 1985 (2015).

125. Garboczi, D. N., P. Ghosh, U. Utz, Q. R. Fan, W. E. Biddison, and D. C. WIley. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. Nature 384:134-141 (1996).

126. Baba, T., Nakamoto, Y. & Mukaida, N. Crucial Contribution of Thymic Sirpα+; Conventional Dendritic Cells to Central Tolerance against Blood-Borne Antigens in a CCR2-Dependent Manner. *J. Immunol.* **183,** 3053 LP-3063 (2009).

127. van Blijswijk, J , Schraml, BU , Reis e Sousa, C. Advantages and limitations of mouse models to deplete dendritic cells. Eur J Immunol. 2013; 43(1): 22–6.

128. Liston, A. *et al.* Differentiation of regulatory Foxp3⁺ T cells in the thymic cortex. *Proc. Natl. Acad. Sci.* **105,** 11903 LP-11908 (2008).

129. Guerau-de-Arellano, M., Martinic, M., 1. Bornstein, C. et al.ist, C. & Mathis, D. Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity. *J. Exp. Med.* **206,** 1245 LP-1252 (2009).

130. Bogue M, Gilfillan S, Benoist C, Mathis D.. Regulation of n-region diversity in antigen receptors through thymocyte differentiation and thymus ontogeny. Proceedings of the National Academy of Sciences 89:11011– 11015 (1992).

131. Robey, I. F. *et al.* Terminal Deoxynucleotidyltransferase Deficiency Decreases Autoimmune Disease in Diabetes-Prone Nonobese Diabetic Mice and Lupus-Prone MRL- Fas lpr Mice. *J. Immunol.* **172,** 4624 LP-4629 (2004).

132. Conde, C. *et al.* Terminal Deoxynucleotidyl Transferase Deficiency Reduces the Incidence of Autoimmune Nephritis in (New Zealand Black × New Zealand White)F1 Mice. *J. Immunol.* **161,** 7023 LP-7030 (1998).