

Australian National University

# **Regulatory roles of IL-10-producing human follicular T cells**

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A thesis submitted for the degree of Doctor of Philosophy of The Australian National  
University

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*To my beautiful wife Francesca*

## Statement

This thesis is original work undertaken at the Department of Immunology and Infectious diseases, John Curtin School of Medical Research, Australian National University. The results herein, except where stated otherwise, were performed by me under the supervision of Professor Carola G Vinuesa.

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## Abbreviations

|         |   |
|---------|---|
| 7-AAD   | 7-amioactinomycin D                         |
| a       | Alexa fluor                                 |
| Ab      | Antibody                                    |
| ANU     | The Australian National University          |
| APC     | Antigen presenting cell                     |
| APF     | Australian Phenomics Facility               |
| B6      | C57BL/6 strain of mice                      |
| BCL6    | B cell lymphoma 6 protein                   |
| BCR     | B cell receptor                             |
| BLIMP-1 | B lymphocyte induced maturation protein 1   |
| BRF     | Bimolecular Resource Facility               |
| BSA     | Bovine serum albumin                        |
| BUV     | Brilliant Ultraviolet                       |
| BV      | Brilliant violet                            |
| CD      | Cluster of differentiation                  |
| CD40 L  | CD40 ligand                                 |
| cDNA    | Complementary deoxyribonucleic acid         |
| CNS     | Conserved non-coding sequence               |
| CSIF    | Cytokine-synthesis inhibitory factor        |
| CTLA4   | Cytotoxic T lymphocyte-associated antigen 4 |
| CXCL13  | CXC motif chemokine ligand 13               |
| CXCR5   | C-X-C chemokine receptor type 5             |
| Cy5.5   | Cyanine 5.5                                 |
| Cy7     | Cyanine 7                                   |
| DAPI    | 4',6-diamidino-2-phenylindole               |
| DEPC    | Diethylpyrocarbonate                        |
| DNA     | Deoxyribonucleic acid                       |
| dNTP    | Deoxynucleotide or Nucleoside triphosphate  |

|                   |  |
|-------------------|--|
| DTT               | Dithiothreitol   |
| DZ                | Dark zone  |
| FACS              | Fluorescent activated cells sorting                                    |
| FITC              | Fluorescein isothiocyanate   |
| FOXP3             | Forkhead/winged-helix transcription factor box P3                      |
| FSC               | Forward light scatter  |
| g (n, $\mu$ , m)  | Grams (nano, micro, mili)  |
| GC                | Germinal centre  |
| GITR              | Glucocorticoid-induced tumour necrosis factor receptor-related protein |
| GLT               | Germline transcript  |
| Ig                | Immunoglobulin   |
| IgA               | Immunoglobulin A   |
| IgG               | Immunoglobulin G   |
| IgE               | Immunoglobulin E   |
| IL                | Interleukin  |
| iT <sub>reg</sub> | Induced T regulatory cell  |
| IV                | Intravenous  |
| JCSMR             | The John Curtin School of Medical Research                             |
| KO                | Knockout   |
| L ( $\mu$ ,m)     | Litre (micro, mili-)   |
| LAG3              | Lymphocyte activating gene 3   |
| LZ                | Light zone   |
| MCRF              | Microscopy and Cytometric Resource Facility                            |
| MFI               | Mean fluorescent intensity   |
| MHC               | Major histocompatibility complex                                       |
| mRNA              | Messenger ribonucleic acid   |
| ns                | Not significant  |
| tT <sub>reg</sub> | Thymic T regulatory cell   |
| qPCR              | Quantitative Polymerase chain reaction                                 |

|                   |   |
|-------------------|---|
| PD-1              | Programed cell death protein 1                                    |
| PD-L1             | Programed cell death protein 1 ligand 1                           |
| PE                | Pycoerythrin  |
| PerCP             | Peridinin-chlorophyll a-protein complex                           |
| PMA               | Phorbol 12-myristate 13-acetate                                   |
| pSTAT3            | Phosphorylated signal transducer and activator of transcription 3 |
| pT <sub>reg</sub> | Peripherally-derived T <sub>reg</sub>                             |
| qPCR              | Quantitative Polymerase chain reaction                            |
| r                 | Recombinant   |
| RNA               | Ribonucleic acid  |
| RPMI              | Roswell Park Memorial Institute                                   |
| RU                | Relative units  |
| SAP               | Signalling lymphocyte activation molecule-associated protein      |
| SEB               | Staphylococcal Enterotoxin B                                      |
| SH2D1A            | Src homology 2 domain containing protein 1A                       |
| SLAM              | Signalling lymphocyte activation molecule                         |
| SRBC              | Sheep red blood cell  |
| STAT3             | Signal transducer and activator of transcription 3                |
| T <sub>FH</sub>   | T follicular helper   |
| T <sub>FR</sub>   | T follicular regulatory   |
| T <sub>H1</sub>   | T helper type 1   |
| T <sub>H2</sub>   | T helper type 2   |
| T <sub>r1</sub>   | Type 1 regulatory cell  |
| T <sub>reg</sub>  | T regulatory cell   |
| U                 | units   |
| WT                | Wild type   |



# Abstract

Mucosal lymphoid tissues like human tonsils are colonized by bacteria and exposed to ingested and inhaled antigens, requiring tight regulation of immune responses. Antibody responses are regulated by follicular helper T cells ( $T_{FH}$ ) and  $Foxp3^+$  follicular regulatory T cells ( $T_{FR}$ ). Here we describe a subset of human tonsillar follicular T cells ( $T_F$ ) identified by expression of  $T_{FH}$  cell markers and CD25 that are the main source of T cell-derived IL-10 in the follicle. Despite lack of FOXP3 expression,  $CD25^+$   $T_F$  cells resemble  $T_{regs}$  in high CTLA4 expression, low IL-2 production, and their ability to repress T cell proliferation. IL-10 boosts conversion of human tonsil  $T_{FH}$  cells into FOXP3-expressing cells, possibly promoting peripherally- induced  $T_{FR}$  cells at mucosal sites.  $CD25^+$   $T_F$  cell-derived IL-10 inhibits B cell class-switching to IgE. In children, serum total IgE titers were inversely correlated with the frequencies of tonsil  $CD25^+$   $T_F$  cells and IL-10-producing  $T_F$  cells but not with total  $T_{regs}$ ,  $T_{FR}$  or IL-10-producing T cells. Thus,  $CD25^+$   $T_F$  cells emerge as a subset with unique T and B cell regulatory activities that may help prevent atopy.



# **Chapter 1: Introduction**

## 1. Overview of the adaptive immune system

The environment we live in is populated by an alarming number of harmful microorganisms, toxins and allergens that pose a risk to the body's homeostasis. Such exposure provides a great threat to humans and many higher vertebrates, as infections and allergies can quickly ensue upon contact with these substances. To overcome this threat, natural selection has designed an ingenious army that defends the body against infectious agents. This army is known as the immune system, and it comprises a highly evolved set of organs, tissues, cells, molecules and chemical mediators that aim to recognize, neutralize and clear foreign invaders. The immune system is composed of two broad arms, namely innate and adaptive immunity (Bonilla and Oettgen, 2010). The innate immune system provides the first line of defense. It is comprised of physical barriers, such as the skin and mucosal surfaces that protect the sterile environment of the body, as well as an array of immune cells, including myeloid cells, natural killer cells and innate lymphoid cells that recognize and respond to a broad range of microbial substances. Innate immune cells express receptors able to recognize generic patterns distinctive of parasites, bacteria or viruses; such as microbial DNA or RNA, bacterial lipopolysaccharides, peptidoglycans and lipoproteins (Chaplin, 2010). The adaptive immune system consists of T and B cells that specifically recognize antigens through surface receptors and mount a defensive antibody or cytotoxic response to eliminate the microbe (Bonilla and Oettgen, 2010).

Antigen presenting cells (APCs) including macrophages, B cells and dendritic cells (the latter considered the "professional APCs"), can recognize and take up foreign proteins for presentation to T cells. These foreign proteins are known as antigens, which are essentially substances exogenous to the organism, capable of generating antibody or cell-mediated responses. APCs can recognize antigens, internalize them and break them down into smaller peptides (Rossjohn et al., 2015). After such antigen processing, APCs can then load these peptides onto the Major Histocompatibility Antigen complex (MHC class II) and present them to T lymphocytes. This process is known as antigen presentation, and serves as the first step by which adaptive immune responses are initiated (Heath and Carbone, 2001). Not only

can the adaptive immune system react and respond to millions of different antigens, but it can also perpetuate immune responses for decades or even the lifetime of the host through immunological memory.

The most extraordinary characteristic of the adaptive immune system is its ability to generate a diverse repertoire of cells that can respond to countless numbers of antigens. Such diversity comes from the unique properties of antigen specific receptors expressed on the surface of B and T lymphocytes. These, namely T cell and B cell receptors (TCR and BCR), are encoded by polygenic and polymorphic gene segments that randomly recombine - via a process called V, (D), J recombination - to assemble a unique B or T cell receptor DNA sequence (Schatz and Ji, 2011). In essence, as lymphocytes develop in primary lymphoid organs each clone commits to an individual antigen specificity.

### 1.1. Overview of B and T lymphocytes

B cells comprise the humoral arm of the immune system, which is critical in providing effective immunity and pathogen clearance in the form of antibody production. The BCR, also known as antibody, can be membrane-bound – which acts to recognize and bind to native foreign antigens, or secreted, which is produced once a B cell has undergone differentiation to become a plasmablast or in its terminally-differentiated form, a plasma cell. Antibodies bind to molecular structures, known as antigens, found on foreign invaders or toxins, and can either directly neutralize them or opsonize them so as to tag them for ingestion and destruction by phagocytes and other innate cells, thus preventing them from harming the host (Maity et al., 2018). Structurally, antibodies are composed of two large heavy chains and two small light chains that assemble together to form a Y-shaped immunoglobulin. These can be further broken down into the crystallizable fragment (Fc) region and the antibody-binding fragment (Fab) (Lu et al., 2018). The latter contains the variable region responsible for antigen-antibody interactions, whilst the former is responsible for determining antibody effector functions.

There are many different types of heavy chains that make up the five different Fc classes, namely antibody isotypes. The default isotypes on naïve B cells are IgM and IgD. However, activated B cells can undergo a process known as class switch recombination (CSR), whereby antibodies are able to switch to a different isotype whilst retaining the antigen-specific variable region (Schroeder and Cavacini, 2010). The other three isotypes, namely IgG, IgA and IgE carry out important immunological functions, and the choice of the isotype to which a B cell switches to is governed by the cytokine milieu in which B cell activation occurs. For instance, IgA is important in mediating immunity at mucosal sites, whilst IgE plays a central role in protecting parasitic and helminth infections and is responsible for many allergic conditions by triggering a chain of events leading to the release of histamine and other chemical mediators by mast cells and basophils (Stavnezer et al., 2008).

B cells can increase the affinity of the antibodies they produce during the process of affinity maturation that occurs in germinal centers (GCs), reviewed below. It is widely accepted that high affinity antibodies are crucial for effective humoral protection, as these can readily bind to their target antigen even at very low concentrations. High affinity antibodies are produced by memory B cells and long-lived plasma cells. Such long-lived plasma cells typically reside in the bone marrow and can live for decades or even the lifetime of an individual (Manz et al., 2005). These cells act as factories of high affinity antibodies and provide the host with a continuous supply of very powerful molecular weapons, which can effectively neutralize foreign bodies before they can even establish an infection. Production of long-lived high affinity antibodies is the ultimate goal of B cell responses. It determines the success of most immunization regimens and forms the basis of humoral memory. The establishment, maturation and maintenance of humoral immune responses are, at least in part, directed and orchestrated by specialized helper T cells (Brynjolfsson et al., 2018).

T cells originate from bone marrow progenitors, which migrate to the thymus for maturation and eventual release to peripheral tissues. Unlike B cells, which recognize native antigen, T cells recognize antigen in the context of peptide coupled to the MHC complex (pMHC). This peptide

is typically derived from exogenous or endogenous foreign antigens after being broken down and processed by cells expressing MHC. The type of MHC that a single clone of naïve T cell recognizes will determine its fate, as either cytotoxic CD8<sup>+</sup> T cells or helper CD4<sup>+</sup> T cells. The former subset recognizes pMHC class I, which is ubiquitously expressed by all cells of the body (except for non-nucleated cells), and is usually loaded with foreign endogenous peptides. As such, the main role of CD8<sup>+</sup> T cells is to kill infected, cancerous or damaged cells. CD4<sup>+</sup> T cells predominantly recognize exogenous foreign antigens presented on MHC class II. Expression of this complex is restricted to APCs, which include monocytes, macrophages, dendritic cells and even B cells. Interaction of the TCR with its cognate antigen, and in the presence of appropriate co-stimulatory signals, results in T cell activation (Murphy, 2016).

The context in which T cell activation occurs is crucial, as specific cytokine environments induce distinct transcriptional networks that govern and direct T cell differentiation towards a specific cell subset. There are a growing number of well-established CD4<sup>+</sup> T cell subsets, which include but are not limited to T helper 1 (T<sub>H1</sub>), T helper 2 (T<sub>H2</sub>), follicular helper T (T<sub>FH</sub>), T helper 17 (T<sub>H17</sub>) and regulatory T cells (T<sub>regs</sub>). These subsets orchestrate immune responses to specific types of threats through the release of soluble mediators and cell contact-dependent mechanisms. Amongst others, CD4<sup>+</sup> T cell responses direct macrophage activation (T<sub>H1</sub>), help clear parasitic infections (T<sub>H2</sub>), assist immune responses at mucosal sites (T<sub>H17</sub>), help B cells (T<sub>FH</sub>) and suppress autoimmunity and allergy (T<sub>regs</sub>) (Zhu et al., 2010).

## 2. Tolerance

Lymphocyte diversity is a product of the stochastic and combinatorial assembly of TCRs and BCRs. As such, the body is able to generate a large repertoire of cells that, individually are able to recognize a unique antigen specificity, but collectively respond to virtually an endless number of molecular structures. Nevertheless, such ability to generate diversity will inevitably produce receptors that recognize self-antigens, as well as innocuous foreign antigens such as foods, commensal bacteria and harmless inhaled substances. Activation of B cells or T cells bearing such specificities can eventually lead to life threatening conditions, such as autoimmune pathology and

severe allergic reactions. To overcome this problem, the adaptive immune system has evolved a series of checkpoints that preclude self-reactive lymphocytes from targeting and destroying self-tissues or reacting against harmless substances. Together, these mechanisms comprise immunological tolerance.

### 2.1. Historical context

The term tolerance refers to the ability of the immune system to remain unresponsive to self or innocuous antigens. Ray Owen first described this phenomenon after his immunization experiments on dizygotic twin cows (Owen, 1945). Unlike adult cows, which generated antibody responses when injected with other unrelated adult red blood cells, the twin cows did not respond when injected with each other's RBCs. Given that twin cows experienced placental blood exchange during embryonic development, Ray Owen concluded that this unresponsiveness was related to the twins having a mixture of each other's hematopoietic blood cells. This observation was later confirmed in experiments involving tissue engraftments across allogeneic mice. Medawar and colleagues observed that, unlike adult mice, which rejected the allogeneic grafts by day 12, newborn mice tolerated the transplant (Billingham et al., 1953). These results proposed the notion that tolerance is an acquired ability of the body, which is learnt upon antigen exposure throughout development.

Given that Medawar and colleagues did not set out to study the immunological consequences of tolerance, it was later that F.M. Burnet put these results into the context of clonal selection theory (Burnet, 1957). Medawar and Burnet were eventually credited for the discovery of acquired immunological tolerance and were awarded the Nobel Prize for physiology or medicine in 1960. Their theory proposed that each lymphocyte had unique antigen receptors on their surface. Activation would ensue if these receptors were occupied by a foreign substance, but would lead to clonal deletion if bound to self-antigens during development. Over the past decades, a large body of knowledge has been generated regarding immunological tolerance (Kyewski and Klein, 2006). We now know that tolerance comprises a series of checkpoint mechanisms aiming to reduce the chances of self-reactive lymphocytes to participate in immune responses. These

include, but are not limited to, central tolerance, clonal deletion, anergy and peripheral tolerance mechanisms.

## 2.2. Central tolerance

Central tolerance refers to a series of checkpoints that lymphocyte precursors undergo throughout development, which typically lead to clonal deletion of self-reactive cells before being able to be released as mature lymphocytes in the periphery. This occurs in the thymus and the bone marrow for T cells and B cells respectively. In essence, specialized cells in primary lymphoid tissues are able to present self-antigens and test for self-reactivity on maturing lymphocytes, whereby binding of self-proteins to self-reactive clones results in clonal deletion or anergy.

B cell tolerance predominantly occurs in the bone marrow, whereby interaction of BCRs with self-antigens generally results in receptor editing. Failure to assemble an “edited” BCR that does not bind to self-antigen results in programmed cell death (Kurosaki et al., 2010). In contrast, T cell tolerance is a more sophisticated process that begins when T cell precursors migrate to the thymus. In here, T cells begin to rearrange their TCR-encoding genes, with failure to assemble a productive TCR leading to the first wave of apoptosis. Those cells that successfully re-arrange a productive TCR undergo a sequential education processes that constitute T cell selection. This selection ensures that T cells: 1) T cells can recognize autologous MHC complexes, and 2) clones that bind to self-reactive peptides are selected against (Koch and Radtke, 2011). The former process is known as positive selection, and this guarantees that T cells are able to recognize either MHC class I or class II in order to elicit immune responses. T cells that survive positive selection migrate to the medulla of the thymus and interact with medullary thymic epithelial cells (mTECs) (Klein et al., 2014). The most fascinating characteristic of these cells is their ability to express tissue-specific peripheral antigens that are distal to the thymus, such insulin (Derbinski et al., 2001). Expression of these genes is controlled, at least in part, by the transcription factor Autoimmune Regulator (AIRE) (Anderson et al., 2002). Binding of T cells to self-antigen represents a potential threat to the host, as immune responses against self-tissues could ensue

upon release of self-reactive thymocytes to the periphery. Thus, self-antigen recognition typically leads to negative selection and programmed cell death. Additionally, a subset of CD4<sup>+</sup> T cells that bind strongly to self-antigens will emerge as regulatory T cells, which are the key players in mediating peripheral tolerance.

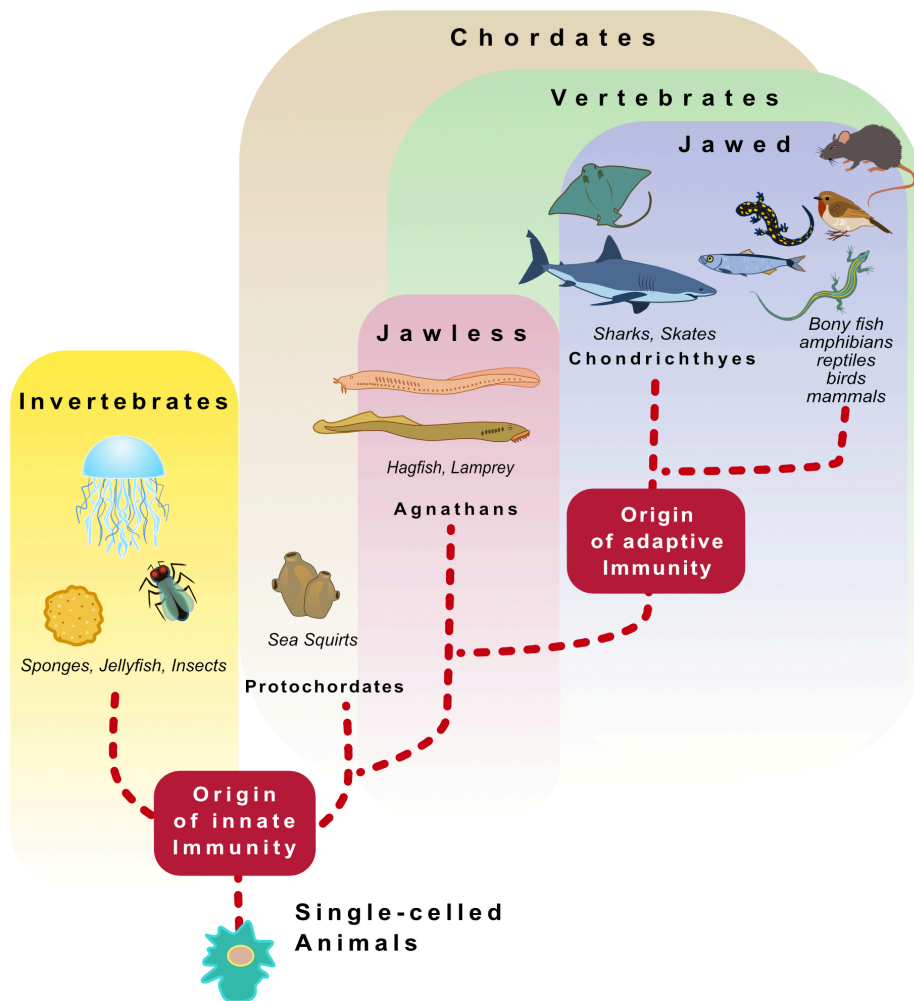
### 2.3. Peripheral tolerance

The mechanisms outlined above provide a framework for the immune system to evaluate the presence of self-reactive lymphocytes and to preclude them from generating immune responses, so as to minimize autoimmune pathologies. Nevertheless, this primary checkpoint is not bulletproof; the complexity that underpins this process can lead to tolerance breaches, which is the basis for many autoimmune diseases and allergies. As such, the immune system has evolved additional checkpoints that suppress self-reactive and exaggerated immune responses in the periphery.

Evolution has come up with a clever way to ensure T cell tolerance in the periphery, which is the need for co-stimulatory signals in T cell activation. T cell activation ensues after the TCR binds to pMHC on the surface of APCs. This interaction triggers a signaling cascade that leads to the activation of transcription factors important for cell survival, differentiation and proliferation (Smith-Garvin et al., 2009). In addition to strong TCR signaling, T cells require additional secondary signals to become fully activated and be able to mount effector functions. In the case of helper T cells this signal is delivered by binding of CD28 to CD80/CD86 (often referred to as B7.1/B7.2), the latter are normally expressed on APCs. Whereas CD28 is constitutively expressed on T cells, CD80/CD86 are only expressed when APCs encounter danger signals from pathogens or adjuvants in immunizations (Chen and Flies, 2013). This is crucial in preserving immune homeostasis, as it ensures that T cell responses are mounted only in the context of infection and/or immunization. As a result, T cell activation in the absence of CD28 signaling induces a state of unresponsiveness. This phenomenon is known as anergy, and it is characterized by an inability of T cell clones to mount immune responses against the target antigen (Fathman and Lineberry, 2007).



The evolutionary significance of co-stimulation in T cell activation can be illustrated by studies that compared the genetics of CD28 and its ligand in different classes of vertebrates. Vertebrates are comprised of two subphyla: jawed (Gnathostomata) and jawless (Agnatha) vertebrates (Hofmann et al., 2010). The consensus amongst scholars suggests that the adaptive immune system originated in the common ancestor that preceded early jawed-vertebrates (Flajnik, 2002, Sunyer, 2012) (**Figure 1**). This ancestor further bifurcated and gave rise to two distinct clades, cartilaginous fishes (i.e. sharks) and bony vertebrates. Within the later clade, the teleost family of fish is often referred to as the oldest living family of fish with true bones that contain an adaptive immune system akin to that of mammals (Cooper and Alder, 2006) (**Figure 1**). Thus, comparative analyses using these organisms are commonly used to illustrate the evolutionary timeline of the immune system. These phylogenetic analyses have revealed that the CD28-B7 co-stimulatory axis can be traced back as early as teleost fish (Cooper and Alder, 2006), and the binding motifs of CD28 to its ligands are highly conserved from virtually all bony fish to tetrapods (Hansen et al., 2009). Furthermore, rudimentary T cells and TCRs have been observed in sharks (Hofmann et al., 2010), and a CD28 homologue was discovered upon sequencing of the elephant shark's genome (Venkatesh et al., 2014). These findings suggest that the need for co-stimulation in lymphocyte activation originated and evolved hand in hand with adaptive immunity, and highlight that peripheral tolerance is just as important as generating lymphocyte diversity.



**Figure 1. Origins of adaptive immunity.**

Phylogenetic tree showing the evolutionary origins of the adaptive immune system. Illustration performed by J.M Fernandez De Canete Gomez Rubiera.

Pertinent to maintaining peripheral tolerance, the immune system is equipped with a variety of receptors capable of inhibiting co-stimulatory pathways, thereby preventing T cell activation. These are commonly referred to as co-inhibitory receptors, and of these PD-1 and CTLA4 have been thoroughly investigated in their ability to repress T cell immunity (Chen and Flies, 2013). PD-1 knockout mice develop lupus-like autoimmunity (Nishimura et al., 1998), and concomitant interaction of TCR and PD-1 to its ligands (PD-L1 and PD-L2) leads to the induction of phosphatases that attenuate T cell activation signals (Riley, 2009). Similarly, CTLA4 can regulate antigen presentation and T cell priming, and it appears to be particularly important in providing

$T_{\text{regs}}$  with capabilities to mediate peripheral tolerance. The biology of CTLA4 will be discussed in detail later in this chapter.

### 3. Regulatory T cells

#### 3.1. Discovery and historical context

The idea that  $CD4^+$  T cells are able to mediate peripheral tolerance can be traced back as early as 1969. This notion was first observed when female neonate mice that underwent thymectomy (NTx mice) experienced destruction of ovarian tissue (Nishizuka and Sakakura, 1969). Initial predictions attributed these lesions as a consequence of a lack of a purported thymic-derived ovarian growth factor. However, later investigations demonstrated that NTx mice exhibited other severe inflammatory conditions (Kojima and Prehn, 1981), which could be reversed when NTx mice were transferred with thymocytes from non-thymectomized mice (Sakaguchi et al., 1982). Consequently, it was concluded that thymocytes contained a cell subset responsible for preventing autoimmune pathology. It was not until 1995 that these suppressor T cells re-gained interest amongst immunologists, when Sakaguchi and colleagues proved that a particular subset of  $CD4^+$  T cells, characterized by high expression of CD25 (the alpha subunit of the high affinity IL-2 receptor), could suppress induced autoimmunity in BALB/c athymic nude mice (Sakaguchi et al., 1995). As such, the discovery that the thymus continuously produced a subset of  $CD25^+CD4^+$  T cells capable of suppressing immune responses to self and foreign antigens, prompted immunologists to refer to these cells as  $T_{\text{regs}}$ .

Several breakthroughs followed this discovery, including the observation that  $T_{\text{regs}}$  could potently suppress polyclonal T cell proliferation *in vitro* (Takahashi et al., 1998, Thornton and Shevach, 1998). Similarly, such studies revealed that, unlike conventional  $CD4^+$  T cells,  $T_{\text{regs}}$  are unable to produce IL-2 (a cytokine required for T cell priming and proliferation) and take up IL-2 through their high expression of CD25. This straightforward and reproducible *in vitro* assay, together with the identification of CD25 as a surface marker, allowed for the recognition and study of human  $T_{\text{regs}}$  (Shevach, 2001). It was then evident and universally accepted that the main

role of  $T_{\text{regs}}$  was to restrain fatal auto-inflammatory responses. However, given that CD25 upregulation is also a characteristic of activated  $CD4^+$  T cells, the field was still in search for a unique genetic marker responsible for differentiation and function of  $T_{\text{regs}}$ .

### 3.2. FOXP3

Genetic characterization of the Scurfy mouse strain provided researchers with a critical lead into the discovery of a unique marker for  $T_{\text{regs}}$ . This strain was revealed to harbor a 2 base-pair frameshift mutation in the Forkhead box transcription factor 3 (*Foxp3*) that resulted in a truncated and dysfunctional protein (Brunkow et al., 2001). Male mice hemizygous for the *Foxp3*<sup>sf</sup> allele succumbed to a  $CD4^+$  T cell-intrinsic lymphoproliferative autoimmune disorder, which was characterized by multi-organ lymphocyte infiltrates. Similarly, the genetic etiology of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans, a condition akin to the Scurfy phenotype, was mapped to various mutations in the human *FOXP3* homologue (Chatila et al., 2000, Bennett et al., 2001, Wildin et al., 2001).

In light of the strong connection between *Foxp3* deficiencies and autoimmunity, Rudensky's and Skakaguchi's groups studied the role of *Foxp3* in  $T_{\text{regs}}$ . Real time PCR experiments reported unique and selective expression of FOXP3 by  $T_{\text{reg}}$  cells across immune cell subsets. Similarly, generation of *Foxp3* knockout mice revealed that *Foxp3* is indispensable for the generation of thymic  $CD4^+CD25^+$  regulatory T cells. Additionally, ectopic expression of *Foxp3* was sufficient to activate suppressor functions employed by  $T_{\text{regs}}$  (Fontenot et al., 2003) and prevent inflammatory bowel disease in mice (Hori et al., 2003). Furthermore, manifestations of autoimmunity in *Foxp3*-deficient mice were shown to be  $T_{\text{reg}}$ -intrinsic and not due to aberrant T effector cell proliferation. Consequently, *Foxp3* became the universally accepted master regulator of  $T_{\text{regs}}$ , and its discovery provided a solid foundation on which further fascinating discoveries regarding T cell regulation would be built on.

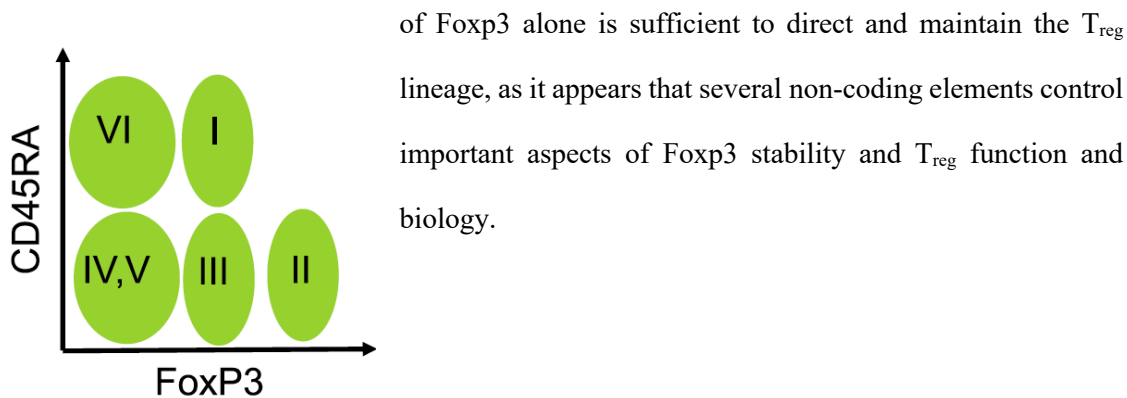
We now know that *Foxp3* is at the core of an intricate transcriptional network that governs  $T_{\text{reg}}$  identity. *Foxp3* is known to control over 2000 genomic loci, which approximately

corresponds to 700-1400 genes that are important for  $T_{reg}$  biology at any point in time throughout their lifetime (Lu et al., 2017). It appears that the predominant function of Foxp3 is to repress genes that are up regulated upon TCR stimulation (Marson et al., 2007). Interestingly, Foxp3 is known to bind only a small fraction of genes that are known to be under its control (Zheng et al., 2007). As such, Foxp3 is capable of indirectly regulating gene expression via binding to more than 300 different interacting partners, which include transcription factors and chromatin-modifying factors (Rudra et al., 2012). For instance, association of Foxp3 with the *IL2* and *IFN $\gamma$*  loci results in de-acetylation of H3 at these sites, which in turn triggers gene silencing. Lack of IL-2 and IFN $\gamma$  expression in  $T_{regs}$  is key to maintaining their phenotype and function. Consequently, Foxp3 is not only important in driving or repressing expression of  $T_{reg}$  associated genes, but it can also mediate important epigenetic modifications that modify the chromatin landscape needed to maintain  $T_{reg}$  identity (Lu et al., 2017).

Having established that Foxp3 deficiencies lead to fatal autoimmune disorders, researchers realized that tight control of this locus was of critical interest in order to ensure immune homeostasis. As such, elucidating the structure of this locus and how it controls the transcriptional network governing  $T_{reg}$  development and function has been a major focus of research over the past decades. Comparative genomics that aligned the *Foxp3* locus to other mammals have revealed 3 major non-coding conserved sequences (CNS). These sequences contain a promoter, two enhancers and an additional intronic enhancer. Following these observations, Rudensky and colleagues strived to characterize the function of each CNS region on  $T_{reg}$  biology (Zheng et al., 2010). Selective deletion of each region revealed that, whilst CNS1 is dispensable for the generation of thymic  $T_{regs}$  (t $T_{regs}$ ), it is fundamental for the generation of peripherally-induced  $T_{regs}$  (p $T_{regs}$ ), including those that mediate maternal-fetal tolerance in mammals (La Rocca et al., 2014). By contrast, deletion of CNS3 highlighted its importance in directing differentiation of t $T_{regs}$ . CNS2 contains a series of CpG islands whose de-methylated status is considered as the most reliable marker for fully committed  $T_{regs}$  (Floess et al., 2007, Wieczorek et al., 2009). A de-methylated CNS2 is crucial for stable Foxp3 expression as it creates a chromatin structure that is permissible to transcription factors and other regulatory elements that drive optimal Foxp3

expression. Whereas deletion of CNS2 does not compromise  $T_{reg}$  cell output, it does reduce Foxp3 stability and expression in the progeny of proliferating  $T_{regs}$  (Zheng et al., 2010).

These findings have been applied to human  $T_{regs}$  by Sakaguchi's laboratory in an attempt to delineate the distinct  $T_{reg}$  fractions observed in human peripheral blood on the basis of CD45RA and CD25 expression (Miyara et al., 2009) (**Figure 2**). Indeed, it was revealed that whilst fraction II and I  $T_{regs}$  exhibited a demethylated CNS2, this region was completely methylated in the remaining fractions. Not surprisingly, de-methylation of CNS2 correlated with a superior suppressive capacity of  $T_{regs}$ , along with a heightened FOXP3 expression. In summary, expression



**Figure 2. Human peripheral blood  $T_{reg}$  fractions.**

Functional  $T_{reg}$  delineation according to expression of FOXP3 and CD45RA. Adapted from Miyara et al, 2009, immunity.

### 3.3. $T_{reg}$ mechanisms of suppression

Elucidating how  $T_{regs}$  operate in suppressing immune responses has been a major focus of intense research over the past decades. Multiple mechanisms of effector T cell suppression have been thoroughly documented, both *in vitro* and *in vivo*. These can be broadly categorized into cell-to-cell contact, secretion of soluble factors, cytotoxicity and metabolic disruption mechanisms. To date, CTLA4, IL-10, TGF- $\beta$ , granzymes, perforin, CD39, CD79, TGIT and even

non-coding RNAs have been demonstrated to play a role in  $T_{reg}$ -mediated suppression (Zhao et al., 2017).

a. CTLA4

One of the best-characterized receptors that mediate  $T_{reg}$  suppression is CTLA4, which is expressed constitutively by  $T_{regs}$  even at the resting state. CTLA4 is a CD28 homologue and thus it is able to interact and bind to co-stimulatory molecules on APCs – CD80/CD86. In essence, competition of CTLA4 for the latter ligands is thought to suppress T cell activation (Alegre et al., 2001).

The biological function of CTLA4 was first described by James Allison and colleagues in the early 90's. His initial experiments, which revealed that this receptor acted as a T cell break, were carried out with the use of a CTLA4 antagonist (Krummel and Allison, 1995). Blockade of CTLA4 with this antibody boosted CD28-driven T cell stimulation. Soon after, the function of CTLA4 *in vivo* was first reported by Arlene Sharpe's and Tak Mak's groups. The severe autoimmune phenotype in *Ctla4<sup>-/-</sup>* mice sparked enormous enthusiasm due to its potential use in the clinic (Tivol et al., 1995, Waterhouse et al., 1995). Whilst scientist at the time largely focused on utilizing this mechanism for the treatment of autoimmune diseases, Allison thought of applying it to treating tumors, an idea that completely revolutionized cancer immunotherapy. The rationale behind this was that CTLA4 blockade would prevent T cell suppression in tumor environments, which in turn would enhance T cell immunity against cancerous cells. *In vivo* studies soon followed, which revealed a spectacular reduction of tumors in several strains of mice treated with anti-CTLA4 antibodies (Leach et al., 1996). In 2010 its use in clinical trials showed remarkable results, including complete remission of cancer in many cohorts of melanoma patients. James Allison, together with Tasuku Honjo, whose discovery of PD-1 has allowed successful cancer treatments using PD-1 blocking antibodies (Ishida et al., 1992), were awarded the 2018 Nobel Prize in Physiology or Medicine "for their discovery of cancer therapy by inhibition of negative immune regulation" (Ledford et al., 2018).

Much of our understanding of CTLA4 has been aided by transgenic mouse models. CTLA4-deficient mice develop abnormal lymphoproliferative disorders, and succumb to fatal autoimmunity by 2-3 weeks of age (Tivol et al., 1995, Waterhouse et al., 1995). The comparable phenotypes observed in CTLA4- and Foxp3-deficient mice sparked interest in whether the former played an important role in T<sub>reg</sub> suppression. The scurfy phenotype, as a result of Foxp3 deficiency, appears to be cell extrinsic, as it is corrected when Rag<sup>-/-</sup> mice are transplanted with a mix of wildtype and scurfy bone marrow. In other words, the autoimmune pathology manifested by a Foxp3 deficiency is not due to uncontrolled proliferation of Foxp3-deficient cells, but rather it is due to a Foxp3 deficient T cell that is unable to control external T cell proliferation in its environment (Godfrey et al., 1991). Early attempts to find connections between CTLA4 and T<sub>regs</sub> employed this same mixed bone marrow chimera strategy (Bachmann et al., 1999). The results were astonishing: CTLA4 deficiency could be fully compensated by the addition of wild type bone marrow. More strikingly, the fact that CTLA4-deficient T cells remained in an unactivated status in such chimeras clearly illustrated that CTLA4 could regulate bystander T cell activation in a cell-extrinsic manner.

These results have been extensively replicated in the literature (Chikuma and Bluestone, 2007, Read et al., 2006, Friedline et al., 2009, Tai et al., 2012), and more recently, advanced genetic engineering tools have allowed for definitive proof showing a role of CTLA4 in T<sub>regs</sub> (Wing et al., 2008). In 2014, Arlene Sharpe and Shimon Sakaguchi created conditional knockout mouse models bearing a T<sub>reg</sub> specific deletion of CTLA4 (Wing et al., 2014, Sage et al., 2014b). These animals not only exhibited autoimmune phenotypes that resembled those observed in scurfy and CTLA4-deficient mice, but also revealed key regulatory properties of CTLA4 in controlling humoral immunity and germinal center responses. Furthermore, these findings have been complemented by human studies that interrogated the role of CTLA4 in autoimmune diseases. Analysis of point mutations in the CTLA4 locus has proved that CTLA4 haploinsufficiency results in severe autoimmune disorders (Schubert et al., 2014). Although these conditions often resemble those described in IPEX patients, incomplete penetrance and variable



age of onset can be observed in some pedigrees. Nonetheless, the information gained from a combination of *in vivo*, *in vitro* and human genetic studies have uncovered a well-defined role of CTLA4 in T<sub>reg</sub>-mediated immune regulation.

CTLA4 is a CD28 homologue and thus it is able to interact and bind to co-stimulatory molecules on APCs – CD80/CD86. Researchers have always concurred in that the function of CTLA4 is to suppress T cell activation by regulating CD28 signaling. These conclusions were derived from the observation that crossing CTLA4-deficient animals to knockout mice for CD80 and/or CD86 completely abrogates autoimmunity (Mandelbrot et al., 1999). The severe immune dysregulation syndrome observed in CTLA4 deficient mice is solely dependent on CD28. Nevertheless, the mechanism by which CTLA4 operates has been controversial and subjected to a strong dichotomy between T cell-intrinsic and extrinsic modes of action. Advocates for an intrinsic mechanism have observed that CTLA4 delivers a negative signal in T effector cells themselves (Krummel and Allison, 1995, Krummel and Allison, 1996, Walunas et al., 1996), and these assertions have been supported by the use of CTLA4-specific agonistic antibodies in *in vitro* studies. However, it is important to note that physiologically there is no equivalent to CTLA4-specific agonists, as to date, there is no ligand that binds exclusively to CTLA4 and not CD28 (Walker and Sansom, 2015). Furthermore, in spite of the vast amount of information on the signaling events following CTLA4 stimulation, a single unified signaling pathway for this event still remains elusive.

In contrast to the above claims, bone marrow chimera experiments described earlier were irrefutable evidence for a T cell extrinsic mechanism of action of CTLA4. Many theories and explanations have been put forward to attempt to provide a plausible mechanism of action (Walker and Sansom, 2015). However, CTLA4-mediated downregulation of CD80/CD86 has been observed in many experimental settings and is regarded as the most plausible mechanism of action. Constitutive expression of CTLA4 in T<sub>regs</sub> allows them to exploit this interaction to suppress immune responses, through the direct downregulation of these co-stimulatory molecules. Doing so decreases the magnitude of antigen presentation and thus attenuates T cell-

driven immune responses. This phenomenon has been elegantly demonstrated to occur, at least in part, through transendocytosis, whereby CTLA4 is able to physically remove CD80/CD86 ligands off the membrane of antigen presenting cells (Qureshi et al., 2011). Such observation has finally provided a convincing mechanism as to how  $T_{\text{regs}}$  are able to suppress bystander T cell activation in a cell-extrinsic manner.

#### b. CD25

Most  $T_{\text{regs}}$  are able to express the high affinity IL-2 receptor alpha subunit (CD25). This receptor is not only a phenotypic marker of  $T_{\text{reg}}$  identification, but it also plays several important roles in shaping  $T_{\text{reg}}$  biology. One of the best well characterized functions of  $T_{\text{reg}}$ -derived CD25 is IL-2 deprivation (Scheffold et al., 2007). Given that this cytokine is a key mediator for T cell proliferation and survival, rapid consumption of soluble IL-2 allows  $T_{\text{regs}}$  to suppress bystander T cell activation and thus attenuate immune responses. Interestingly, although  $T_{\text{regs}}$  themselves are unable to produce this cytokine, their dependency on IL-2 for stable Foxp3 expression is unquestionable (Furtado et al., 2002, Almeida et al., 2002, Fontenot et al., 2005). Many researches have proposed that this inability to produce IL-2 permits CD25 to remain unbound, and it is this unbound state of CD25 that allows  $T_{\text{regs}}$  to mediate IL-2 deprivation (Pandiyani et al., 2007, Yamaguchi et al., 2013). Additionally,  $T_{\text{regs}}$  are also dependent on IL-2 for survival and maturation signaling cues in the thymus. It is well established that maturing  $T_{\text{regs}}$  have a dependency for IL-2 signaling, as ablation of STAT5 or any of the IL-2 receptors, including CD25, severely impairs  $T_{\text{reg}}$  differentiation (Furtado et al., 2002, Fontenot et al., 2005, Malek et al., 2002, Yao et al., 2007). Furthermore, IL-2 signaling works in cooperation with TGF- $\beta$  to induce  $T_{\text{regs}}$  in the periphery (Chen et al., 2003).

#### c. IL-10

Most cells of the innate and adaptive immune system are able to produce IL-10, including dendritic cells, macrophages, natural killer cells, eosinophils, neutrophils, mast cells, B cells, CD8<sup>+</sup> T cells, CD4<sup>+</sup> T helper and  $T_{\text{reg}}$  cells (Sky et al., 2013). Because IL-10 has

immunosuppressive effects that inhibit Th1 cytokine secretion, it was initially denominated as cytokine-synthesis inhibitory factor (CSIF) (Fiorentino et al., 1989, Jinquan et al., 2000, Taga et al., 1993). Multiple studies over the last decades have investigated the function of T cell-derived IL-10 in the regulation of immune responses. T<sub>reg</sub>-derived IL-10 appears to be particularly important in modulating immune homeostasis and maintaining tolerance, as mice with selective deficiency of IL-10 in Foxp3-expressing cells develop spontaneous colitis associated with the presence of commensal bacteria (Rubtsov et al., 2008).

Not only is T<sub>reg</sub>-derived IL-10 crucial in mediating immune tolerance, but also its production by other non-Foxp3-expressing T cells is essential in modulating immune responses. For instance, a prominent Foxp3<sup>-</sup> IL-10 secreting T cell subset with potent suppressive capacity, denominated as type 1 regulatory T cells (T<sub>r1</sub>), has been extensively studied (Groux et al., 1997). T<sub>r1</sub> cells do not express Foxp3 and thus do not depend on it for gaining suppressive privileges, yet can exert potent suppressive functions. It appears that T<sub>r1</sub>-derived IL-10 inhibits expression of co-stimulatory molecules in antigen-presenting cells (APCs), which in turn curtails immune responses by limiting T cells activation (Fujio et al., 2010). Furthermore, in vivo studies have demonstrated that under chronic inflammation, a subset of T<sub>H2</sub> cells (T<sub>H2i</sub>) that expresses IL-10 can adopt regulatory properties (Altin et al., 2012). These cells express other regulatory molecules, such as CTLA4 and the cytotoxic molecule Granzyme B. T<sub>H2i</sub> cells can suppress T cell proliferation and IL-4 production in a partially IL-10-dependent manner (Altin et al., 2012). Together, these experiments suggest that T cell-derived IL-10 plays a critical immunosuppressive role in different immunological contexts.

### 3.4. T<sub>reg</sub> ontogeny

T<sub>regs</sub> can emerge from two distinct sources of Foxp3<sup>+</sup> T cells. T cell clones that bind to self-peptides in the thymus can express Foxp3 and exit the thymus as fully committed T<sub>regs</sub>. These are commonly referred to as tT<sub>regs</sub>. In contrast, T<sub>regs</sub> can also originate from conventional T cells (T<sub>conv</sub>)

cells in the periphery as a response to specific environmental cues, such as chronic antigen exposure (Apostolou and von Boehmer, 2004, Kretschmer et al., 2005), administration of oral antigens (Curotto de Lafaille et al., 2008) or in the presence of helminth infection (Finney et al., 2007, Curotto de Lafaille et al., 2008, Grainger et al., 2010). These  $T_{\text{regs}}$  require specific cytokine stimulation, which can vary between mice and humans, and are known as peripherally-induced  $T_{\text{regs}}$  ( $pT_{\text{regs}}$ ) in the literature.

Researches have strived to elucidate the relative contributions of these two types of  $T_{\text{regs}}$  to the  $T_{\text{reg}}$  compartment, and whether they exert different biological functions. A popular hypothesis is that they utilize separate TCR specificities, with  $tT_{\text{regs}}$  and  $pT_{\text{regs}}$  recognizing self and innocuous specificities respectively. Indeed, elegant experiments demonstrated that commensal bacterial antigens in the colon instruct  $pT_{\text{reg}}$  conversion from  $T_{\text{conv}}$  precursors. Generation of transgenic mouse models bearing those commensal bacterial specific TCRs revealed that these specificities did not facilitate thymic  $T_{\text{reg}}$  development. These observations concluded that  $pT_{\text{regs}}$  preferentially develop in response to environmental antigens, thus allowing them to control colon homeostasis (Lathrop et al., 2011).

Contrary to this notion, several studies have suggested that  $pT_{\text{regs}}$  and  $tT_{\text{regs}}$  do not differ in antigen specificity, as comparison of TCR sequences revealed minimal differences between these two subsets (Hsieh et al., 2006, Pacholczyk et al., 2006) and even when compared to effector T cells (Wong et al., 2007). Similarly, transcriptional signatures were highly comparable between  $T_{\text{regs}}$  obtained from the thymus and those from peripheral tissues (Feuerer et al., 2010, Haribhai et al., 2009). Furthermore, analysis of TCR repertoires via single cell sequencing revealed that  $T_{\text{reg}}$ -mediated tolerance in the colon is predominantly facilitated by  $tT_{\text{regs}}$  (Cebula et al., 2013). A caveat to all these sequencing approaches was the use of transgenic TCR mouse models, which may have restricted the diversity of the TCR repertoire (Cebula et al., 2013). Indeed, it was later observed that a full repertoire of TCRs is necessary for  $T_{\text{regs}}$  to exert protective tolerance and maintain colon homeostasis (Nishio et al., 2015). Helios expression was proposed to identify  $tT_{\text{regs}}$  (Thornton and Shevach, 1998), albeit several lines of evidence have shown Helios expression in

activated T cells and pT<sub>regs</sub> too (Gottschalk et al., 2012, Darce et al., 2012). As such, the fact that, to date, there is no specific marker that separates tT<sub>regs</sub> from pT<sub>regs</sub> may have confounded all these tTreg vs pT<sub>reg</sub> comparative analyses, and thus makes one take these assertions with a pinch of skepticism.

Despite the many gaps that exist in the literature pertaining to pT<sub>reg</sub> ontogeny, the molecular cues leading to their conversion have been well documented in mice and humans. T<sub>regs</sub> can develop from naïve T cells *in vitro* upon exposure to TGF- $\beta$  and IL-2 (Chen et al., 2003). However, the functional consequences of this observation *in vivo* have been controversial due to instability of Foxp3 expression (Huehn et al., 2009) and the fact that their suppressive ability varies across different studies (Benoist and Mathis, 2012). However, given that pT<sub>regs</sub> differentiate in the periphery in response to exogenous antigens, these are commonly thought to exert regulatory functions against non-harmful foreign antigens, such as commensal microbial and/or food or innocuous antigens. To this end, Rudensky and colleagues reported that pT<sub>reg</sub>-deficient mice, via disruption of the CNS1 element in the *Foxp3* locus, exhibited inflammatory lesions at mucosal sites - especially those associated with continuous exposure of environmental antigens (Josefowicz et al., 2012b). Similarly, experiments conducted with elegant mouse models, that were either raised germ-free and had no commensal microbiota, or fed a chemical diet devoid of nutrient-derived macromolecules and food antigens, revealed further diversity within the pT<sub>reg</sub> compartment in the gut (Kim et al., 2016). pT<sub>regs</sub> induced by dietary antigens were short-lived and disappeared as soon as the food antigen supply ceased. Additionally, exposure of food antigens in chemically fed mice resulted in aggressive immune responses that were partly due to an absence of dietary antigen-induced pT<sub>regs</sub>. Moreover, these pT<sub>regs</sub> appeared to be different to those induced by microbiota in terms of ROR $\gamma$ T expression: microbiota-induced pT<sub>regs</sub> appear to express high levels of ROR $\gamma$ T, whereas T<sub>regs</sub> induced by food antigens do not. Together, these findings reinforced the notion that pT<sub>regs</sub> appear to be particularly important in controlling mucosal inflammatory conditions and maintaining tolerance to innocuous, commensal and food antigens.

### 3.5. T<sub>reg</sub> plasticity and diversity

Preserving T<sub>reg</sub> cell identity is crucial to maintain immune homeostasis, as loss of T<sub>regs</sub> leads to serious autoimmune pathology. However, whether T<sub>regs</sub> are functionally or phenotypically stable remains a matter of controversy amongst researchers (Sakaguchi et al., 2013). There is a wealth of data that suggests that T<sub>regs</sub> can permanently shut down Foxp3 expression and adopt T cell effector phenotypes and functions (Zhou et al., 2009). Transfer of GFP-labeled T<sub>regs</sub> into T cell deficient recipients revealed that 30-60% of these T<sub>regs</sub> had lost Foxp3 expression by 4 weeks post-transfer (Komatsu et al., 2009, Duarte et al., 2009). Furthermore, these cells not only secreted the effector cytokines IFN $\gamma$ , IL-4 and IL-17 - characteristic of T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 cells respectively, but were also pathogenic in triggering inflammatory lesions in the lungs (Duarte et al., 2009). Additionally, elegant experiments performed by the Fagarassan group demonstrated that in the Payer's patches T<sub>regs</sub> can lose Foxp3 expression and become T<sub>FH</sub> cells that provide B cell help for IgA secretion (Tsuji et al., 2009). Furthermore, fate mapping analysis of Foxp3 expression in a dual reporter system bearing a *FoxP3<sup>cre</sup> : Rosa26<sup>lox.STOP.lox-YFP/+</sup>* transgene revealed that 20% of effector T cells had, at some point during their life time, expressed Foxp3. Expression of Foxp3 in this model leads to an irreversible DNA rearrangement that can be measured by YFP activity, and thus one can interrogate whether Foxp3 has been active at any time in practically any cell type (Zhou et al., 2008). Taken together, these observations have questioned the stability of Foxp3 expression and suggest that T<sub>regs</sub> are able to convert to effector T cells.

Notwithstanding, the above experiments have been subjected to criticism amongst several scholars. First, the transfer experiments using GFP-labeled T<sub>regs</sub> outlined above were performed using lymphopenic recipient mice, and such environment may artificially force expansion of small numbers of contaminating effector T cells present in cell sorting preparations (Benoist and Mathis, 2012). Indeed, loss of Foxp3 expression was not reported when GFP-labeled T<sub>regs</sub> were transferred into recipients with intact immune systems (Rubtsov et al., 2010). Similarly, continuous expression of the tracer transgene in the above fate mapping strategy would also tag effector T cells with transient Foxp3 expression. Indeed, pulse labeling of Foxp3 expression via tamoxifen-driven *FoxP3<sup>Cre-ERT2</sup>* in adult mice failed to detect effector T cells that had lost Foxp3

expression. To reconcile these discrepancies it has been proposed that these ex-T<sub>regs</sub> represent a small fraction of uncommitted T<sub>regs</sub>, and has been supported by the unstable methylation status of the CNS2 region in these cells (Miyao et al., 2012).

Despite the above controversy, most researches agree that T<sub>regs</sub> possess the ability to adapt to their environment and tailor their suppressive functions towards specific immune responses. There is a wealth of data indicating that T<sub>regs</sub> are able to direct repression against specific CD4<sup>+</sup> T cell responses. In doing so T<sub>regs</sub> are able to reprogram their transcriptional network, via expression of the same key transcription factors as the targeted effector CD4<sup>+</sup> T cells. For instance, Tbet<sup>+</sup> T<sub>regs</sub> repress T<sub>H</sub>1-driven immune responses (Koch et al., 2009), whilst IRF4<sup>+</sup> T<sub>regs</sub> appear to be important in modulating T<sub>H</sub>2 responses (Zheng et al., 2009). Similarly, BCL6-expressing T<sub>regs</sub>, or T<sub>FR</sub> cells, have been shown to repress antibody responses, whereas Stat3 expression confers T<sub>regs</sub> the ability to dampen T<sub>H</sub>17 cells (Chaudhry et al., 2009). Furthermore, T<sub>H</sub> subset-specific reprogramming of T<sub>regs</sub> appears to be dynamic, as several studies have documented inter-conversion of Tbet<sup>+</sup> T<sub>regs</sub> into Gata3<sup>+</sup> T<sub>regs</sub> and *vice versa* (Yu et al., 2015).

It is also worth mentioning that T<sub>regs</sub> do not constitute a cell subset whose sole purpose is to repress immune responses. Indeed, there are few instances where T<sub>regs</sub> have been observed to promote certain effector functions so as to refine the efficacy of the immune response. Ablation of T<sub>regs</sub> in an acute model of herpes infection revealed a decrease in interferon production that failed to restrain an uncontrolled inflammation (Lund et al., 2008). Similarly, A. Ballesteros-Tato and B. Leon reported that Foxp3<sup>+</sup> T<sub>regs</sub> augmented the quality of influenza-specific antibody titers (Leon et al., 2014). Additionally, selective deficiencies in T<sub>FR</sub> cells via mixed bone marrow chimeras revealed a decrease in the output of antigen specific antibodies (Wollenberg et al., 2011a, Chung et al., 2011). Furthermore, T<sub>reg</sub> depletion led to a decreased ability of macrophages to carry out apoptotic cell clearance, which led to uncontrolled inflammation (Proto et al., 2018). Such observation occurred as a failure of T<sub>regs</sub> to promote macrophage-derived IL-10 secretion that would normally activate macrophage cell engulfment. Collectively, these findings highlight

that  $T_{\text{regs}}$  are not only mediators of immune tolerance, but can also facilitate proper immune homeostasis and exert functions that extend far beyond immune repression.

## 4. Germinal center responses

### 4.1. Overview

The first description of germinal centers (GCs) was documented by Fleming. After observing that a large proportion of lymphocytes were undergoing mitosis in the follicles of secondary lymphoid organs, he predicted these to be sites of lymphocyte generation (Nieuwenhuis and Opstelten, 1984). This observation sparked fervent interest in the field, which disproved Fleming's predictions and demonstrated that GCs were linked with T-dependent antibody responses (Allen et al., 2007). Today, we know that GCs are specialized microenvironments responsible for production of high-affinity antibodies, memory B cells and long-lived plasma cells. These are transient microanatomic structures that form within B cell follicles when activated B cells interact with cognate T cells at the T:B border of secondary lymphoid organs. At this stage, B cells can adopt one of two possible fates: terminal differentiation into short-lived plasma cells that secrete low affinity antibody, or 2) migration into the B cell follicle in order to establish the GC reaction and increase their affinity for the immunizing antigen (Victora and Nussenzweig, 2012).

GCs can be further divided into two functionally distinct sections, namely the dark zone (DZ) and light zone (LZ). In the DZ, GC B cells undergo rapid and intense clonal expansion and somatic hypermutation (SHM), that allows acquisition of random mutations in their antibody coding genes. This is possible due to expression of the enzyme activation-induced deaminase (AID), which deaminates cytidine residues and introduces point mutations in the variable region of the VDJ locus. Doing so allows for some B cells to improve affinity for antigen, which, upon cell cycle exit, migrate to the LZ. This compartment contains abundant follicular dendritic cells (FDCs) that present native antigen complexed to antibodies bound to FDCs' Fc receptors (Victora and Nussenzweig, 2012).



#### 4.2. T<sub>FH</sub> cells

Competitive fitness of B cell clones is the driving force of affinity maturation in the GC, akin to a Darwinian mode of natural selection. High affinity GC B cells are more likely to collect the highest amount of antigen, and thus are capable of presenting maximal concentration of peptide-MHC complexes to helper T cells. Subsequent interaction with a limiting number of B-helper T follicular (T<sub>FH</sub>) cells results in affinity-based selection. T<sub>FH</sub> cells not only express CXCR5, which allows migration to the follicle, but also contain preformed CD40L ligand, which quickly translocates to the cell membrane and provides cognate B cell help (Vinuesa et al., 2016). Limiting T<sub>FH</sub> cells is crucial to promote competition amongst B cell clones, which ensures that only high affinity B cells preferentially survive and dominate the GC reaction. T<sub>FH</sub>:B cell interactions are thought to result in one of three possible outcomes: 1) Terminal differentiation of high affinity GCB cells into long-lived plasma cells and/or memory B cells, 2) cyclic re-entry into the DZ for further rounds of division, mutation and BCR diversification, 3) apoptosis of low affinity or self-reactive clones (Vinuesa et al., 2016). However, how and if the same follicular T cell subset controls cell cycle entry, vs exit as well as terminal differentiation is still unclear for scientists working in the field.

Even though it was widely accepted that CXCR5<sup>+</sup> T cells were preferentially recruited to GCs, the hypothesis that these cells constituted a distinct T cell subset remained somewhat controversial for several years. Gene expression profiles of CXCR5-expressing T cells, both from mice and humans, were then generated in order to interrogate whether such cells constituted a unique T cell subset. T<sub>FH</sub> cells turned out to be different to other T helper lineages in terms of transcription factors and cytokine expression (Chtanova et al., 2004). Not only did T<sub>FH</sub> cells not express *GATA3* or *Tbet*, but were also found to selectively express genes such as *BCL6*, *IL21*, *ICOS* and *ASCL2* (Kim et al., 2004). Additionally, PD-1 together with CXCR5 were found to reliably identify T<sub>FH</sub> cells (Haynes et al., 2007), and has now become the gold standard strategy to identify T<sub>FH</sub> cells in mice and humans. It was not until 2009, in which three independent groups demonstrated that the transcription factor Bcl6 orchestrated commitment to T<sub>FH</sub> phenotype, that

T<sub>FH</sub> cells were recognized as a unique T cell subset (Nurieva et al., 2009, Johnston et al., 2009, Yu et al., 2009).

#### 4.3. T<sub>FR</sub> cells

Even though SHM is crucial to diversify and improve affinity for antigen, its stochastic nature makes the generation of self-reactive clones a likely consequence of the GC reaction. Regulation of mutated B cell clones within the GC is therefore crucial, particularly given the potential for formation of self-reactive long-lived plasma cells that could lead to serious autoimmune disease. As discussed previously, Foxp3 deficiencies lead to severe immune dysregulation, including auto-antibody-mediated autoimmunity. The connection between T<sub>reg</sub> deficiencies and antibody dysregulation inspired researchers to uncover a novel T<sub>reg</sub> cell subset that could participate in the GC reaction. In 2011, three independent groups discovered a Bcl6<sup>+</sup> follicular T cell population that co-expressed Foxp3, which they named follicular regulatory T (T<sub>FR</sub>) cells upon observing that they could limit T<sub>FH</sub> cell and GC B cell responses (Linterman et al., 2011, Wollenberg et al., 2011a, Chung et al., 2011). Even though the full significance of this repression is yet to be elucidated, our understanding of T<sub>FR</sub> cell biology has grown exponentially over the past years.

Much like T<sub>FH</sub> cells, T<sub>FR</sub> cells emerge during the response to immunization and appear to require very similar differentiation cues as T<sub>FH</sub> cells, not only in their requirement of Bcl6 expression, but also in their dependence on priming by DCs and SAP-mediated B cell interactions (Linterman et al., 2011, Wollenberg et al., 2011a, Chung et al., 2011). Additionally, antigen presentation is key in driving T<sub>FR</sub> cell generation, as depletion of DCs significantly reduced T<sub>FR</sub> cell numbers (Sage et al., 2014a). Phenotypically, T<sub>FR</sub> cells resemble both T<sub>FH</sub> cells and T<sub>regs</sub>, and express markers commonly used for T<sub>FH</sub> cell identification, such as ICOS, CXCR5, PD-1 and BCL6. CXCR5 grants T<sub>FR</sub> cells unrestricted access to the GC, and unlike T<sub>FH</sub> cells, its expression appears to be driven by the transcription factor NFAT2 (Vaeth et al., 2014). Bcl6 drives the transcriptional network that orchestrates follicular T cell differentiation, as we and others have observed that ablation of *Bcl6* in Foxp3 expressing cells completely abrogates T<sub>FR</sub> cells (Wu et al., 2016, Botta et al., 2017, Fu et al., 2018). Furthermore, PD-1 expression serves as checkpoint

that restrains excessive  $T_{FR}$  activity, as PD-1 or PD-L1 deficient mice exhibited increase frequencies of  $T_{FR}$  cells (Sage et al., 2013).

Despite the high degree of similarity between  $T_{FH}$  and  $T_{FR}$  cells, mouse  $T_{FR}$  cells also share characteristics with thymic-derived regulatory T cells ( $tT_{regs}$ ) (Linterman et al., 2011, Wollenberg et al., 2011a, Aloulou et al., 2016, Chung et al., 2011). In addition to Foxp3, mouse  $T_{FR}$  cells also express high levels of GITR, BLIMP1, CTLA4, as well as moderate CD25 and *IL-10* mRNA. Dual expression of Blimp1 and Bcl6 is a characteristic, yet puzzling, property of  $T_{FR}$  cells, and it still remains unclear how two transcription factors that antagonize each other can co-exist in the same cell. Ballesteros-Tato and colleagues have proposed that Blimp1 signaling is important in reducing CD25 expression in  $T_{FR}$  cells, together with a concomitant decrease in IL-2 signaling (Botta et al., 2017). This, in turn, allows  $T_{FR}$  cells to participate in the GC reaction, as it has been previously demonstrated that IL-2 signaling dampens follicular T cell differentiation (Ballesteros-Tato et al., 2012). Furthermore, it has been suggested that  $CD25^+$  and  $CD25^-$   $T_{FR}$  cells are two functionally and transcriptionally distinct subsets, with the latter representing the true  $T_{FR}$  cell population (Wing et al., 2017b). The authors proposed that the same phenomenon occurs in human tonsils, but the lack of functional data renders this assertion nothing more than a testable hypothesis. Additionally, the co-inhibitory receptor CTLA4 is critical for repressing excessive  $T_{FR}$  cell activity (Wing et al., 2014). Conditional deletion of CTLA4 in  $T_{regs}$  revealed a stark increase in  $T_{FR}$  cell numbers, together with abnormally high IgE titers. However, seeing that these mouse models exhibited a concomitant expansion of  $T_{FH}$  and GCB cells, whether CTLA4 directly represses  $T_{FR}$  cells or whether they expand in an attempt to restraint exacerbated GC responses is still unknown.

Functionally,  $T_{FR}$  cells have been shown to limit the magnitude of germinal center reactions (Linterman et al., 2011, Wollenberg et al., 2011a, Chung et al., 2011, Aloulou et al., 2016, Sage et al., 2014b, Wing et al., 2014), as well as reduce antigen-specific antibody responses, although the reported magnitude of this effect has varied in different studies (Linterman et al., 2011, Wollenberg et al., 2011a, Chung et al., 2011, Sage et al., 2014b, Wing et al., 2014, Sage et al.,

2014a, Sage et al., 2016, Maceiras et al., 2017, Botta et al., 2017). T<sub>FR</sub> cells were initially thought to emerge exclusively from the thymus as Foxp3<sup>+</sup> precursor cells, and as such were predicted to predominantly regulate immune responses against self-antigens. Indeed, selective ablation of T<sub>FR</sub> cells in *FoxP3<sup>cre</sup>:BCL6<sup>lox/lox</sup>* revealed autoantibody-mediated pathology, including the presence of anti-nuclear antibodies (ANAs) (Wu et al., 2016, Botta et al., 2017, Fu et al., 2018), and seropositivity against salivary glands (Fu et al., 2018). Nevertheless, it has been recently demonstrated that T<sub>FR</sub> cells can also be induced outside the thymus from naive T cells (Aloulou et al., 2016) in response to foreign antigens. These “induced” T<sub>FR</sub> cells have been shown to enter follicles and exert similar repressive effects as thymic-derived T<sub>FR</sub> cells (Aloulou et al., 2016). Furthermore, T<sub>FR</sub> cells have been shown to exert a durable repressive effect on GC B cells, which not only include transcriptional changes but also extend to metabolic disruptions and epigenetic modifications (Sage et al., 2016). These effects however, can be rescued at least in part by IL-21, suggesting a role for T<sub>FR</sub> cells in mediating IL-21 suppression.

## 5. Regulation of Immunoglobulin of class E

### 5.1. Historical context

Allergy is a growing epidemiological problem. Despite being classified as the epidemic of the 21<sup>st</sup> century, occurrences of this condition can be traced as early as the beginnings of written history, where King Menses of Egypt presumably succumbed to an aggressive anaphylactic shock after being stung by a wasp (Platts-Mills et al., 2016). Scholars later proved this account to be nothing more than an urban myth, and the first scientific description of anaphylaxis was only documented last century in 1901. Coined by the zoologist Charles Richet, in collaboration with Paul Portier, the term refers to a phenomenon that “opposes *phylaxis*” or protection (Portier P., 1902). Their observations were derived from experiments that evaluated the administration of a jellyfish toxin into dogs. To their surprise, Richet and his team observed that the dogs that had survived the first injection rapidly succumbed to a violent death upon re-injection of the toxin (May, 1985). A few years later in 1903, Clemens Von Pirquet, a French pediatric resident, published a revolutionary idea, which he termed *Allergy* (*Allos*-other, *ergy*-work) in an attempt to explain the unusual clinical symptoms manifested in his patients (Von Pirquet C, 1903).

Contrary to the paradigm at the time, which believed that the immune system was purely protective, von Priquet proposed that the immune system could actually cause harm to the host when repeatedly exposed to the same foreign substance (Igea, 2013). It was not until the 1950s, when two independent groups discovered immunoglobulin of class E, together with its ability to mediate fast and aggressive inflammatory responses, that the field began to gain a deeper understanding of the molecular mechanisms that underpin allergic diseases (Platts-Mills et al., 2016).

## 5.2. Overview of IgE as the driver of allergy

We now know that allergic reactions are fast inflammatory immune responses against innocuous antigens. The hallmark of allergic responses is sudden hypersensitivity upon antigen re-exposure. These reactions are elicited by antigen specific IgE, and usually manifest themselves at the sites of antigen contact (Xiong et al., 2012a). Allergic reactions owe their rapid nature to the unique properties of IgE responses. Crosslinking of IgE bound to FcεRI on mast cells and basophils leads to the release of inflammatory and vasoactive mediators (Galli et al., 2008). Allergic pathology is often located at epithelial and mucosal sites, and consists of type 2 immune responses, in which signature cytokines IL-4 and IL-13 are derived from innate lymphoid-like 2 (ILC2) cells, basophils, or helper CD4<sup>+</sup> T (T<sub>H</sub>) cells (Licona-Limon et al., 2013, Hammad and Lambrecht, 2015, Voehringer et al., 2006). These signature cytokines are known drivers of B cells to undergo class switch recombination (CSR) to IgE.

There is evidence that IgE-producing plasma cells can arise both during the extrafollicular antibody response, and upon interaction with T cells within the epithelial lesions or in secondary lymphoid tissues, as a result of sequential CSR in IgG memory B cells that arose in GCs (He et al., 2013b, Xiong et al., 2012b, Erazo et al., 2007). Although rare, IgE<sup>+</sup> B cells can also be found in GCs (He et al., 2013b) and several lines of evidence have suggested that T<sub>FH</sub> cells contribute to IgE production (Reinhardt et al., 2009, King and Mohrs, 2009, Glatman Zaretsky et al., 2009) (Coquet et al., 2015, Ballesteros-Tato et al., 2016a). This is not surprising given that T<sub>FH</sub> cells are specialized B cell helpers required to elicit both extrafollicular and GC antibody responses. Bcl6-

dependent T<sub>FH</sub> cells interact with B cells first at the time of B cell priming by antigen at the T:B border (Lee et al., 2011), and then after repeated cycles of division and mutation within germinal centers. Recently, a dependency of T<sub>FH</sub> cells has been confirmed for mouse IgE responses induced by airborne-antigens (Kobayashi et al., 2016, Ballesteros-Tato et al., 2016b).

### 5.3. Control of IgE responses

The scarcity of IgE in normal serum (normally present at 100 ng/ml, several orders of magnitude lower than IgG found at 2-10 mg/ml) suggests that tight regulatory mechanisms limit B cells to switch to this isotype or overall limit the survival of IgE<sup>+</sup> B cells. Indeed, a number of B cell-intrinsic mechanisms have been shown to limit IgE production from GCs, including BCL6-mediated repression of transcription from the germline epsilon promoter (Harris et al., 1999), reduced survival of IgE<sup>+</sup> GC B cell due to lowered B cell receptor (BCR) expression (He et al., 2013b), and unique characteristics of the IgE heavy chain (Yang et al., 2012) that alters signaling downstream of CD19 and BLNK (Haniuda et al., 2016), or preferential Fas-mediated apoptosis (Butt et al., 2015). B cell-extrinsic mechanisms regulating IgE production have also been suggested. Amongst these, Allen and colleagues proposed that IgE<sup>+</sup> B cells have increased dependency on T<sub>FH</sub> cell help for their survival (Yang and Robinson, 2016).

Amongst other B cell extrinsic mechanisms that curtail IgE responses, mouse models of allergy have revealed a role for IL-10 in mediating tolerance to allergens. For instance, systemic administration of OVA in alum often results in an allergic reaction known as airway hyper sensitivity (AHR) driven by pathogenic T<sub>H</sub>2 cells. However, exposure to OVA intranasally prior to systemic immunization ameliorates AHR severity, and this protection is associated with the induction of OVA-specific IL-10-secreting T cells (Akbari et al., 2001). Additionally, IL-10 blockade during the protective antigen exposure period failed to protect animals from AHR, and resulted in an increase of OVA-specific IgE titers (Vissers et al., 2004). Not surprisingly, clinical studies that successfully procured allergen-specific immunotherapy reported an increase in IL-10 secreting CD4<sup>+</sup> T cells (Francis et al., 2003, Jutel et al., 2003). Furthermore, human in vitro studies

have revealed that IL-10 represses IgE production and switching to IgE (Jeannin et al., 1998). Similarly, defects in IL-10 signaling and/or production have been recognized in patients with hyper IgE syndrome, which is characterized by an aberrant increase of circulating IgE (Saito et al., 2011) (Avery et al., 2008). Altogether, there is plenty of evidence suggesting a direct role of IL-10 in the control of IgE and IgE-related pathology.

The notion that natural IgE titers are elevated in  $T_{reg}$  deficient mice suggested a role for  $T_{regs}$  in controlling IgE. Indeed, this hypothesis has been demonstrated by numerous independent studies and by using a variety of mouse models (Palomares, 2013, Calzada and Baos, 2018, Satitsuksanoa et al., 2018) and in humans (Bacher et al., 2016). Nonetheless, whether  $T_{FR}$  cells control IgE responses still remains to be tested. Follicular regulatory T ( $T_{FR}$ ) cells are tentative candidates for repression of high affinity IgE responses, given their FOXP3 expression, abundant IL-10 mRNA expression and location close to antigen-primed B cells, GC B cells and memory B cells (Linterman et al., 2011, Wollenberg et al., 2011b, Chung et al., 2011, Aloulou et al., 2016).

## **6. Preliminary findings, aims and hypotheses**

The bulk of  $T_{FR}$  cell research has been conducted in mouse models, with very little contribution of functional human studies. To date, although regulatory  $CD25^+$  T cells and follicular  $FOXP3^+$  T cells have been reported in humans (Chung et al., 2011, Lim et al., 2004, Carreras et al., 2006) and circulating follicular  $FOXP3^+$  regulatory populations have been described (Wing et al., 2017b, Fonseca et al., 2017) the human equivalent of mouse  $T_{FR}$  cells in human tonsil, the most accessible human secondary lymphoid tissue, remains uncharacterized. Only two reports have studied FOXP3-expressing cells in human tonsils (Wing et al., 2017a) and peripheral lymph nodes (Sayin et al., 2018), albeit their observations remained descriptive due to the difficulty of sorting purified  $FOXP3^+$  cells. Furthermore,  $CD25^+$  follicular T cells have been described in human tonsils, and based on their lack of FOXP3 expression these cells are still presumed not to carry out regulatory roles despite lacking functional data (Li and Pauza, 2015).

Additionally, studies attempting to characterize human  $T_{FR}$  cells may have over-estimated the ability of FOXP3 to identify human  $T_{regs}$ , as unlike in mice, FOXP3 can either 1) be present in activated effector cells (Wang et al., 2007), or 2) be completely absent in some crucial  $T_{reg}$  populations such as  $T_H1$  cells (Battaglia et al., 2006).

Back in 2012, Dr. Rebecca Sweet (a past member of the Vinuesa group) was able to identify a putatively related human  $T_{FR}$  cell population, by using flow cytometry markers associated with human  $T_{regs}$  ( $CD25^{hi} CD127^{lo}$ ) (Liu et al., 2006), in combination with markers characteristic of murine  $T_{FR}$  cells ( $PD-1^{hi} CXCR5^{hi} GITR^{hi} CTLA4^{hi}$ ). Unlike murine  $T_{FR}$  cells, these  $CD25^{+} CD127^{lo} PD-1^{hi} CXCR5^{hi}$  cells did not express FOXP3, but expressed the  $T_{reg}$  associated marker glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR). Interestingly, data obtained by Dr R. Sweet suggest that these putative human  $T_{FR}$  cells are the practically the only T cell-derived source of IL-10 in the GC, with  $T_{FH}$  cells just expressing background levels of this cytokine. Interestingly, STAT3 is distinctly phosphorylated downstream of IL-10 in GC B cells, but only subtly phosphorylated in  $T_{FH}$  cells (Rebecca Sweet, unpublished), suggesting that IL-10 is likely to signal directly to GC B cells. These preliminary findings first suggested a likely regulatory T cell subset characterized by expression of IL-10 in human GCs.

Following up from this work, one of the aims of this thesis is to characterize the function and phenotype of such putative regulatory T cell population in human tonsils. First, by use of a series of functional *in vitro* tools, flow cytometric analyses, comparative transcriptomics and bisulfate sequencing techniques we aim to determine whether this T cell population possesses regulatory properties. We hypothesize that based on preliminary findings that demonstrate resemblance to *bona fide*  $T_{regs}$ , and due to lack of FOXP3 expression, this cell population is a novel  $T_{reg}$  subset likely to arise in the periphery in response to the abundance of environmental antigens in tonsillar tissue. Second, we attempt to uncover the role of IL-10 produced by this cell subset. IL-10 is known to boost peripheral  $T_{reg}$  conversion, and thus we predict that IL-10 helps mediating p $T_{reg}$  differentiation in tonsil. Additionally, given the scarcity of  $IgE^{+}$  cells in the GC, and that IL-10 is



a known repressor of IgE switching, we hypothesize that IL-10-producing follicular T cells repress IgE responses.

The mechanisms that control of germinal center antibody output are of critical interest, since efficient B cell selection in GCs is key for the development of long-lasting antibody responses. By elucidating the role of T cell-derived IL-10 in the GC, and how it controls pathogenic antibody responses, we hope to contribute to uncovering the complex and fascinating regulation of the GC reaction. Additionally, a demonstration of key differences between murine and human  $T_{\text{regs}}$  obtained from secondary lymphoid organs will help us re-evaluate our pre-conceived ideas of what constitutes a  $T_{\text{reg}}$ . Furthermore, I hope this thesis will help us appreciate the importance of conducting human *in vitro* studies despite being far more challenging due to limited resources and technical limitations.

## **Chapter 2: Material and Methods**

## 1. Human tonsil and lymph node cells

Human tonsils were obtained from children undergoing routine tonsillectomy. Tonsillar lymphocyte single cell suspensions were prepared by mechanical disruption of the tissue followed by cell separation using Ficoll Hypaque (GE Healthcare Life Sciences) gradient and frozen until further use, except for RNA sequencing where fresh samples were used. Human mesenteric lymph nodes were obtained as discarded tissue from non-malignancy gastrointestinal surgery. Informed consent was obtained from all subjects. For human tonsil immunohistochemistry, tissue samples were retrieved from the paraffin and cryopreserved archives of the Pathology Unit of San Raffaele Scientific Institute and utilized following IRB-approved institutional rules. All experiments with human samples were approved by the Australian National University's Human Experimentation Ethics Committee and the ACT Health Human Research Ethics Committee.

## 2. Flow cytometry

Tonsillar lymphocytes were stained with the following anti-human antibodies: anti-CD4 APC-Cy7 (RPA-T4, BD Biosciences), anti-CD8 FITC (RPA-T8, BD Biosciences) or PE (SK1, BD Biosciences), anti-CD19 FITC (SJ25C1, Biolegend) or PE Cy7 (SJ25C1, BD Biosciences), anti-CXCR5 Alexa 488 or Alexa 647 or PerCP-Cy5.5 (RF8B2, BD Biosciences) or PerCP-Cy5.5 (J252D4, Biolegend), anti-CD45 RA (HI100, Biolegend), anti-CD45RO (UCHL1, Biolegend), anti-CTLA4 PE (BNI3, BD Biosciences) or PE Cy7 (L3D10, Biolegend). anti-PD-1 PE (MIH4, eBioscience) or BV605 or BV421 (EH12.2H7, Biolegend) or (J105, eBioscience), anti-CD127 FITC (11-1278, eBioscience) or BV421 (A019D5, Biolegend), anti-CD25 biotin (BC96, eBioscience or Biolegend) or PE-Cy7 (BC96, BD Biosciences or Biolegend) or APC (2A3 BD Biosciences), anti-CD127 FITC (A019D5, eBioscience) or BV421 (A109D5, Biolegend) or BV510 (HIL-7R-M21, BD Biosciences), anti-FOXP3 Alexa 647 (259D, eBioscience), anti-GITR PE (110416, R&D Systems), anti-CTLA4 PE-CF-594 (BNI3, BD Biosciences) or PE Cy7 (L3D10, Biolegend), anti-CD40L Pacific Blue or FITC (24-31, Biolegend), anti-BCL6 Alexa 647 or PE-Cy7 (K112-91, BD Biosciences), anti-BLIMP-1 Alexa 647 (646702, R&D systems), anti-

CXCL13 APC (53610, R&D Systems), anti-HELIOS PE (22F6, Biolegend), anti-IL-10 PE (JES3-19F1, Biolegend) or APC (JES3-19F1, BD Biosciences), anti-IL-10R PE (3F9, iCyt), anti-IL-21 PE (3A3-N21, BD Biosciences), anti-IL2 a488 (MQ1-17H12), anti-CD3 APC (HIT3a, BD Biosciences) or Alexa 700 (UCHT1, BD Biosciences) or Pacific Blue (OKT3, Biolegend) or BV510 (OKT3, Biolegend), anti-CD27 FITC or APC (M-T271, BD Biosciences), anti-CD38 FITC (HIT2, BD Biosciences) or PE (HB7, BD Biosciences), anti-LAG3 PE or FITC (#2319-L3-050, R&D systems) anti-pSTAT3 Alexa 647 (4/P-STAT3, BD Biosciences). Intracellular staining was performed using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) or Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. LAG3 was stained at 37°C for 15 minutes in the dark.

Mesenteric lymph nodes were stained with the following anti-human antibodies – anti-CD3 BUV395 (UCHT1, BD Biosciences), anti-CD4 PerCP (RPA-T4, Biolegend), anti-CD8 APC-C7 (RPA-T8, Biolegend), anti-CD19 APC-Cy7 (HIB19, Biolegend), anti-CD45RA PE-TR (MEM-56, Invitrogen), anti-CXCR5 BV510 (RF8B2, BD Biosciences), anti-PD-1 BV421 (EH12.2H7, Biolegend), anti-CD127 BV650 (HIL-7R-M21, BD Biosciences), anti-CD25 PE-C7 (BC96, Biolegend), anti-FOXP3 PE (259D/C7, BD Biosciences), anti-CD27 BV711 (L128, BD Biosciences). Intracellular staining was performed using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions.

### **3. Double immunohistochemistry**

Heat-induced antigen retrieval in Tris EDTA buffer (pH 9.0) for 30 minutes at 97 °C was used, followed by blocking of endogenous peroxidase with 3% H<sub>2</sub>O<sub>2</sub> and incubation with 3% normal bovine serum. Primary antibodies (anti-FOXP3 (236A/E7, Abcam), anti-CD25 (4C9, Ventana-Roche), anti-IgD (polyclonal, Ventana-Roche) were incubated for 1 hour at room temperature, followed by detection with HRP conjugate-polymer (Thermo Scientific, Fremont, CA, USA) and developed with DAB chromogen. The second reaction was performed after a quick round of blocking in Tris EDTA buffer (pH 9.0), overnight incubation with the second primary

antibody at 4°C and developed using AP conjugate-polymer and Fast Red chromogen (Thermo Scientific, Fremont, CA, USA). Tissue sections were counterstained with haematoxylin.

#### **4. CpG methylation analysis by Bisulfite sequencing.**

Genomic DNA was prepared using the NucleoSpin Tissue XS kit (Macherey-Nagel). After Sodium Bisulfite treatment (MethylEasy Xceed, Human Genetic Signatures), modified DNA was amplified by PCR and subcloned into PCR2.1- TOPO Vector (Invitrogen). PCR primers used were TTGGGTAAAGTTTGTGTAGGATAG and ATCTAAACCCTATTATCACAACCCC. The colonies (16-48 colonies/region) were directly amplified using the Illustra™ TempliPhi™ Amplification Kit (GE Healthcare), and sequenced.

#### **5. In vitro stimulation**

Intracellular cytokine staining was performed following 4-6 hours of PMA (50 ng/ml) and Ionomycin (500 ng/ml) stimulation with GolgiStop (BD Biosciences) or Brefeldin A (Biolegend) in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin-streptomycin, 0.1 mM non-essential amino acids, 100 mM Hepes, and 44 µM 2-mercaptoethanol. 200,000 sorted naïve B cells were stimulated with 50 ng/ml recombinant human IL-10 (Peprotech) for 30 minutes with or without 5µg/ml of anti-IL-10 blocking antibody (3F9, Biolegend).

#### **6. Secreted Cytokine Surface Capture**

Live CD4<sup>+</sup> human tonsillar lymphocytes were stained with the anti-human IL-10 catch reagent (Miltenyi) at 9 million cells per 100 ul. After 10 minutes on ice, the cells were diluted 1:20 in RPMI supplemented as above. Stimulation was with PMA (100 ng/ml) and Ionomycin (500 ng/ml). Cells were incubated for 2 hours in a 37°C incubator with 5% CO<sub>2</sub>, rotating slowly

using a MacsMix (Miltenyi). Cells were then stained as above using the anti-human IL-10 detection Ab (Miltenyi).

## 7. Microarray RNA analysis

IL-10 positive and negative T<sub>FR</sub> cells were sorted from 3 human subjects. mRNA was extracted, and samples were sent to the Ramaciotti Centre for Genomics (Sydney, Australia) for analysis with Affymetrix GeneChip® Human Gene 2.0 *ST* microarrays. The resulting 9 CEL files were imported into Partek Genomics Suite (Version 6.6) using the RMA algorithm with RMA background correction, quantile normalization and median polish probeset summarization. Differentially-expressed probesets were identified in Partek GS using a 2-factor ANOVA model with the factors being “SubjectID”. GEO NCBI accession number GSE79887.

## 8. Autologous human tonsillar T<sub>FH</sub>:B cell co-cultures and IgE

### detection

20,000 human FACS purified T<sub>FH</sub> and/or T<sub>FR</sub> cells were co-cultured with 100,000 germinal center B (CD4<sup>+</sup>CD19<sup>+</sup>CD38<sup>+</sup>CD27<sup>int</sup>) cells or memory B (CD4<sup>+</sup>CD19<sup>+</sup>CD38<sup>+</sup>CD27<sup>+</sup>) cells in the presence of SEB (500ng/ml) (Sigma Aldrich) for either 3, 5 or 8 days. For IgE-inducing conditions recombinant IL-4 and IL-13 (Peprotech) were used at 40 ng/ml. Culture supernatants were used to measure IgE and IgG using the Cytometric Bead Array (CBA) flex sets (#558682 and #558679, BD Bioscience) according to manufacturer’s instructions. For proliferation assays cells were stained with CTV (Thermo Fisher) according to manufacturer’s instructions. IL-10R blockade was achieved using anti IL-10R blocking antibody (3F9, Biolegend) or isotype control (RTK2758, Biolegend) at 5µg/ml. For epsilon germline transcript detection, 100,000 naïve B cells (IgD<sup>+</sup> CD19<sup>+</sup> cells) were incubated with recombinant IL-4 and IL-13 together with SEB with or without T<sub>FH</sub> and or T<sub>FR</sub> cells for 24 hours. RNA was extracted using phenol/chloroform extraction and cDNA was synthesized. Epsilon germline transcripts were detected using (Forward: 5’ TGCATCCACAGGCACCAAAT and

Reverse: 5' ATCACCGGCTCCGGGAAGTA-3) and normalised to RPL13 (Forward: 5' CTCAAGGTGTTTGACGGCATCC, Reverse: 5' TACTTCCAGCCAACCTCGTGAG).

## 9. Human T<sub>reg</sub> suppression assays

10,000 CTV labeled FACS purified responder T cells (CD4<sup>+</sup>CD3<sup>+</sup>CD25<sup>-</sup>) were co-cultured in the presence or absence of serially diluted follicular or non-follicular T regulatory cells starting with 10,000 cells. Cells were stimulated with CD3/CD28 microbeads (Miltenyi) at a 1:1 bead to T<sub>responder</sub> cell ratio. After 3 days, cells were stained with 7-AAD and subsequently analyzed.

## 10. RNA sequencing (RNA-seq) and analysis.

The different subsets of human follicular T cells were FACS purified from 3 fresh tonsils. mRNA was extracted and sent to the ACRF Biomolecular Resource Facility, The John Curtin School of Medical Research, Australian National University for Library construction using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina). Library samples were sequenced on a HiSeq2000 with a coverage of 25 million reads. The data was then sent to the Genome Discovery Unit (ANU Bioinformatics Consulting Unit, JCSMR, ANU) for analysis. Initial quality control checks with FastQC revealed that none of the 12 samples were problematic. All reads were aligned to the *H. sapiens* genome reference sequence using TopHat version 2.0.13 with default parameters. Read counts were then generated for each gene in each sample using featureCounts version 1.4.6-p1 by using annotated gene locations. Differential expression analysis was performed using the edgeR package version 3.10. Read counts per gene were normalized by trimmed mean of M-values (TMM). As edgeR uses the negative binomial distribution as its basic model for differential expression data, dispersion estimates were obtained using the quantile-adjusted conditional maximum likelihood (qCML) method for single factor experiments. Then, the qCML-based exact test for the negative binomial distribution was performed to test for differentially expressed genes in our groups of samples. We used a Benjamini-Hochberg adjusted

p-value threshold of 0.05 to identify significantly differentially regulated genes. NCBI Sequence read archive accession number (SRP072739)

## 11. Mathematical modelling

The GC model LEDAX (Meyer-Hermann et al. Cell Reports 2012) was complemented by dynamic switching (He et al. JEM 2013), dynamic number of divisions (Meyer-Hermann J Immunol 2014), and a continuous influx of founder B cells (Tas et al. Science 2016). Here, only the model assumptions specific to IgE are discussed. As determined by experiment and simulation, a deficiency of IgE GC-B cells in acquisition of the LZ phenotype was assumed (He et al. JEM 2013): All IgE GC-B cells have a B cell receptor expression level reduced to 30% plus a defect in the upregulation of CXCR5, i.e. in 75% of the cases IgE GC-B cells remain sensitive to CXCL12. Reduced impact of  $T_{FR}$  cell was modelled by increasing the number of  $T_{FH}$  from 250 to 400, by reducing the K-value of the Hill-function describing the dynamic number of divisions (Meyer-Hermann J Immunol 2014) by a factor of 2/3, and by increasing the probability of positively selected GC-B cells to differentiate to output cells from 0.23 to 0.7. All simulations were repeated 100 times with random number generator seed from CPU time. The simulations software was programmed in C++ and data analysis was performed using R.

## 12. Human $T_{reg}$ differentiation assays

$T_{reg}$  differentiation was carried out as previously described (Hsu et al., 2015). Briefly, 20,000 FACS-sorted  $CD25^{-}$   $T_{FH}$  cells were stimulated with aCD3aCD28 beads (miltenyi) together with 100 ng/ml of IL-2 and 5 ng/ml of TGF- $\beta$  in the presence or absence of IL-10 (20 ng/ml). Cytokines were added on days 0 and 3, and cultures were analysed on day 7.

## 13. Statistical Analyses

All data were analyzed with non-parametric Mann-Whitney test (U test) except for for some human cell culture experiments, in which paired Student's t test was used. Paired analyses were performed using the Wilcoxon test. All statistical analysis was performed with Prism software



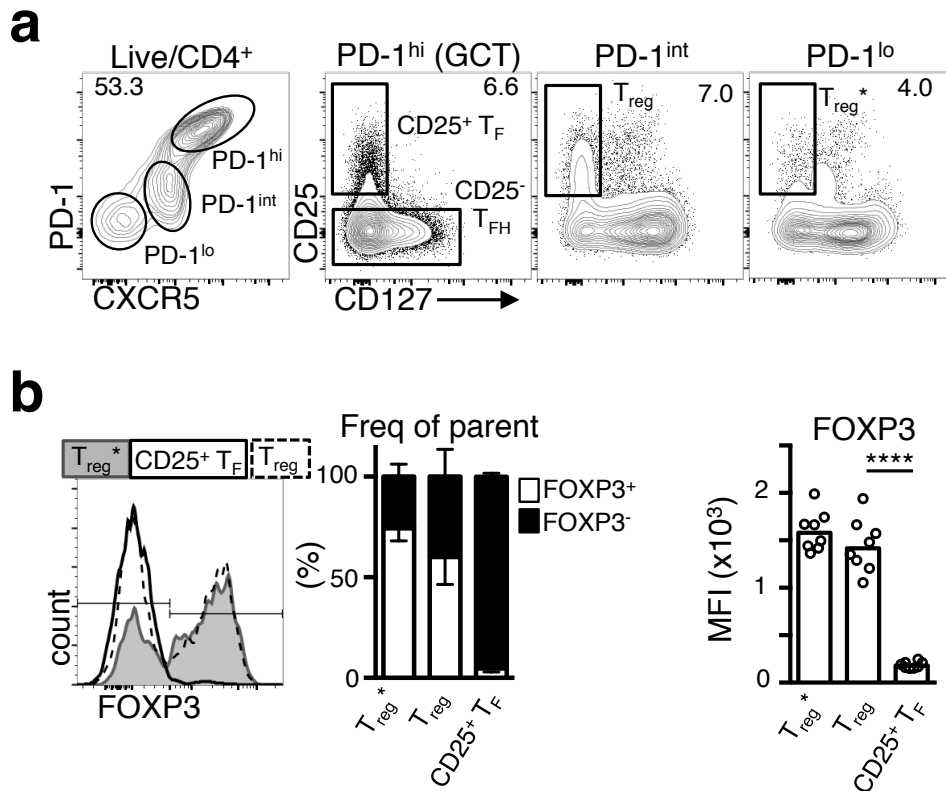
## Chapter 2: Materials and methods

(version 6, GraphPad Software). Statistically significant differences are indicated as \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , and \*\*\* $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$  and ns = not significant.

## **Chapter 3: Results**

## 1. Identification of IL-10-producing follicular T cells in human tonsil

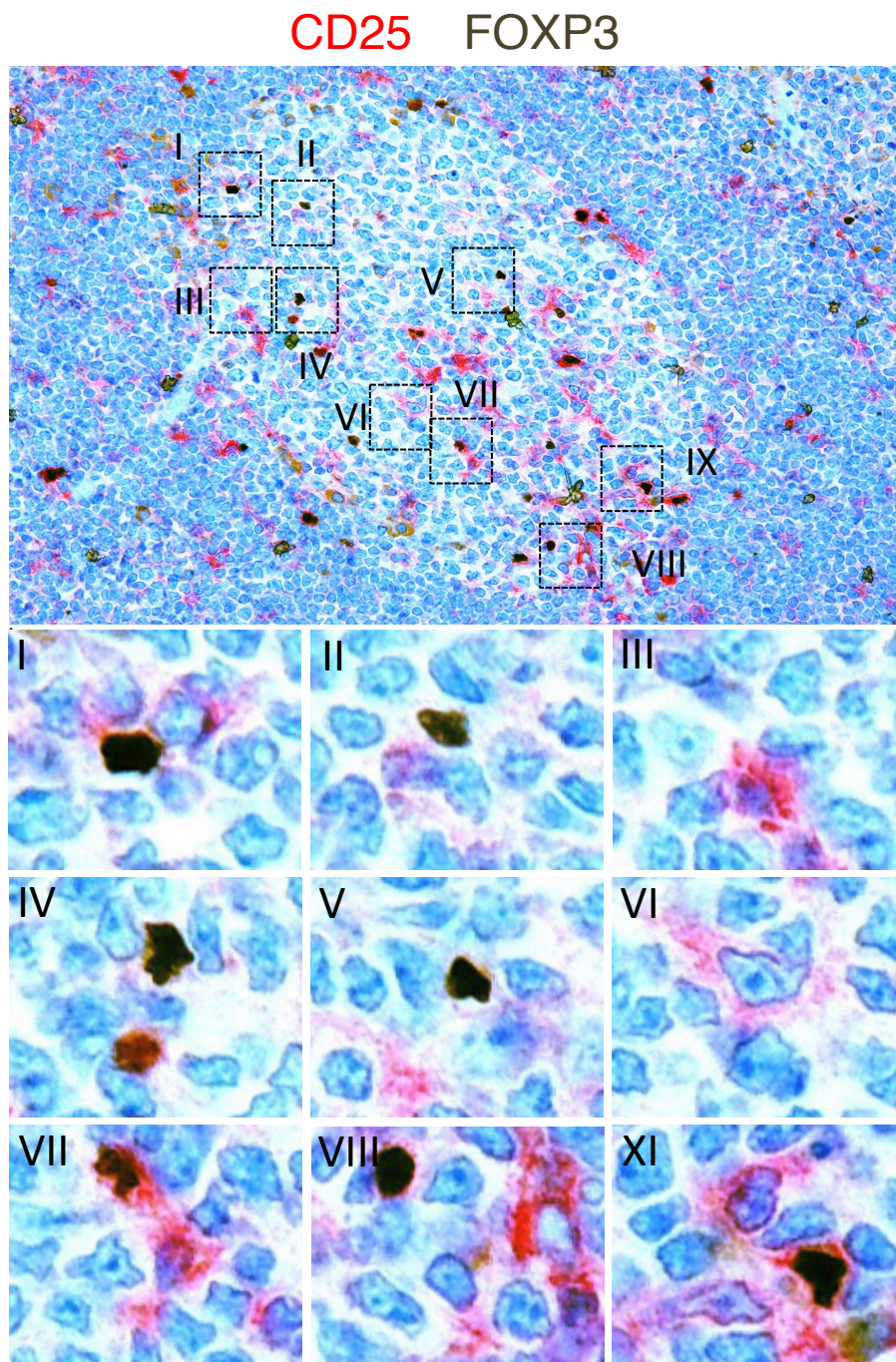
In an effort to identify the human equivalent of mouse  $T_{FR}$  cells, we stained cells from the most accessible human secondary lymphoid tissue, tonsil, for markers of follicular T cells and  $T_{regs}$ . Total  $T_{regs}$  were identified by expression of CD25 in the absence of CD127 (Seddiki et al., 2006) (**Figure 3a**). The majority of  $CD25^+ CD127^- T_{regs}$  found within the non-follicular T effector gate ( $CXCR5^{int} PD-1^{int}$ ) expressed FOXP3, and as such constitute the conventional  $T_{reg}$  population (**Figure 3a-b**). A  $CD25^+ CD127^-$  subset was also identified within the follicular  $CXCR5^{hi} PD-1^{hi}$  population, which we refer to as “ $CD25^+ T_F$ ” cells (**Figure 3a**). We speculated that these might correspond to the  $T_{FR}$  cell population identified in mice, though we were surprised to find that the majority (~95%) of these tonsillar  $CD25^+ CD127^-$  follicular T cells lacked FOXP3 expression ( $p = <0.0001$ ) (**Figure 3b**).



**Figure 3. Identification of FOXP3<sup>-</sup> human follicular regulatory T cells in human tonsils.**

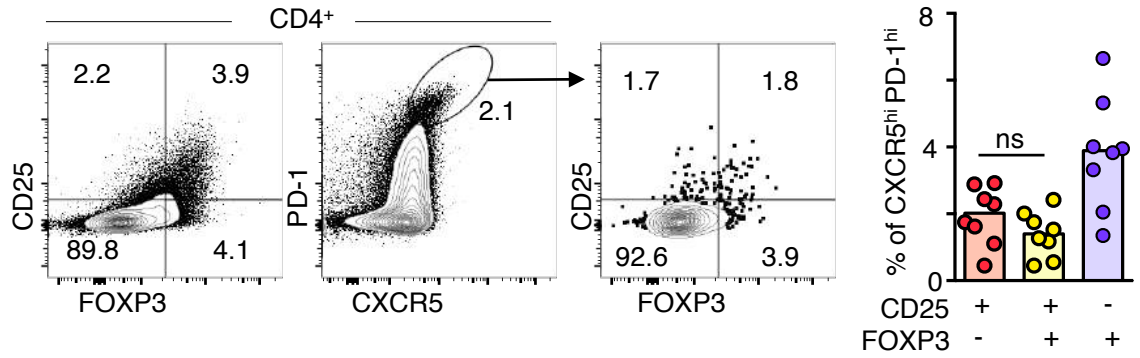
(a) Flow cytometric plots showing gating strategy to identify the indicated populations. (b) Flow cytometric plots and quantification (n=8) of percentage of FOXP3<sup>+</sup> cells and FOXP3 MFI within the indicated subset according to (a). Bars represent means (left graph) or medians (right graph) and error bars sd. Data is representative of 10 independent experiments. Each dot represents an individual donor (n=8). ns, not significant, (\*p ≤ 0.05), (\*\* p ≤ 0.001), (\*\*\*\* p ≤ 0.0001) non-parametric Mann-Whitney test (U test).

To investigate the localization of CD25<sup>+</sup> and Foxp3<sup>+</sup> cells in human tonsil, we stained human tonsil sections using immunohistochemistry. We could easily identify CD25<sup>+</sup> cells lacking FOXP3 expression (**Figure 4**) within germinal centers, in the follicular mantle and in perifollicular areas. We also identified CD25<sup>+</sup> FOXP3<sup>+</sup> and CD25<sup>-</sup> FOXP3<sup>+</sup> cells (**Figure 4**). CD25<sup>+</sup> FOXP3<sup>-</sup> follicular T cells were also present in human mesenteric lymph nodes, albeit these were less frequent than those seen in the tonsil and found at comparable proportions to CD25<sup>+</sup> FOXP3<sup>+</sup> T cells ( $p = 0.2236$ ) (**Figure 5**).



**Figure 4. Expression of Foxp3 and CD25 in human tonsils.**

Representative double immunohistochemistry on human tonsil tissue showing localization of CD25<sup>+</sup> cells (Red) and FOXP3 (brown). Tissue sections were counterstained with haematoxylin (blue). Original magnification x200. Data is representative of two independent experiments. Results courtesy by Claudio Dondglioni.

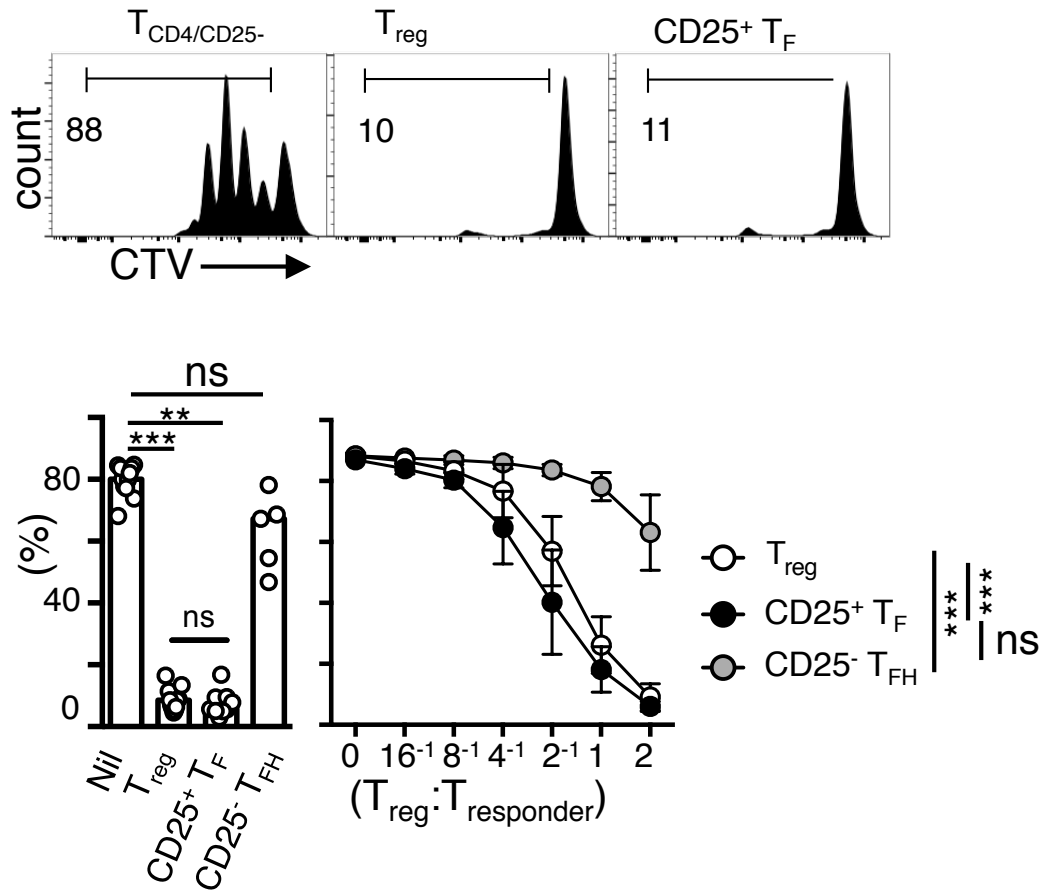


**Figure 5. Human CD25<sup>+</sup> T follicular cells in human lymph nodes**

(a) Flow cytometric plots and quantification of CD25 and FOXP3 expressing cells amongst follicular T cells from human mesenteric lymph nodes (n=8). Data are representative of two independent experiments. Bars represent medians and each dot an individual LN donor. ns, not significant, non parametric Mann-Whitney test (U test). Results courtesy by Ismail Sayin and David H Canaday.

In view of absent FOXP3 expression in the majority of human tonsil CD25<sup>+</sup> T<sub>F</sub> cells, we considered that these could be highly activated T<sub>FH</sub> cells that had upregulated CD25, as previously proposed (Li and Pauza, 2015) rather than regulatory cells with a function similar to conventional CD25<sup>+</sup> CD127<sup>-</sup> FOXP3<sup>+</sup> T<sub>regs</sub>. Surprisingly, analysis of their ability to suppress T cell proliferation *in vitro* indicated that these cells were regulatory, rather than activated effectors. Unlike typical helper T<sub>FH</sub> cells (CD25<sup>-</sup> CD127<sup>-</sup>), human CD25<sup>+</sup> T<sub>F</sub> cells and T<sub>regs</sub> were equally effective in suppressing conventional (CD25<sup>-</sup> CD4<sup>+</sup>) T cell proliferation (**Figure 6**), as previously described for mouse T<sub>FR</sub> cells (Linterman et al., 2011, Chung et al., 2011). Together, these results demonstrate that human CD25<sup>+</sup> CD127<sup>-</sup> follicular T cells are not simply activated T<sub>FH</sub> cells, but can behave like regulatory T cells.

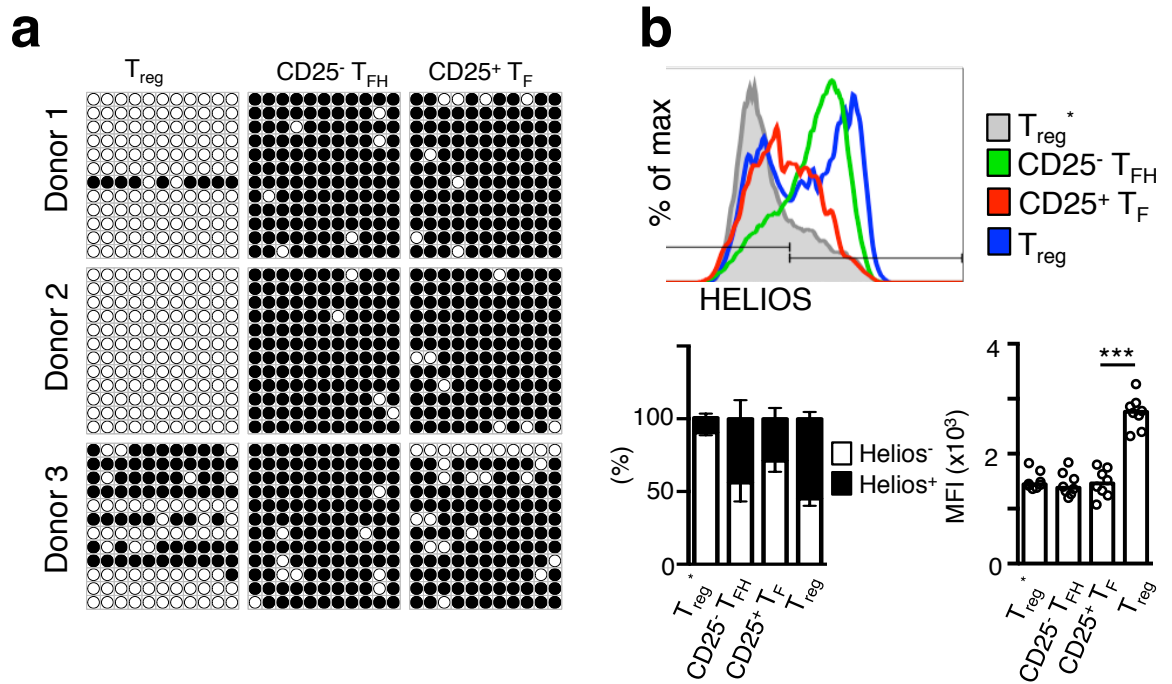




**Figure 6. Human  $CD25^+$   $T_F$  cells are  $T_{regs}$  in the conventional sense.**

Flow cytometric plots and quantification of proliferating  $CD4^+CD25^-$  responder T cells. Proliferation is measured as dilution of the cytoplasmic fluorescent dye (CTV) after 3 days of  $\alpha$ -CD3 and  $\alpha$ -CD28 stimulation in the presence or absence of  $T_{regs}$  ( $n=10$ ),  $CD25^+$  follicular T ( $n=8$ ) and  $CD25^-$  follicular helper T cells ( $n=5$ ). Each dot represents the mean value of cultures set up in triplicate from a single donor. Data is representative of five independent experiments. ns, not significant, (\*\*  $p \leq 0.01$ ), (\*\*\*)  $p \leq 0.001$ ), non-parametric Mann-Whitney test (U test) (left panel) or 2-way ANOVA (right panel).

To gain insights into CD25<sup>+</sup> T<sub>F</sub> cell ontogeny, we investigated the methylation of the CNS2 locus of the *FOXP3* promoter, as demethylation of this locus is critical for stable FOXP3 expression characteristically seen in tT<sub>regs</sub> (Zheng et al., 2010). The CNS2 locus was over 90% methylated in human CD25<sup>+</sup> T<sub>F</sub> cells (**Figure 7a**), suggesting a peripheral rather than thymic origin. Similarly, compared to T<sub>regs</sub>, CD25<sup>+</sup> T<sub>F</sub> cells expressed 40% less HELIOS (**Figure 7b**,  $p = 0.0002$ ), a known target of FOXP3 (Fu et al., 2012) that is abundant in, although not exclusive of, tT<sub>regs</sub> (Thornton et al., 2010, Szurek et al., 2015). Although mouse T<sub>FR</sub> cells were originally thought to be exclusively of thymic origin, peripherally induced T<sub>FR</sub> cells have also been recently described (Aloulou et al., 2016, Fu et al., 2012). Together, these data demonstrate the existence of human CD25<sup>+</sup> FOXP3<sup>-</sup> follicular regulatory T cells that appear to be enriched in tonsillar tissue.



**Figure 7. Human  $CD25^+$   $T_F$  cells may be peripherally induced  $T_{regs}$ .**

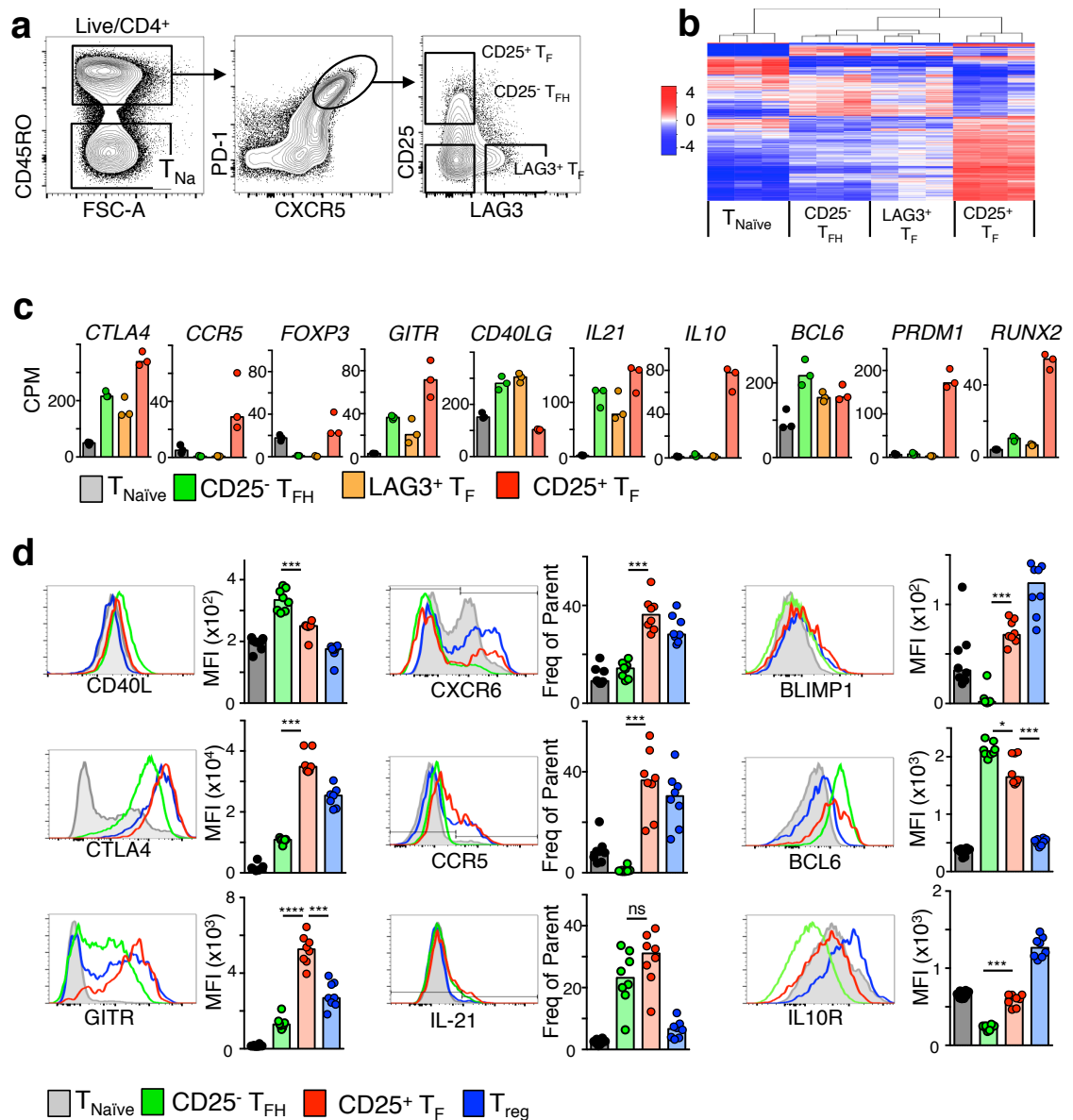
(a) Bisulfite sequencing of the 11 CpG islands in the *FOXP3* CNS2 locus with 12 representative clones per population per donor (filled circle = methylated, open circle = demethylated). Results courtesy by R.A. Sweet. Experiment was conducted by N.Ohkura and S.Skagaguchi. (b) Flow cytometric plots and quantification showing HELIOS expression in the indicated cell subsets. Bars represent means (left) or medians (right) and error bars sd. Each dot represents an individual donor (n=8). Data is representative of three independent experiments. ns, not significant, (\*\*\*)  $p \leq 0.001$ , non-parametric Mann-Whitney test (U test).

## 2. Human CD25<sup>+</sup> follicular T cells resemble mouse T<sub>FR</sub> cells

To investigate the extent to which human CD25<sup>+</sup> T<sub>F</sub> cells resembled mouse T<sub>FR</sub> cells, we determined the transcriptional signature of CD25<sup>+</sup> T<sub>F</sub> cells. Type 1 regulatory (T<sub>r</sub>1) cells that co-express lymphocyte-activation gene 3 (LAG3) have been previously described (Gagliani et al., 2013). We noted that amongst follicular T cells, CD25<sup>+</sup> T<sub>F</sub> cells did not overlap with LAG3<sup>+</sup> cells. Thus, we sorted naïve T cells and the three major follicular T cell subsets according to CD25 and LAG3 expression: CD25<sup>+</sup> LAG3<sup>-</sup> T<sub>FR</sub> cells, CD25<sup>-</sup> LAG3<sup>+</sup> T<sub>F</sub> cells and CD25<sup>-</sup> LAG3<sup>-</sup> T<sub>FH</sub> cells (Figure 8a), and performed RNA sequencing. Paired analyses from 3 different donors revealed that CD25<sup>+</sup> T<sub>F</sub> cells were fundamentally different from naïve, T<sub>FH</sub>, and LAG3<sup>+</sup> T<sub>FH</sub> cells (Figure 8b) and were remarkably similar to the phenotype described for mouse T<sub>FR</sub> cells (FOXP3<sup>+</sup> follicular T cells).

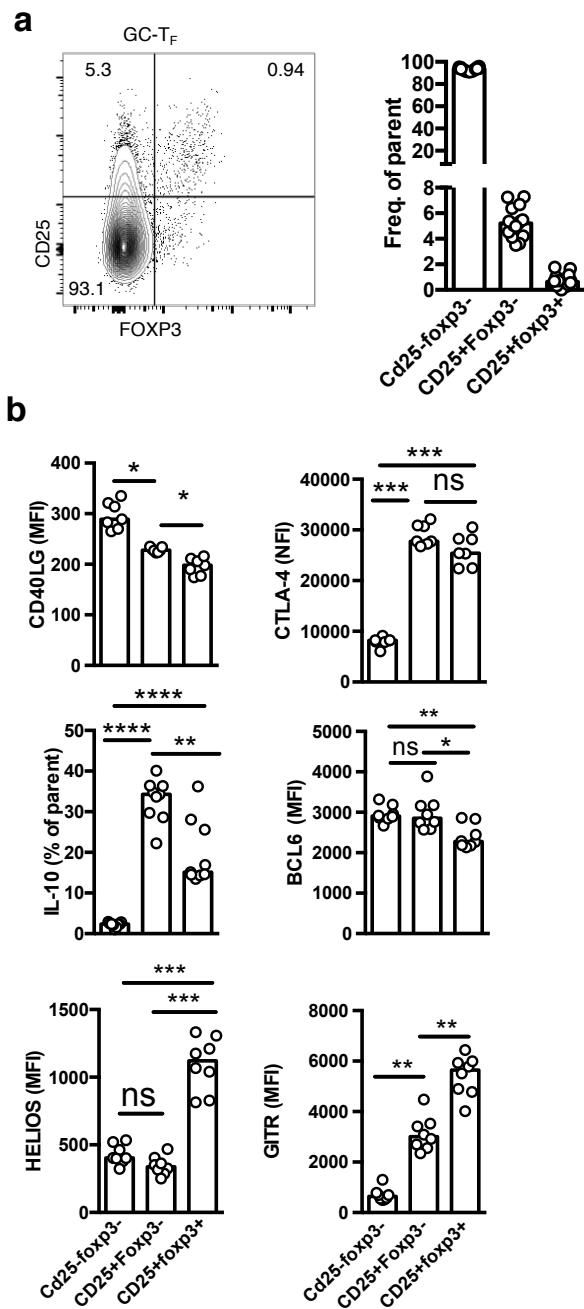
Human CD25<sup>+</sup> T<sub>F</sub> cells expressed key molecules required for T<sub>FH</sub> cell development including *BCL6*, and showed the highest expression of transcripts associated with effector T<sub>regs</sub> including *CTLA4*, *GITR*, *PRDMI*, *RUNX2*, *CCR5*, and *IL10* (Figure 8c) (Fu et al., 2012). Similar to mouse T<sub>FR</sub> cells, human CD25<sup>+</sup> T<sub>F</sub> cells expressed the lowest amount of the key B cell helper molecule *CD40LG*, but abundant *IL21*, which is low in mouse T<sub>FR</sub> cells but has also been shown to be expressed in T<sub>FR</sub> cells from macaques (Chowdhury et al., 2015). Flow cytometric analysis of protein expression confirmed the similarities between CD25<sup>+</sup> T<sub>F</sub> cells and T<sub>regs</sub> (Figure 8d).

Similarly, amongst tonsil follicular T cells, minimal differences were observed between the small fraction of FOXP3<sup>+</sup> CD25<sup>+</sup> and the more abundant FOXP3<sup>-</sup> CD25<sup>+</sup> T cells. The latter expressed more IL-10 and BCL6, whereas FOXP3<sup>+</sup> cells expressed more HELIOS and GITR (both direct targets of FOXP3) (Figure 9). Together, these results suggest that human CD25<sup>+</sup> T<sub>F</sub> cells have the gene expression profile that allows follicular migration and T<sub>reg</sub> function.



**Figure 8. Human CD25<sup>+</sup> T follicular cells' transcriptome resemble that of murine T<sub>FR</sub> cells.**

(a) Flow cytometric plots showing the gating strategy used to sort-purify each cell subset. (b) Heat-map analysis of RNA-seq showing the RNAs differentially-expressed in CD25<sup>+</sup> T<sub>F</sub> cells compared to the indicated T cell populations (Log<sub>2</sub> value of counts per million) extracted from the tonsils of 3 individuals. (c) Selected transcripts from (b) in the indicated subsets (RNA counts per million). (d) Flow cytometric plots and quantification (n=8) of the indicated proteins. Data is representative of at least 3 independent experiments. In all graphs bars represent medians; each dot represents a single tonsil donor. ns, not significant, (\* p ≤ 0.05), (\*\* p ≤ 0.001) and (\*\*\* p ≤ 0.0001) non-parametric Mann-Whitney test (U test).



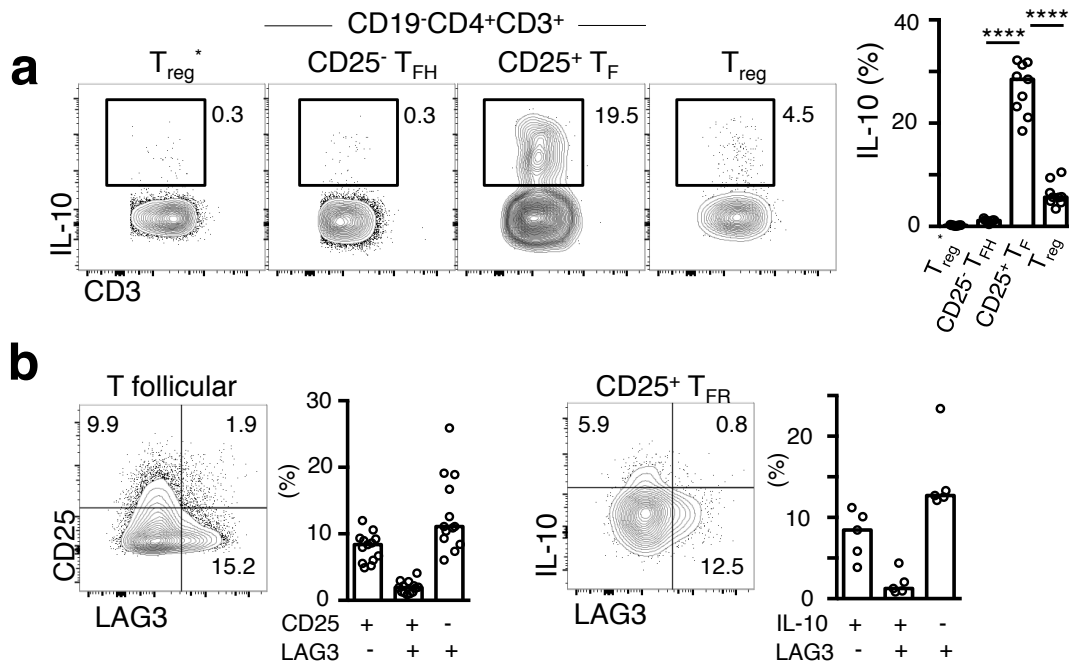
**Figure 9. Phenotypic comparison between CD25<sup>+</sup>FOXP3<sup>-</sup> and CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>F</sub> cells**

(a) Flow cytometric plots and quantification (n=12) of the indicate population within tonsillar T follicular cells (GC-T<sub>F</sub>). Data is representative of 3 independent experiments. (b) Flow cytometric quantification of the indicated protein within the indicated tonsillar cell subset (n=7). Data is representative of 2 independent experiments. ns, not significant (\*p ≤ 0.05), (\*\*p ≤ 0.01), (\*\*p ≤ 0.001), (\*\*\*\*p ≤ 0.0001), non-parametric Wilcoxon test.

### 3. Human CD25<sup>+</sup> T<sub>F</sub> cells constitute the major follicular T cell subset producing IL-10

We were intrigued by the selective production of IL-10 by human Foxp3<sup>-</sup> CD25<sup>+</sup> T<sub>F</sub> cells, compared to human T<sub>FH</sub> cells, which contrasts with mice in which both T<sub>FH</sub> and T<sub>FR</sub> cells express IL-10. Besides conventional T<sub>regs</sub>, T<sub>r1</sub> cells and terminally-differentiated T<sub>H1</sub> and T<sub>H2</sub> effectors (Jankovic et al., 2007, Altin et al., 2012) are also characterized by production of IL-10 (Gagliani et al., 2013). We therefore investigated the relative ability of the different tonsil T cell subsets to produce IL-10.

Strikingly, and consistent with the RNAseq data, staining for IL-10 revealed that FOXP3<sup>-</sup> CD25<sup>+</sup> follicular T cells were the subset containing the largest fraction of IL-10-producing T cells in human tonsil (**Figure 10a**): 20-30% expressed IL-10 compared with 3-12% of conventional T<sub>regs</sub> ( $p = <0.0001$ ), and barely any T<sub>FH</sub> cells ( $p = <0.0001$ ) (**Figure 10a**). Unlike IL-10-producing T<sub>r1</sub> cells, IL-10-producing follicular T cells did not express LAG3 (**Figure 10b**), suggesting that CD25<sup>+</sup> T<sub>F</sub> cells are not T<sub>r1</sub> cells licensed to enter the follicle.

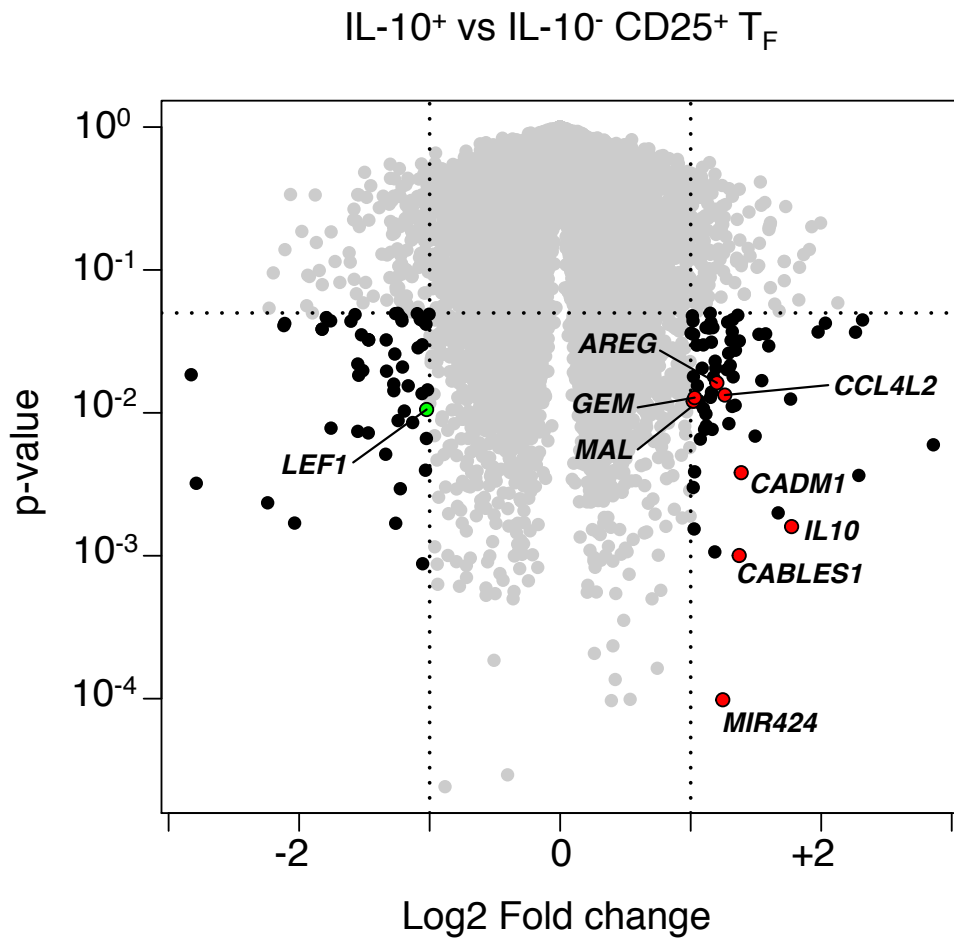


**Figure 10. Human CD25<sup>+</sup> T<sub>F</sub> cells express abundant IL-10.**

(a) Flow cytometric plots and quantification of PMA/Ionomycin-stimulated tonsillar cell suspensions showing IL-10 expression in the indicated subset (n=9). (b) Flow cytometric plot and quantification showing LAG3 and CD25 expression in total T follicular cells (n=8) (left panel), and IL-10 and LAG3 in CD25<sup>+</sup> T<sub>F</sub> cells (n=5) (right panel). Data is representative of 5 independent experiments. In all graphs bars represent medians and each dot represents a single tonsil donor. ns, not significant, (\* p ≤ 0.05), (\*\* p ≤ 0.001), (\*\*\*\* p ≤ 0.0001) non-parametric Mann-Whitney test (U test).



Having demonstrated that only a subset of CD25<sup>+</sup> T<sub>F</sub> cells expressed IL-10, we asked whether IL-10<sup>+</sup> and IL-10<sup>-</sup> T<sub>F</sub> cells were fundamentally different subsets. Transcriptional profiling using Affymetrix RNA microarrays of IL-10-producing vs non-producing CD25<sup>+</sup> T<sub>F</sub> cells revealed highly comparable transcriptomes (**Figure 11**) with only a few differentially expressed transcripts including *IL-10*, *CCL4L2*, *MAL* (T-Lymphocyte Maturation-Associated Protein), *MIR424* – reported to be an activator of TGF- $\beta$  signaling (Li et al., 2014), and *LEF1*, a transcription factor important for T<sub>FH</sub> differentiation (Choi et al., 2015). These data suggest that IL-10 producing and non-producing CD25<sup>+</sup> T<sub>F</sub> cells are closely related, with IL-10 expression likely to occur upon activation.

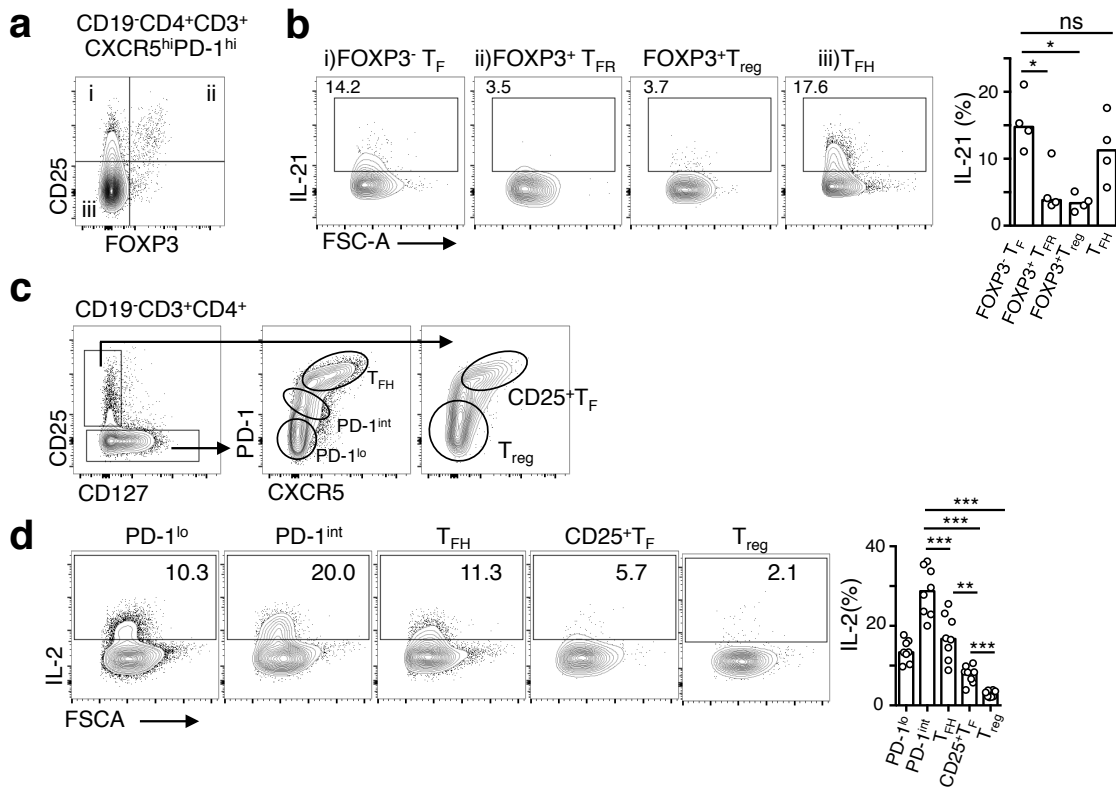


**Figure 11. Transcriptomic differences between human CD25<sup>+</sup> IL-10<sup>+</sup> and CD25<sup>+</sup>IL-10<sup>-</sup> T<sub>F</sub> cells.**

Volcano plot of Affymetrix RNA microarrays comparing gene expression between IL-10-producing and non-producing CD25<sup>+</sup> T<sub>F</sub> cells (n=3). IL-10 staining was performed using the IL-10 catch reagent kit (Miltenyi). Results courtesy by S.Ohms, R.A. Sweet and JA Roco.

In view of the high amounts of IL-21 seen in CD25<sup>+</sup> T<sub>F</sub> cells, we asked whether the less abundant FOXP3<sup>+</sup> T<sub>FR</sub> cell subset was also capable of secreting IL-21. Intracellular flow cytometric staining of tonsil cells confirmed abundant IL-21 expression in CD25<sup>+</sup> T<sub>F</sub> cells, comparable to that of T<sub>FH</sub> cells (**Figure 12a-b**). Interestingly, all FOXP3<sup>+</sup> T cells regardless of expression of follicular markers lacked IL-21 expression. This is consistent with reports that FOXP3 enforces repression of IL-21 (Gavin et al., 2007). Thus, CD25<sup>+</sup> T<sub>F</sub> cells are distinct from the rarer tonsillar CD25<sup>+</sup> FOXP3<sup>+</sup> T<sub>FR</sub> cells and the mouse Foxp3<sup>+</sup> T<sub>FR</sub> cells in their expression of IL-21.

CTLA4 is one of the most important targets of FOXP3 in T<sub>regs</sub>, and high amounts of CTLA4 expression plus absence of IL-2 production are good markers of T<sub>reg</sub> function even in the absence of FOXP3 expression (Yamaguchi et al., 2013). As previously shown in our RNA-seq and flow cytometry experiments, CD25<sup>+</sup> T<sub>F</sub> cells had the highest amount of CTLA4 amongst tonsil T cell subsets (**Figure 8c-d**). IL-2 production was only detected in approximately 5% of human tonsil CD25<sup>+</sup> T<sub>F</sub> cells (**Figure 12c, d**), further suggesting this is indeed a regulatory cell subset. In summary, human CD25<sup>+</sup> T<sub>F</sub> cells express abundant IL-10, CTLA4 and IL-21 but little to no IL-2.



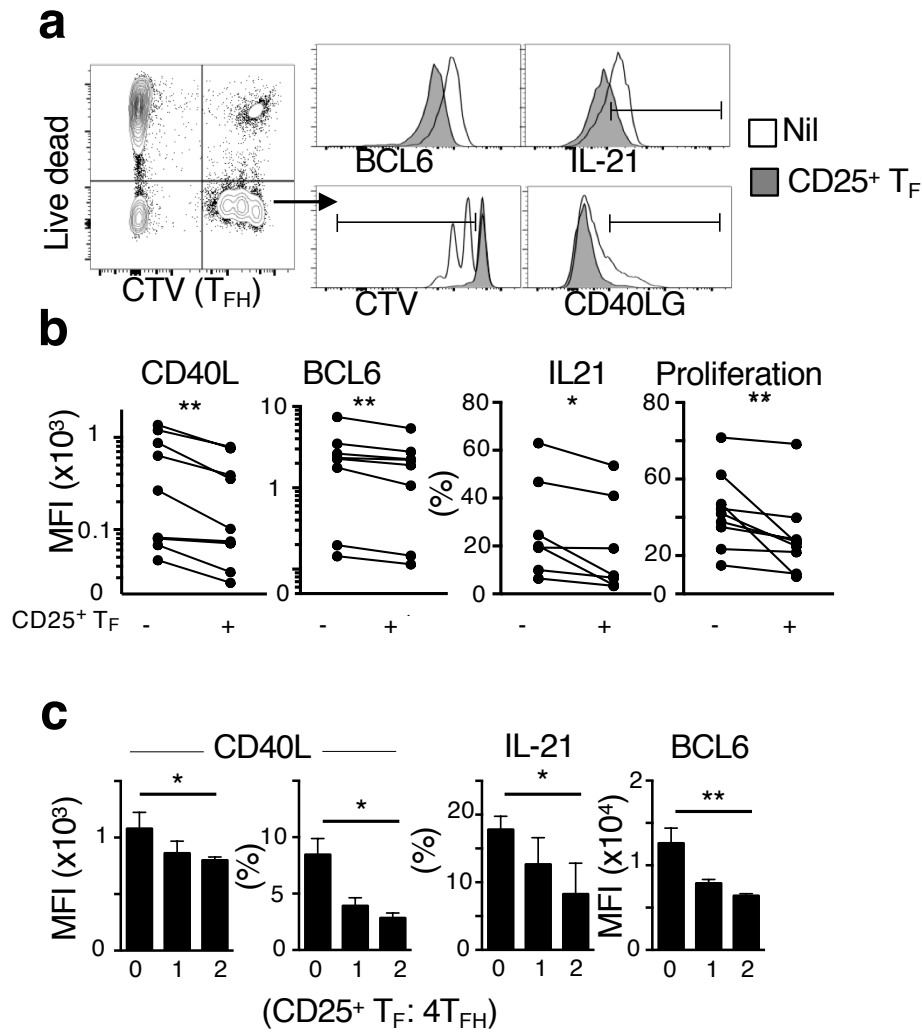
**Figure 12. Human CD25<sup>+</sup> T<sub>F</sub> cells express IL-21 but little to no IL-2.**

Flow cytometric plots and quantification showing (a) gating strategy and (b) IL-21 expression of PMA/Ionomycin-stimulated tonsillar cell suspensions in the indicated subset (n=4). Data is representative of 2 independent experiments. (c-d) Flow cytometric plots and quantification showing (c) gating strategy and (d) IL-2 expression of PMA/Ionomycin-stimulated tonsillar cell suspensions in the indicated subset (n=8). Data is representative of 2 independent experiments. In all graphs bars represent medians and each dot represents a single donor. ns, not significant, (\*  $p \leq 0.05$ ), (\*\*  $p \leq 0.01$ ), (\*\*\*)  $p \leq 0.001$ ), (\*\*\*\*  $p \leq 0.0001$ ) non-parametric Mann-Whitney test (U test).

#### 4. Human CD25<sup>+</sup> T<sub>F</sub> cells regulate T<sub>FH</sub> cells

In mice, both B cells and T<sub>FH</sub> cells have been suggested to be targets of T<sub>FR</sub> cell suppression (Sage and Sharpe, 2016). To test whether human tonsillar CD25<sup>+</sup> T<sub>F</sub> cells could regulate T<sub>FH</sub> cell function, we analyzed CTV-labeled T<sub>FH</sub> cells after three days in culture with autologous memory B cells and CD25<sup>+</sup> T<sub>F</sub> cells in the presence of Staphylococcus enterotoxin B (SEB). Addition of CD25<sup>+</sup> T<sub>F</sub> cells suppressed T<sub>FH</sub> cell proliferation (**Figure 13a**).

We next looked for potential regulatory roles of CD25<sup>+</sup> T<sub>F</sub> cell molecules required for helper function. We observed that CD25<sup>+</sup> T<sub>F</sub>:T<sub>FH</sub> cell co-cultures reduced the percentage of CD40L-expressing T<sub>FH</sub> cells ( $p = <0.0001$ ) and also reduced CD40L expression per T<sub>FH</sub> cell by 50% ( $p = <0.0001$ ) (**Figure 13b**), as well as IL-21 production ( $p = <0.0001$ ) and BCL6 expression ( $p = 0.0010$ ) (**Figure 13b**). Reduction of each of these T<sub>FH</sub> cell molecules is known to limit T<sub>FH</sub> cell help for B cells (Vinuesa et al., 2016). Similar results were obtained in cultures in which B cells were not included and T cells were activated with  $\alpha$ -CD3/ $\alpha$ -CD28 antibodies (**Figure 13c**), suggesting that CD25<sup>+</sup> T<sub>F</sub> cells act directly on T<sub>FH</sub> to suppress their function. These effects did not seem to be mediated by IL-10, as IL-10 blockade did not rescue repression of helper molecules (data not shown).

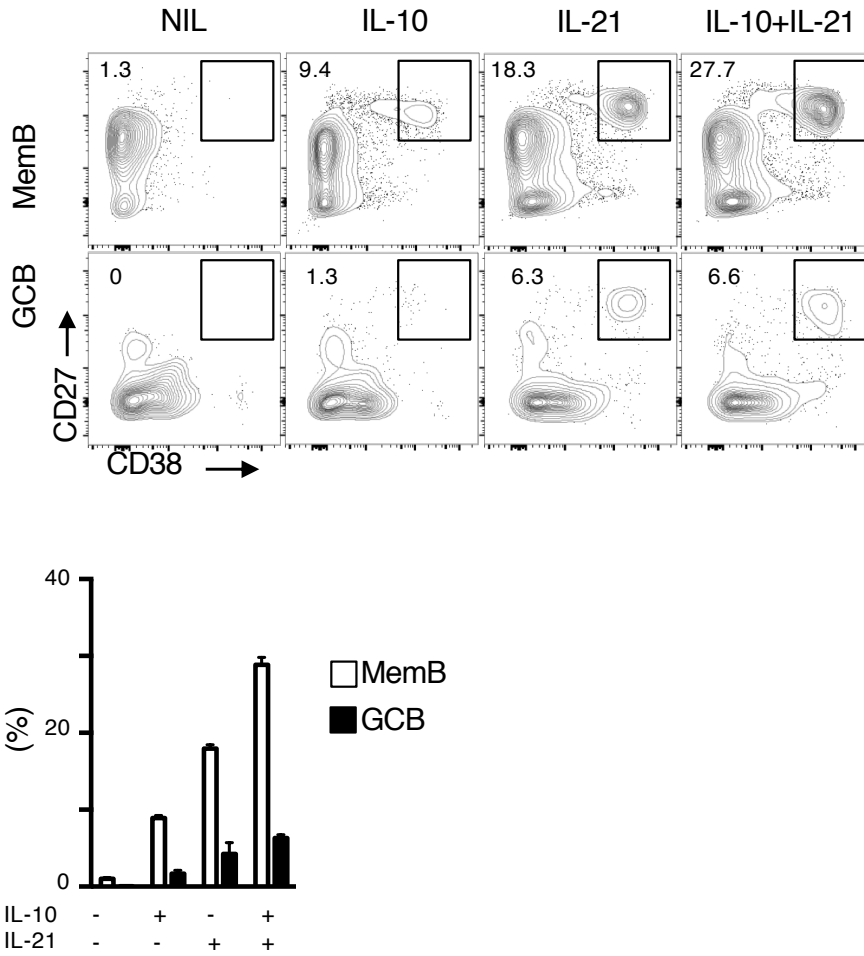


**Figure 13. Human CD25<sup>+</sup> T<sub>F</sub> cells repress T<sub>FH</sub> cells.**

(a) Flow cytometric plots and (b) quantification (n=8) of CTV-labeled-T<sub>FH</sub> cells, co-cultured with memory B cells and CD25<sup>+</sup> T<sub>F</sub> cells, showing expression of the indicated proteins after 3 days. Each dot represents a single tonsil donor, and data were pooled together from 4 independent experiments. (c) Flow cytometric plots and quantification of CTV-labeled-T<sub>FH</sub> cells, co-cultured with or without CD25<sup>+</sup> T<sub>F</sub> cells, showing expression of the indicated proteins after 3 days of  $\alpha$ -CD3 and  $\alpha$ -CD28 stimulation. Data is representative of 2 independent experiments. Bars represent means of 3 technical replicates and error bars represent standard deviations. ns, not significant (\*p  $\leq$  0.05), (\*\*p  $\leq$  0.01), (\*\*p  $\leq$  0.001), (\*\*\*\*p  $\leq$  0.0001), non-parametric paired Wilcoxon test (b) and two-tailed Students t-test (c).

## 5. Human CD25<sup>+</sup> T<sub>F</sub> cells regulate B cells and IgE secretion

We originally hypothesized that CD25<sup>+</sup> T<sub>F</sub> -derived IL-10 was important in driving plasma cell differentiation, given that early reports described a role for human IL-10 in driving this effect (Arpin et al., 1995). Consistent with this notion, we first cultured memory and GC B cells with  $\alpha$ -CD40, together with rIL-21 in the presence or absence of rIL-10. Plasma cell differentiation was measured by high expression of surface CD38 and CD27. Indeed, we could modestly induce plasma cell differentiation in IL-10-treated cultures, and we successfully induced plasma cell differentiation upon addition with IL-21 alone (**Figure 14**). Not surprisingly, addition of IL-10 boosted IL-21-mediated plasma cell differentiation. While our data underwent several rounds of review, the finding that CD25<sup>+</sup> T<sub>F</sub> cell-derived IL-10 promoted plasma cell differentiation ended up being published by the Craft group in 2017 (Laidlaw et al., 2017). As a result, we concentrated our efforts in investigating additional biological roles of CD25<sup>+</sup> T<sub>F</sub> cell-derived IL-10.

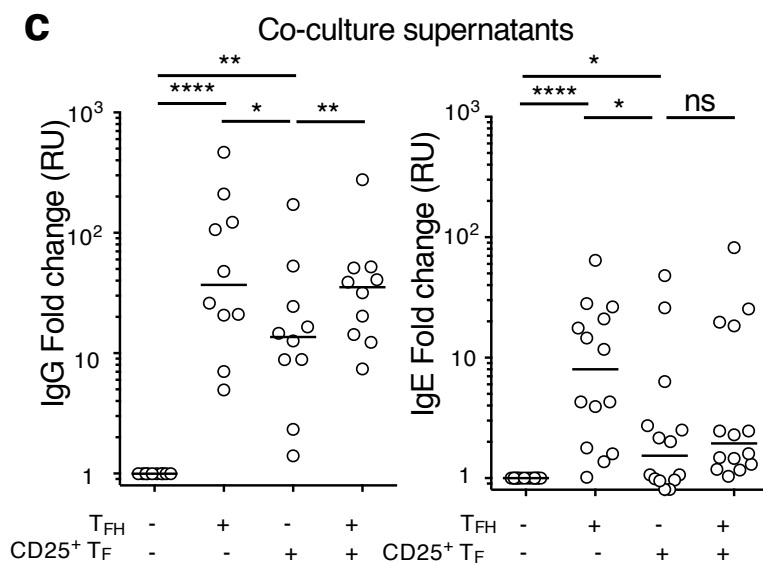
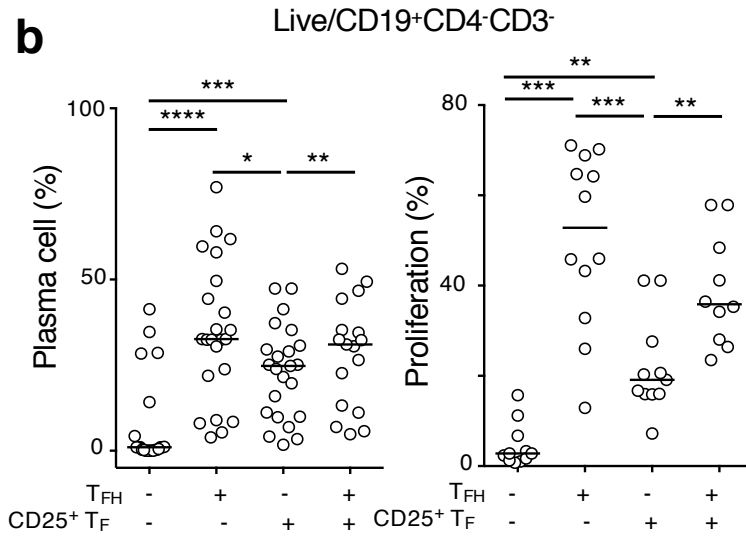
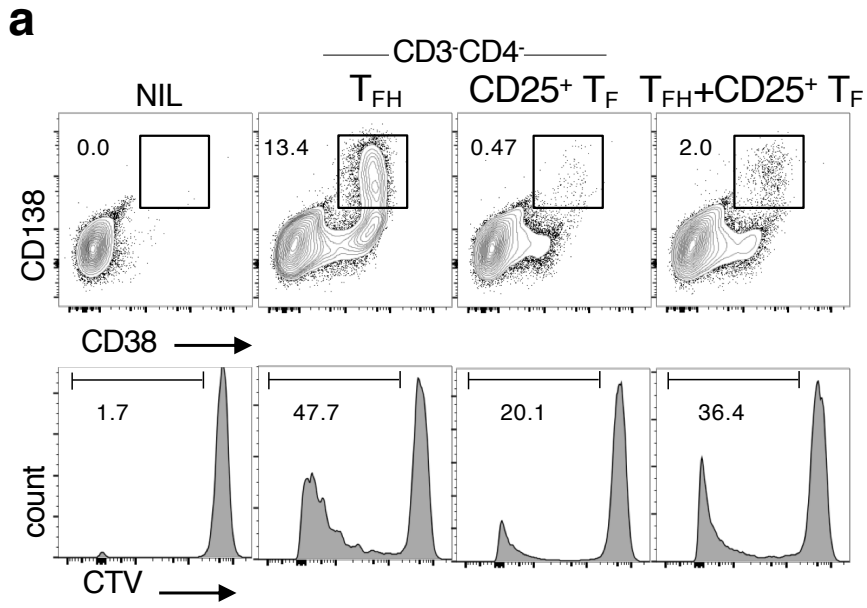


**Figure 14. IL-10 boosts plasma cell differentiation of memory and GC B cells.**

Representative flow cytometric plots and quantification of plasma cell (CD27+ CD38+) differentiation from memory or GC B cells cultured with  $\alpha$ -CD40 (2ug/ml) together with rIL21 (20 ng/ml) and or rIL-10 (20 ng/ml) for 5 days. Data is representative of 5 independent experiments.



Next, we compared the ability of human  $T_{FH}$  cells and  $CD25^+$   $T_F$  cells to induce B cell responses. Human memory B cells were co-cultured with autologous  $T_{FH}$  cells or  $CD25^+$   $T_F$  cells for seven days in the presence of (SEB). Co-culture with  $T_{FH}$  cells successfully induced cell division, differentiation of plasma cells (**Figure 15a-b**), and secretion of IgG and IgE (**Figure 15c**). Co-culture with  $CD25^+$   $T_F$  cells induced plasma cell differentiation, although to a lesser extent (**Figure 15b left panel**), modest B cell proliferation (**Figure 15b right panel**), and resulted in 5.2-fold decrease in IgE secretion and 2.7-fold decrease in IgG compared to  $T_{FH}$  co-cultures (**Figure 15c**). Addition of  $T_{FH}$  cells to the  $CD25^+$   $T_F$ : B cell co-cultures at equal ratios rescued plasma cell production, B cell proliferation, and IgG production, but not IgE production (**Figure 15b-c**). Together, these data suggest that human  $CD25^+$   $T_F$  cells are able to regulate  $T_{FH}$  cell function and IgE production from B cells.



**Figure 15. Human CD25<sup>+</sup> T<sub>F</sub> cells repress IgE secretion.**

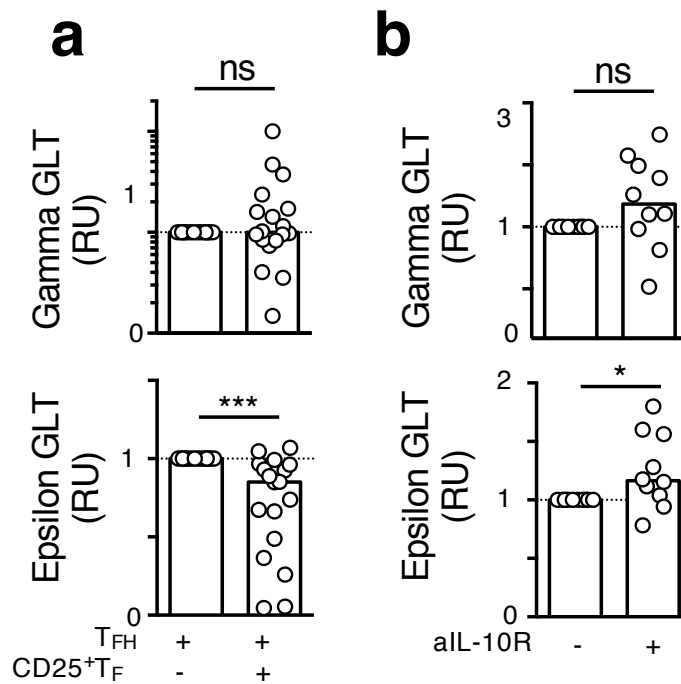
(a) Flow cytometric plots and (b) quantification of plasma cell (CD27<sup>+</sup> CD38<sup>+</sup>) differentiation (n=25) or proliferation (n=12) from memory B cells co-cultured with either T<sub>FH</sub> cells, CD25<sup>+</sup> T<sub>F</sub> cells or both, in the presence of SEB (500 ng/ml) IL-4 and IL-13 (40 ng/ml) for 9 days. (c) IgG (n=10) or IgE (n=14) in co-culture supernatants of co-cultures as in d. Data were normalized to values from cultures cultured without T cells. Each dot represents a single tonsil donor, and bars represent medians. ns, not significant (\* p ≤ 0.05), (\*\* p ≤ 0.01), (\*\*\*) p ≤ 0.001), (\*\*\*\* p ≤ 0.0001), non-parametric paired Wilcoxon test (b bottom panels, d-g).

## 6. Human CD25<sup>+</sup> T<sub>F</sub> cell-derived IL-10 represses epsilon germline transcription

Early reports from human in vitro studies showed that IL-10 could suppress switching to IgE but not IgG (Akdis and Blaser, 2001, Jeannin et al., 1998). Since IL-10 can have additional effects on B cells such as promoting plasma cell differentiation (Arpin et al., 1995) we also sought to separate the observed action of CD25<sup>+</sup> T<sub>F</sub> cells on plasma cell induction from possible repressive effects on Ig switching to IgE by looking at the earliest event that occurs in cells undergoing class switch recombination (CSR): production of germline transcripts (GLTs).

In order to investigate direct CSR events to IgE from IgM<sup>+</sup> B cells as opposed to sequential CSR, in which an initial IgM to IgG1 CSR event is followed by switching to IgE, we limited our culture to 25 hours. This is sufficient to induce the production of εGLTs, but is not sufficient for naïve B cells to undergo two cell divisions, thus preventing sequential CSR from occurring (Erazo et al., 2007). Naïve CD19<sup>+</sup> IgD<sup>+</sup> human B cells were FACS-purified and co-cultured with T<sub>FH</sub> cells and stimulated with IL-4, IL-13, and SEB, in the presence or absence of CD25<sup>+</sup> T<sub>F</sub> cells. Although addition of CD25<sup>+</sup> T<sub>F</sub> cells did not have a statistically-significant effect on γGLT induction, it consistently reduced εGLTs in T<sub>FH</sub>:B cells co-cultures (**Figure 16a**). Whilst there was high variability across individuals, paired statistics revealed a reduction in εGLT across 18 donors (p=0.0006).

IL-10 has been shown to suppress εGLTs in human B cell cultures (Jeannin et al., 1998). We therefore evaluated the contribution of IL-10 to CD25<sup>+</sup> T<sub>F</sub> cell-mediated suppression of εGLTs. Blocking the IL-10 receptor did not have a profound effect on γGLT, but rescued εGLT expression in CD25<sup>+</sup> T<sub>F</sub>: B cell co-cultures (**Figure 16b**) in 8 out of 10 donors. Taken together, these data suggest that IL-10 contributes to suppression of IgE switching by human CD25<sup>+</sup> T<sub>F</sub> cells.



**Figure 16. Human  $CD25^+$   $T_F$  cell-derived IL-10 represses IgE switching.**

(a-b) Quantification of gamma germline transcripts ( $\gamma$ GLT) (top panel) or epsilon germline transcripts ( $\epsilon$ GLT) in naïve B cells incubated for 24 hours with SEB (250 ng/ml) together with (a)  $T_{FH}$  cells with or without  $CD25^+$   $T_F$  cells (n=19), and (b) naïve B cells co-cultured with  $CD25^+$   $T_F$  cells alone in the presence or absence of an IL-10 blocking antibody (n=10). RNA values were calculated using the  $\Delta\Delta CT$  method, normalized to RPL13 expression, and normalized to the untreated control. Bars represent medians and each dot a single tonsil donor. ns, not significant ( $p \leq 0.05$ ), (\*\* $p \leq 0.001$ ), non-parametric paired Wilcoxon test.

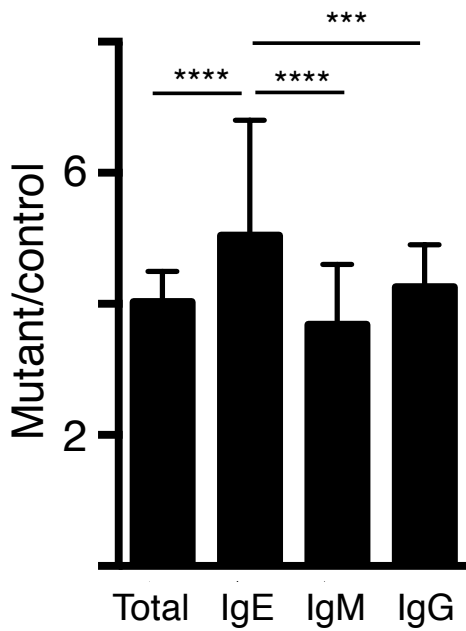
## **7. *In silico* model predicts increased sensitivity of IgE<sup>+</sup> GC B cells to diminished T<sub>FH</sub>-help**

Our data suggest that human tonsil CD25<sup>+</sup> T<sub>F</sub> cells can suppress the earliest events in IgE production – expression of epsilon switch transcripts. T<sub>FH</sub> influence B cell activation at multiple stages, from the earliest B cell priming stages where Ig CSR is initially triggered (Jacob et al., 1991, Toellner, 1996), to selection in germinal centers, and reactivation as memory B cells (Vinuesa et al., 2016). CD25<sup>+</sup> T<sub>F</sub> cells are also likely to interact and influence B cells at all these stages. It has been reported that once in the germinal center, IgE<sup>+</sup> B cells do not thrive and undergo apoptosis at least in part due to their lowered B cell receptor expression and lowered sensitivity to the light zone (LZ) chemokine CXCL13 (He et al., 2013b). Furthermore, T<sub>FH</sub> cells have recently been suggested to play a role in this differential selection (Yang and Robinson, 2016) and we have shown here that CD25<sup>+</sup> T<sub>F</sub> cells regulate the T<sub>FH</sub> cell helper ability directly through reducing CD40L. To date, it is unclear where repression of switching to IgE occurs - either outside or inside the GC. If, however, this repression were to take place within GCs, we turned to a well-validated *in silico* model of GC selection (He et al., 2013b) and postulated that CD25<sup>+</sup> T<sub>F</sub> cells are particularly potent in the repression of IgE<sup>+</sup> GC B cell selection, in part through dampening T<sub>FH</sub> cell help to B cells.

In order to develop and test a model of how loss of CD25<sup>+</sup> T<sub>F</sub> cells help would impact selection of B cells of different isotypes in GCs, Prof. Meyer-Herman introduced differential availability of T<sub>FH</sub> cell help onto his previous GC mathematical model, in which they simulated loss of IgE<sup>+</sup> B cells in GCs (He et al., 2013b). This simulation was based on experimental data showing that IgE<sup>+</sup> GC B cells have a defect in the acquisition of the LZ phenotype and fail to transmigrate to the dark zone because of lack of sensitivity to CXCL13 and reduced expression of the B cell receptor. This would already diminish their antigen-presenting ability and place them at a disadvantage when in competition for T cell help, and in CXCR5 up-regulation, which would predominantly result in retention in the dark zone.

Using this model we interrogated what would happen to Ig output should CD25<sup>+</sup> T<sub>F</sub> cells be absent in the GC reaction. Given our empirical evidence that CD25<sup>+</sup> T<sub>F</sub> cells reduce the proliferation and function of T<sub>FH</sub> cells, a CD25<sup>+</sup> T<sub>F</sub> cell deficiency absence was modeled by increasing T<sub>FH</sub> cell numbers, B cell divisions (attributed to selected B-cells by T<sub>FH</sub> help), and probability of differentiation. This *in silico* manipulation resulted in smaller GCs, delayed affinity maturation, more B cell divisions per round of selection, and a higher production of output cells. We next asked whether the differences in the GC characteristics were specific for the B cell isotypes. A significant difference in the increase of total Ig and IgE was found: while total Ig increased by a factor of 4.0, IgE increased by a factor of 5.0 (

**Figure 17**). While the degree of this difference depended on the details of the assumptions, an enhancement of the impact on IgE output was a robust feature. The model results suggest that facilitation of T<sub>FH</sub>-cell help to B cells favors IgE<sup>+</sup> B cells, which are weak competitors.



**Figure 17. *In silico* simulation of stronger  $T_{FH}$  help to GC B cells results in a selective increase of IgE output.**

State-of-the-art GC simulations were started with an increased number of  $T_{FH}$  cells, a stronger induction of division in selected B cells, and an increased probability of differentiation to output cells (see Methods for quantitative values). The resulting output production at day 21 after GC onset is shown relative to control simulations. Bars represent means and error bars sd from 100 simulations. ns, not significant ( $^{***} p \leq 0.001$ ), ( $^{****} p \leq 0.0001$ ), non-parametric wilcoxon test. Results courtesy by Michael Meyer-Hermann.



## 8. IL-10 producing CD25<sup>+</sup> T<sub>F</sub> cells inversely correlate with circulating IgE in the serum

In order to assess the clinical relevance of CD25<sup>+</sup> T<sub>F</sub> cells, we collected blood from tonsil donors at the time of tonsillectomy, and thus produced a cohort of matched serum and tonsil samples from 50 individuals. We then investigated associations between total IgE in serum and the frequency of CD25<sup>+</sup> T<sub>F</sub> cells in tonsils.

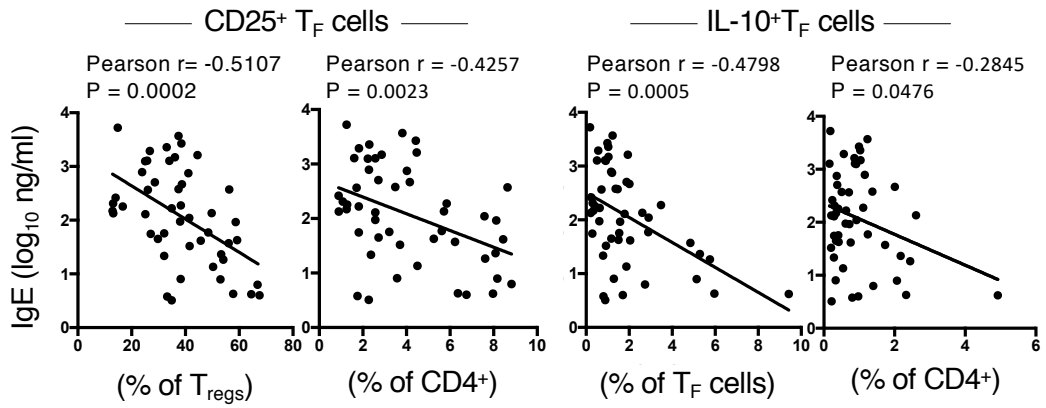
Strikingly, we observed an inverse correlation between the frequency of CD25<sup>+</sup> T<sub>F</sub> cells, measured as either a percentage of T<sub>regs</sub> or of CD4<sup>+</sup> T cells, and the amount of IgE in serum (**Figure 18a**). The more frequent CD25<sup>+</sup> T<sub>F</sub> cells were the less abundant total IgE was present in the serum, suggesting that CD25<sup>+</sup> T<sub>F</sub> cells could regulate IgE production. Although the goodness of fit in this model was somewhat weak ( $r = 0.5107$ ) the deviation from zero in this correlation was highly significant, thus confirming a negative trend between the two variables. Similar results were obtained when we correlated the amount of total IgE and the frequency of IL-10 producing T follicular cells (**Figure 18a**). No correlations were found between total T<sub>regs</sub> or even total IL-10 producing CD4<sup>+</sup> T cells and serum IgE (**Figure 18b right panels**) and even a weak positive correlation was observed between serum IgE and the frequency of FOXP3<sup>+</sup> T follicular cells (**Figure 18b left panels**).

Next, we asked whether the frequency of CD25<sup>+</sup> T<sub>F</sub> cells differed between those donors who exhibited high amounts of IgE vs those with low titers. The clinical value for normal total IgE in children ranges from 2 to 200 IU, or 2.4 to 480 ng/ml (Martins et al., 2014). However, this criterion varies across clinicians, researchers, testing laboratories and even differs individually according to age, ethnicity and the clinical context of test subjects (Martins et al., 2014). Earlier reports observed that the average of total serum IgE in healthy individuals was 100 ng/ml (Gould H.J., 1998), which was the same average value found in our tonsil/serum cohort (**Figure 18c**). As

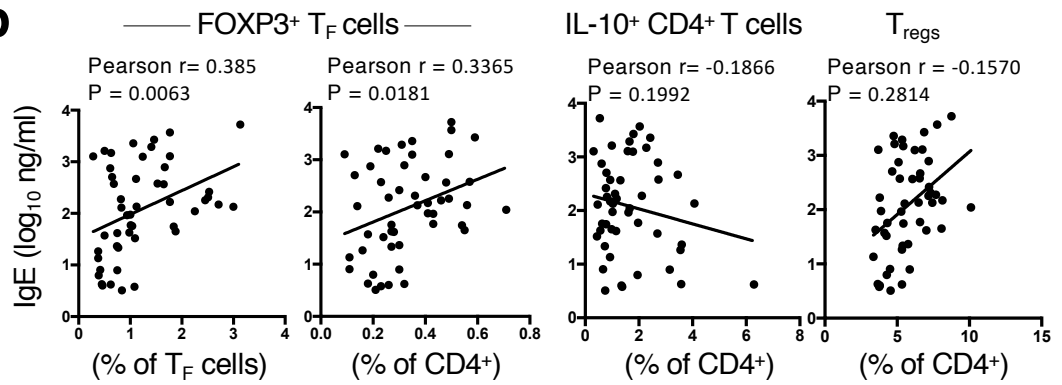
such, we elected 100ng/ml as a cutoff that separated tonsil donors with high vs low IgE titers, and thus interrogated whether the frequency of CD25<sup>+</sup> T<sub>F</sub> cells differed in both groups.

Our analysis revealed that CD25<sup>+</sup> T<sub>F</sub> cells, as a percentage of T<sub>regs</sub>, were significantly less frequent in those donors with higher IgE titres (62% and 42% median decrease respectively) (**Figure 18d**). Similarly, the frequency of IL-10 producing T<sub>F</sub> cells was more abundant in those donors with lower IgE titers (**Figure 18d**). Altogether, these results suggest an inverse correlation between CD25<sup>+</sup> T<sub>F</sub> cells and serum IgE, thus reinforcing the notion of CD25<sup>+</sup> T<sub>F</sub> cells as regulators of IgE production.

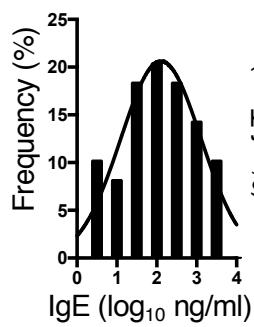
**a**



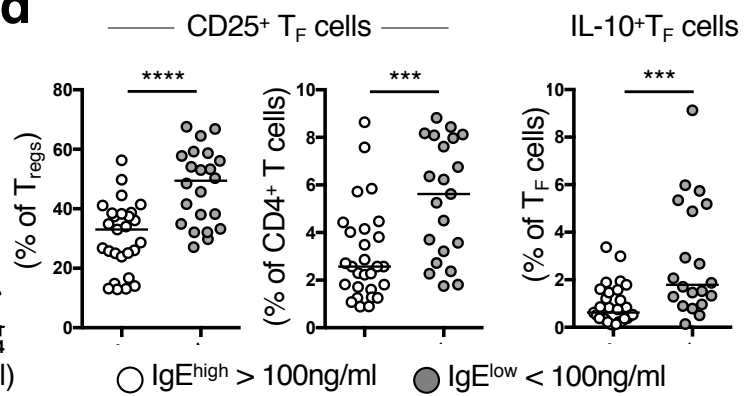
**b**



**c**



**d**



**Figure 18. Tonsillar CD25<sup>+</sup> T<sub>F</sub> cells are inversely proportional to total IgE in the serum.**

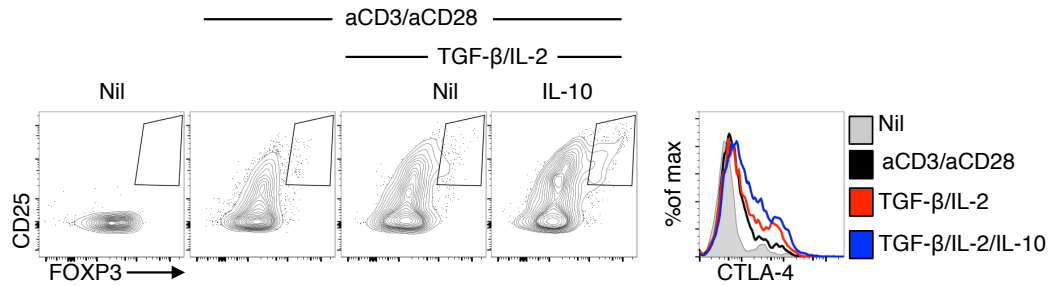
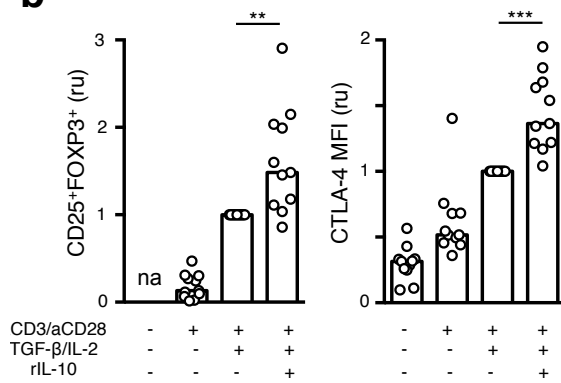
(a-b) Pearson's correlation analyses between serum total IgE and the frequency of the indicated cell subset in tonsil. (c) Histogram showing the frequency distribution of serum total IgE ( $\log_{10}$  ng/ml) from 49 children (mean=2.07). (d) Quantification of the frequency of the indicated cell subset from tonsil donors with high (>100 ng/ml) or low (<100 ng/ml) total serum IgE titers. Bars represent median and dots individual tonsil donors (n=49). Data represents 2 independent experiments that were pooled together. (\*\*  $p \leq 0.001$ ), (\*\*\*)  $p \leq 0.0001$ ) non-parametric Mann-Whitney test (U test) (c).

### **9. IL-10 promotes T<sub>FH</sub> cells to upregulate FOXP3**

Having seen that the strongest repressive effect mediated by CD25<sup>+</sup> T<sub>F</sub> is directed towards T<sub>FH</sub> cells, and that IL-10 can potentiate human T<sub>reg</sub> differentiation (Hsu et al., 2015, Yamagiwa et al., 2001), we asked whether IL-10 could induce T<sub>FR</sub> cell differentiation from T<sub>FH</sub> cells. To do this, we cultured FACS-sorted CD25<sup>-</sup> T<sub>FH</sub> cells in iT<sub>reg</sub>-inducing conditions ( $\alpha$ -CD3/ $\alpha$ -CD28, rTGF- $\beta$  and rIL-2) in the presence or absence of rIL-10 for 7 days. There was a two-fold increase in both the frequency of CD25<sup>+</sup>FOXP3<sup>+</sup> cells and CTLA4 expression when T<sub>FH</sub> cells were cultured with TGF- $\beta$  and IL-2 (

Figure 19a-b). Addition of IL-10 to these cultures further increased the frequency of T<sub>regs</sub> and CTLA4 by a median factor of 1.5. Together, these data demonstrate that IL-10 boosts T<sub>FH</sub> cell differentiation into iT<sub>FR</sub> cells (

Figure 19a-b) in mucosal lymphoid tissues.

**a****b**

**Figure 19. IL-10 induces FOXP3 expression in T<sub>FH</sub> cells.**

Flow cytometric plots (a) and quantification (b) of the proportion of CD25<sup>+</sup>FOXP3<sup>+</sup> cells and intracellular CTLA4 of T<sub>FH</sub> cells cultured with or without  $\alpha$ -CD3/ $\alpha$ -CD28 beads (1:1), or rTGF- $\beta$  (5 ng/ml), or rIL-2 (50 ng/ml) or rIL-10 (20 ng/ml) for 7 days. Bars represent median and dots individual tonsil donors (n=11). Data is representative of 3 independent experiments that were pooled and normalized to the TGF- $\beta$ /IL-2 condition. (\*\*: p < 0.01; \*\*\*: p < 0.001) non-parametric wilcoxon test.

## **Chapter 4: Discussion**

## 1. Summary

There has been enormous recent interest in a novel type of follicular T cell – T follicular regulatory ( $T_{FR}$ ) cells – that the Vinuesa group together with two other groups described a few years ago (Linterman et al., 2011, Chung et al., 2011, Wollenberg et al., 2011a). All groups found they originated from thymic  $Foxp3^+$   $T_{reg}$  cells, co-opted the  $T_{FH}$  differentiation pathway by upregulating *Bcl6*, and repressed germinal center responses, with suppressive effects on both T follicular helper and germinal center B cells. These cells were discovered in mice, and although our knowledge of how these cells operate has grown exponentially over the last years, we still know very little about the human counterpart.

In this thesis we have carefully studied the human T follicular populations in human tonsil, a prominent mucosal-associated lymphoid tissue that is exposed to pathogenic and commensal bacteria, as well as innocuous inhaled and oral antigens. We find an abundant type of follicular T cell identified by *CD25* expression and absent *FOXP3* expression, and thus we designate them  $CD25^+$   $T_F$  cells. These cells are the main IL-10 producers in the follicles. This differs from mice, in which both  $T_{FH}$  and  $T_{FR}$  cells express IL-10. Despite resembling  $T_{regs}$  functionally and phenotypically,  $CD25^+$   $T_F$  cells do not appear to be thymus-derived, since they lack demethylation in the *CNS2* region of the *FOXP3* promoter and *HELIOS* expression. Our proteomic and RNA-seq analysis reveals that  $CD25^+$   $T_F$  cells resemble mouse  $T_{FR}$  cells in many aspects but also differ from them. We have uncovered several important regulatory functions of human  $CD25^+$   $T_F$  cells:

- a.  $CD25^+$   $T_F$  cells are as potent T cell suppressors as  $T_{regs}$ .
- b.  $CD25^+$   $T_F$  cells repress *CD40L*, *BCL6* and *IL-21* expression in  $T_{FH}$  cells
- c.  $CD25^+$   $T_F$  cells regulate B cells, most prominently through inhibition of  $T_{FH}$  cell-induced B cell proliferation and direct inhibition of IgE switching through repression of epsilon germline transcript production. This is further supported by observing an inverse correlation between the frequency of  $CD25^+$   $T_F$  cells and the amount of circulating total IgE in the serum of children.



## Chapter 4: Discussion

- d. Unlike mouse  $T_{FR}$  cells,  $CD25^+$   $T_F$  cells do not repress but rather promote plasma cell differentiation, probably due to co-expression of IL-21.
- e.  $CD25^+$   $T_F$  cell-derived IL-10 specifically enhances FOXP3 expression in  $T_{FH}$  cells, leading to the induction of *bona fide*  $T_{FR}$  cells.

This chapter will consider the fundamental implications that these findings have on our current understanding of tolerance and the regulation of human antibody responses to commensal bacteria and innocuous oral and inhaled antigens. I will also reflect on the possibility that dysregulation of  $CD25^+$   $T_F$  cells may be associated to the development of atopy and excessive IgE-related pathology. Lastly, I will discuss limitations or flaws in our studies, together with the many future research avenues that our data have unveiled.

## 2. Are CD25<sup>+</sup> T<sub>F</sub> cells T<sub>regs</sub>?

This thesis describes a unique human follicular T cell subset, characterized by selective production of IL-10. Several lines of evidence indicate this subset to be functionally related to FOXP3-expressing T<sub>regs</sub>. First, our transcriptomic and proteomic characterization of CD25<sup>+</sup> T<sub>F</sub> cells has revealed that they closely resemble mouse T<sub>FR</sub> cells, except for the absence of FOXP3 expression. A CD25<sup>-</sup> regulatory population that also lacks FOXP3 expression and produces IL-10 has been previously described. These cells regulate B cell responses and, when dysregulated, are associated with autoimmune diseases (Facciotti et al., 2016). One could argue that these cells may be T<sub>r1</sub> cells, which are known to express abundant IL-10 in the absence of FOXP3, that gain access to the GC compartment in humans. However, based on the lack of LAG3 and CD49b expression in CD25<sup>+</sup> T<sub>F</sub> cells, which was shown to identify T<sub>r1</sub> cells in mice and humans, they do not appear to be *bona fide* T<sub>r1</sub> cells. (Gagliani et al, Nat. Medicine, 2013). At face value we do not dismiss this possibility, but we can nonetheless propose that CD25<sup>+</sup> T<sub>F</sub> cells display a distinctive phenotype and localization not described by Roncarolo et al, but akin to follicular regulatory T cells.

Second, human CD25<sup>+</sup> T<sub>F</sub> cell resemble T<sub>reg</sub> cells in the two most important aspect of T<sub>reg</sub> phenotype: high CTLA4 expression in combination with low IL-2 (Yamaguchi et al., 2013). Besides high CTLA4 expression, CD25<sup>+</sup> T<sub>F</sub> cells express only low-level IL-2. These observations are consistent with previous evidence that T cells engineered to express CTLA4 and not IL-2 adopt full T<sub>reg</sub>-like activity even in the absence of FOXP3 (Yamaguchi et al., 2013). Furthermore, we were able to show that CD25<sup>+</sup> T<sub>F</sub> cells were able to exert suppression of T cell proliferation in the same manner and to the same extent as T<sub>regs</sub>. This on its own is the gold standard assay that identifies T<sub>regs</sub> in humans, and thus supports the notion that CD25<sup>+</sup> T<sub>F</sub> cells are regulatory T cells.

We found that human CD25<sup>+</sup> T<sub>F</sub> cells induced plasma cell differentiation, despite the evidence of regulatory activity on T cell proliferation and T<sub>FH</sub> cell function, and their similarity in

phenotype to mouse  $T_{FR}$  cells that effectively repress B cell differentiation (Sage et al., 2016, Maceiras et al., 2017, Botta et al., 2017). One obvious difference between mouse ( $FOXP3^+$ )  $T_{FR}$  cells and the human tonsillar  $FOXP3^- CD25^+$   $T_F$  cells described here is the production of IL-21 by the latter but not the former. IL-21 is known to promote plasma cell differentiation in mice (Ozaki et al., 2002) and humans (Ettinger et al., 2005), so it is possible that this cytokine is contributing to plasma cell differentiation, potentially in conjunction with  $CD25^+$   $T_F$  cell-derived IL-10.

Although we could not see effects of IL-10R blockade on plasma cell differentiation in seven day cultures (data not shown), it is possible that the conditions were not optimal for durable blockade in these assays. Interestingly, unlike in mice in which IL-21 inhibits IgE formation (Ozaki et al., 2002), IL-21 is a potent inducer of IgE in human CD40L-stimulated B cells (Berglund et al., 2013). Thus, there appears to be dissociation between the effects of  $CD25^+$   $T_F$  cells on plasma cell formation, possibly conferred by IL-21 with or without contribution of IL-10, and CSR conferred by IL-10. In aggregate,  $CD25^+$   $T_F$  cells emerge as a unique regulatory cell that controls switching independently of B cell differentiation.

### **3. IL-10-mediated conversion of $T_{FH}$ to $T_{FR}$ cells**

We found a role for IL-10 in boosting  $T_{reg}$  conversion of  $T_{FH}$  cells, as addition of exogenous IL-10 to  $T_{FH}$  cells in combination with TGF- $\beta$  and IL-2 enhanced their FOXP3 expression. This conclusion may seem overall puzzling at first. Seeing that we have demonstrated that the  $CD25^+$   $T_F$  cell population produces generous amounts of IL-10, it would be logical to assume that there would be a significant  $FOXP3^+$  population in the  $CXCR5^{hi}PD1^{hi}$  compartment. This is not the case given that we only see a minority of cells that express FOXP3 in this gate. One possible explanation may relate to the pro-inflammatory milieu within tonsillar tissue. Indeed, given the observation that IL-10 alone (in the absence of TGF- $\beta$ ) was not sufficient to induce FOXP3 expression on its own, we suggest that IL-10 may influence  $T_{reg}$  conversion in pro-tolerogenic

environments where TGF- $\beta$  is readily available. Such environment is unlikely in tonsil tissue, due to its chronic inflammatory nature, and thus may help explain why FOXP3 expression is scarce.

Whilst we did not explore how IL-10 regulates these events, one possibility could be nuclear translocation of FOXO1 as a result of IL-10 signaling, as this has already been documented in B cells (Laidlaw et al., 2017). FOXO1 is a transcription factor that has been shown to directly up regulate Foxp3 (Kerdiles et al., 2010, Harada et al., 2010, Ouyang et al., 2010), and over 300 other genes important for T<sub>reg</sub> biology that are not under the control of FOXP3. Amongst these genes, CTLA4 appears to be a direct target of FOXO1, and selective ablation on FOXO1 in CD4<sup>+</sup> T cells severely reduced global CTLA4 expression in CD4<sup>+</sup> T cells. Therefore, it is conceivable that IL-10 signaling may also act on CD25<sup>+</sup> T<sub>F</sub> cells themselves, and it may endow them regulatory properties via activation of the FOXO1 transcriptional network. Given that we found abundant amounts of FOXO1 mRNA in all follicular T cell subsets in our RNA-seq data set, this idea may not far-fetched and should be pursued further.

#### 4. Ontogeny of CD25<sup>+</sup> T<sub>F</sub> cells

In an attempt to explore parallels between human CD25<sup>+</sup> CD25<sup>+</sup> T<sub>F</sub> cells and tT<sub>regs</sub> in terms of ontogeny, we assessed the methylation status of the CNS2 region of the *FOXP3* promoter. De-methylation of this region is essential for stable and durable expression of FOXP3, and is often used as the best marker for T<sub>regs</sub> of thymic origin. Absence of de-methylation in the *FOXP3* CNS2 promoter, together with low HELIOS expression, suggests that CD25<sup>+</sup> T<sub>F</sub> cells do not have a thymic origin and have been instead induced in the periphery, arising from conventional naïve CD4<sup>+</sup> T cells or from T<sub>FH</sub> cells themselves. The latter is plausible given that ICOS stimulation has been shown to selectively induce IL-10 expression from human CD4<sup>+</sup> T cells (Hutloff et al., 1999), and that T<sub>FH</sub> cells express high amounts of ICOS (Choi et al., 2011).

A methylated CNS2 in CD25<sup>+</sup> T<sub>F</sub> cells is not surprising, given that these cells barely express FOXP3 and that CNS2 controls the stability of FOXP3 expression in a CpG demethylation-

dependent manner (Zheng et al., Nature. 2010). It is not clear whether human CD25<sup>+</sup> T<sub>F</sub> cells express FOXP3 at some point and lose the expression due to instability at the *FOXP3* locus, or whether they are never able to express FOXP3 due to the methylated status of CNS2. Given that, to date, there is no reporter system that allows us to track FOXP3 cells in humans, we are unable to provide an answer to this important question.

HELIOS has also been suggested to identify thymic-derived T regulatory cells in mice and humans (Thornton et al., 2010). However, concerns for using HELIOS as a marker for tT<sub>regs</sub> in both mice and humans have been raised (Himmel et al., 2013, Gottschalk et al., 2012), given that HELIOS could also be detected in induced T<sub>regs</sub> depending on experimental conditions (Himmel et al., 2013). Collectively, the evidence presented in this thesis is suggestive but insufficient to draw the conclusion that these cells have a peripheral rather than thymic origin. We thus present this notion as a possibility rather than a conclusion, and we appreciate that at present we cannot definitely determine whether CD25<sup>+</sup> T<sub>F</sub> cells become regulatory cells in the thymus or the periphery.

## 5. CD25<sup>+</sup> T<sub>F</sub> -mediated regulation of IgE

The origin and regulation of antigen-induced IgE is one of the holy grails in immunology and a very significant clinical problem: excess in high affinity IgE is at the root of the allergy epidemic. The dogma still stands that T<sub>H2</sub> cells are the essential helper T cells for IgE responses, however recent evidence has demonstrated that that T<sub>FH</sub> cells play a crucial role in IgE responses (Kobayashi et al., 2017, Kobayashi et al., 2016, Ballesteros-Tato et al., 2016b). Furthermore, it is thought that IgE<sup>+</sup> B cells do not make it out of germinal center due to B cell-intrinsic defects downstream of the IgE BCR (He et al., 2013a, Yang et al., 2016), or FAS-mediated death (Butt et al., 2015). However, whether B-extrinsic mechanisms operate to control IgE responses, such as the action of T follicular regulatory cells, remains to be explored in the literature.

In this thesis we show that human CD25<sup>+</sup> CD25<sup>+</sup> T<sub>F</sub> cells exert suppressive effects on total T cell and T<sub>FH</sub> cell proliferation, T<sub>FH</sub> function, and B cell CSR. While they appear to reduce overall IgG and IgE responses, we show that they exert a specific repression of εGLT induction, which results in a disproportionate effect on IgE. The effect on IgE switching appears to be mediated by IL-10-mediated inhibition of CSR to IgE, which appears to modulate IgE more than IgG. Indeed, CD25<sup>+</sup> T<sub>F</sub> cells reduce εGLT production in T<sub>FH</sub>:B cell co-cultures, and this effect is rescued by IL-10 blockade. Interestingly, IL-10 appears to act very early, within 25 hours of naïve (unswitched) B cell activation, to inhibit CSR from I<sub>μ</sub>M to I<sub>ε</sub>E. Our experimental system shows this effect is a direct consequence of limiting direct CSR to I<sub>γ</sub>E, rather than on inhibiting γGLTs production and subsequent sequential switching.

These experiments were carried out sorting naïve B cells. Whether memory IgG<sup>+</sup> B cells are equally susceptible to this repression remains to be elucidated. Similarly, it is still unclear whether mouse T<sub>FR</sub> cells predominantly target naïve or GC B cells in vivo. T<sub>FH</sub> cells have been suggested to influence B cells at both maturation stages either at T:B borders or within GCs, so it is likely CD25<sup>+</sup> T<sub>F</sub> cells can also exert regulatory effects at different locations and stages of the immune response.

Our experiments indicate that human tonsil CD25<sup>+</sup> T<sub>F</sub> cells exert a similar function to their murine T<sub>FR</sub> cells, despite the absence of FOXP3 expression. Rare human immune deficiencies arising from monogenic defects has previously provided evidence that T<sub>regs</sub> regulate IgE production in humans (Liston et al., 2008). Given that we observed high expression of CTLA4 in CD25<sup>+</sup> T<sub>F</sub> cells, the data presented here help account for aspects of the phenotype in the recently characterized autoimmune lymphoproliferative syndrome that arises from CTLA4 haploinsufficiency. Hypogammaglobulinaemia is observed in 50-70% of cases, and in a small number of cases, high IgE in the face of IgG deficiency (Kuehn et al., 2014, Schubert et al., 2014). This phenomenon reveals that the antibody phenotype of a global reduction in CTLA4 is variable. Thus, our data predict both of these phenotypes, but their incomplete penetrance suggests that the regulation of these events is dynamic. Since CD25<sup>+</sup> T<sub>F</sub> cells appear the most abundant regulatory

population within tonsil GC T cells (defined by their CXCR5<sup>hi</sup> PD-1<sup>hi</sup> phenotype), and express the highest levels of CTLA4, these cells are likely to regulate Ig production within GC. Given the crucial function of GCs for production of high-affinity Ig, it is conceivable that dynamic regulation of B cell selection by a subset of regulatory GC T cells (CD25<sup>+</sup> T<sub>F</sub> cells) might be more adaptive than regulation by T<sub>regs</sub>, whose abundance is independent of each GC.

Our data suggests that CD25<sup>+</sup> T<sub>F</sub> cells limit B cell differentiation at least by dampening T<sub>FH</sub> cell-mediated selection. Indeed, our data revealed that CD25<sup>+</sup> T<sub>F</sub> cells directly downregulate CD40L, IL-21 and BCL6 on T<sub>FH</sub> cells. An intriguing question is why the effects appear to be more pronounced for IgE compared to IgG production. Apart from the action of IL-10 on repression of εGLT, a possible answer is that IgE<sup>+</sup> B cells, particularly IgE<sup>+</sup> GC B cells, are already at a survival disadvantage due to the different mechanisms operating B cell-intrinsically, including their lower BCR expression and signaling (Yang et al., 2012, He et al., 2013a). Lower BCR density would decrease presentation of antigen to T cells, which together with lowered BCR-mediated survival signals would make IgE<sup>+</sup> germinal center B cells more dependent on T<sub>FH</sub> cell help. If T<sub>FH</sub> cell help is dampened due to CD25<sup>+</sup> T<sub>F</sub> cell-induced downregulation of CD40L, IL-21 and BCL6, IgE<sup>+</sup> cells may suffer disproportionately.

Indeed, our *in silico* model supports this prediction and revealed that diminished T cell help leads IgE<sup>+</sup> B cells to experience a selective disadvantage compared to B cells bearing other isotypes. As such, this model supports a role for CD25<sup>+</sup> T<sub>F</sub> cells in limiting selection of IgE<sup>+</sup> B cells, given that we showed CD25<sup>+</sup> T<sub>F</sub> cells to repress T<sub>FH</sub> cell helping molecules (Miyake et al., 2017, Nakayama et al., 2011). Our results from this model suggest that facilitation of T<sub>FH</sub>-cell help to B cells favors IgE<sup>+</sup> B cells, which are weak competitors. Modeling the effect of CD25<sup>+</sup> T<sub>F</sub>-mediated dampening of T<sub>FH</sub> cell help in the GC enables us to incorporate variables that we cannot control in our cultures, such as the reported lower B cell receptor expression of IgE-expressing germinal center B cells, which is true in mouse and humans (Yang et al., 2016). The model used in this thesis is well respected across the germinal center community, has been used by many groups worldwide (Victora, Nussenzweig, Toellner, Curotto De Lafaille) and has been able to

faithfully predict and confirm the latest advances in GC B cell selection, including modeling the effects of lowered BCR expression and sensitivity to the light zone chemokine CXCL13 of GC IgE<sup>+</sup> cells (Erazo et al., 2007). The trend that diminished T<sub>FH</sub> cell help availability selectively affects IgE<sup>+</sup> B cells has been observed across 100 simulations, all of which highlight a selective increase in IgE output in the absence of CD25<sup>+</sup> T<sub>F</sub> cells. Additionally, the hypothesis that IgE<sup>+</sup> GCB cells require stronger T cell help has been experimentally confirmed by Chris Allen's group (Yang et al., 2016), as they observed IgE<sup>+</sup> GCB cells to experience reduced antigen presentation and to undergo excessively long cell cycles.

We can easily hypothesize on how CD25<sup>+</sup> T<sub>F</sub> cells may have evolved as a regulatory subset separate from the classical T<sub>reg</sub> or T<sub>FR</sub> cell lineages. Thymus-derived T<sub>regs</sub> that stably express FOXP3 are selected on the basis of their ability to recognize self-antigen in the thymus (Hsieh et al., 2012). T<sub>regs</sub> give rise to T<sub>FR</sub> cells in immunized mice (Linterman et al., 2011, Wollenberg et al., 2011a, Chung et al., 2011, Aloulou et al., 2016, Sage et al., 2014b, Wing et al., 2014), thus making them effective suppressors of responses against self. Indeed, ablation of T<sub>FR</sub> cells in vivo leads to autoantibody-mediated pathology (Botta et al., 2017, Fu et al., 2018). By contrast, the CD25<sup>+</sup> T<sub>F</sub> cells described in this study that lack FOXP3 expression do not appear to be thymus-derived. They are abundant in tonsils, which are exposed to oral and inhaled antigens, thus making them particularly good candidates to suppress responses to harmless foreign antigens (Aloulou et al., 2016). If CD25<sup>+</sup> T<sub>F</sub> cells develop in response to the same antigens as T<sub>FH</sub> cells, they may serve as a natural homeostatic mechanism to curtail T<sub>FH</sub> cell responses; comparable IL-10-producing FOXP3- repressive populations have been shown to arise for T<sub>H2</sub> and T<sub>H1</sub> lineages (Altin et al., 2012, Jankovic et al., 2007, Josefowicz et al., 2012a). In other words, human CD25<sup>+</sup> T<sub>F</sub> cells might arise as part of the same immune response that generated the T<sub>FH</sub> cells and GCs, and as an adaptation to curtail the GC and reduce the risk of developing IgE-mediated diseases.

There is evidence that T<sub>FR</sub> cells can be induced against both self and foreign antigens (Aloulou et al., 2016), but it remains unclear whether self- or innocuous environmental antigens such as allergens are more conducive to T<sub>FR</sub> cell development. Indeed, it remains unclear why some



## Chapter 4: Discussion

exogenous antigens are particularly allergenic. Based on our findings we predict that pathogens might be less effective at inducing CD25<sup>+</sup> T<sub>F</sub> cell formation. Thus, predilection to allergy might be influenced by the nature of the GC response, which explains why most allergens are not derived from pathogens (Hsieh et al., 2006). The corollary from our work suggests that defective regulation of CD25<sup>+</sup> T<sub>F</sub> cell homeostasis or function could underpin susceptibility to atopy. Should this be the case, it would open up new avenues for manipulating immunity to reduce the risk of allergy.

## 6. Limitations

This thesis describes a novel type of follicular T cells in humans, characterized by expression of IL-10, and explores its regulatory properties in the context of repression of IgE responses. Whilst the data generated collectively suggest that 1) CD25<sup>+</sup> T<sub>F</sub> closely resemble T<sub>FR</sub> cells, and 2) CD25<sup>+</sup> T<sub>F</sub> cells are as regulators of IgE production, there a number of technical, experimental and conceptual limitations that must be taken into consideration.

Our data does not exclude the possibility that CD25<sup>+</sup> T<sub>F</sub> -mediated dampening of T<sub>FH</sub> cell help occurs through diminishing B cell co-stimulation ability. It is indeed possible that CD25<sup>+</sup> T<sub>F</sub> cell-expressed CTLA4 causes transendocytosis of B cell-expressed CD80 and CD86 (Qureshi et al., 2011) impairing T<sub>FH</sub> activation and provision of help. Indeed, T<sub>regs</sub> have been reported to limit Ig responses in mice through CTLA4 (Sage et al., 2014b, Wing et al., 2014). The only caveat with this scenario is that unlike mouse T<sub>FH</sub> cells that express low amount of CTLA4, most CTLA4 expression being restricted to T<sub>FR</sub> cells in mouse GCs, human GC T<sub>FH</sub> cells express abundant CTLA4. Given that T<sub>FH</sub> cells are potent inducers of Ig responses in co-cultures with human B cells, it is unlikely that CTLA4 is the major or only mechanism of repression.

We also speculated that FASL expression by CD25<sup>+</sup> T<sub>F</sub> cells might have provided plausible mechanisms of IgE repression. Nevertheless, we could not find evidence to support either mode of regulation, as FASL was not expressed by CD25<sup>+</sup> T<sub>F</sub> cells. It is however possible that FASL is expressed very transiently during CD25<sup>+</sup> T<sub>F</sub>:B cell interactions, and this transient expression was not captured in our assays.

CD25<sup>+</sup> T<sub>F</sub> cells may also exert direct actions on B cells through alternative mechanisms. Unfortunately, our experimental systems are not easily amenable to test the direct effects of CD25<sup>+</sup> T<sub>F</sub> cells on B cells in the absence of T<sub>FH</sub> cells in the cultures, given that T<sub>FH</sub> cells are necessary to keep B cells alive in these assays.

We observed a reduction in epsilon CSR mediated by CD25<sup>+</sup> T<sub>F</sub> cells, which could be partly explained by IL-10 signaling. We were able to draw our conclusions by measuring early transcription events that precede CSR. In essence, by showing attenuation of C-epsilon transcription we conclude that CD25<sup>+</sup> T<sub>F</sub> cells inhibit IgE class switching. Whilst this method is the gold-standard technique to measure CSR events by many researchers, germline Cepsilon transcription is an early event that is necessary but not sufficient to define IgM-to-IgE CSR. Once complete, this process further involves the formation of class switch Iepsilon-Cmu transcripts. Quantification of these would strengthen our claim that CD25<sup>+</sup> T<sub>F</sub> cells repress switching to IgE but are technically challenging in primary human B cell cultures. This is because only a small amount of B cells are switching in these cultures, and CSR is not synchronized (synchronization can easily be achieved when using cell lines or BCR transgenic or knock-in mice).

In humans, IL-10 and IL-21 are powerful plasma cell-inducing factors, and thus whether CD25<sup>+</sup> T<sub>F</sub> cells induce plasma cell differentiation of tonsillar germinal center B cells more effectively than T<sub>FH</sub> cells still remains to be elucidated. We have generated informative data obtained from memory B cells as shown in **Figure 15**, but the regulation of memory B cells may be quite different from that of germinal center B cells. Compared to memory B cells, germinal center B cells may need different CD40L signals to generate plasma cells. Indeed, it has been shown that GC B cells, unlike any other B cell subsets, are able to dissociate signals derived from BCR and CD40 signaling (Luo et al., 2018). Our attempt to ascertain whether CD25<sup>+</sup> T<sub>F</sub> cells induce a more robust plasma cell differentiation of germinal center B cells produced inconsistent results. The difficulty to carry out this experiment comes from the highly apoptotic nature of GCB cells together with the need to culture B cells for at least four days to obtain measurable plasma cell differentiation. Indeed, it has been recently documented that the half-life of the entire GCB cell pool *in vivo* is as little as 6 hours (Mayer and Gazumyan, 2017). In my preliminary experiments only less than 10 percent of GCB cells were able to survive for so long in culture, and the observed plasma cells could originate from small contaminating B cells from other non-GC B cell populations in our sorting preparation, such as naïve or memory B cells. As a result, at present we cannot provide an answer to this question with the available tools.

IgE-expressing GC B cells have been reported to be highly prone to apoptosis, which may explain their transient presence in the GC. In the early stages of this thesis I strived to characterize IgE<sup>+</sup> GCB cells by flow cytometry. However, after screening virtually all available commercial antibodies for human surface IgE, we were unsuccessful in detecting IgE<sup>+</sup> GC B cells *ex-vivo* in human tonsils. As such, we have been unsuccessful at answering important questions pertaining to CD25<sup>+</sup> T<sub>F</sub> cell-mediated regulation of IgE, such as whether CD25<sup>+</sup> T<sub>F</sub> -derived IL-10 induces death of IgE class-switched GC B cells.

Correlation analyses between serum IgE and the frequency of human CD25<sup>+</sup> T<sub>F</sub> cells revealed these variables to be inversely proportional. Those tonsil donors with the highest frequencies of CD25<sup>+</sup> T<sub>F</sub> cells exhibited the lowest IgE titers, thus strengthening the notion of CD25<sup>+</sup> T<sub>F</sub> cells as regulators of IgE output. Whilst correlation studies are informative, we should be cautious and avoid overinterpreting these results. Indeed, correlation does not prove causation, and the pattern observed in our analyses could be attributed to some other variable not accounted for. Additionally, we could not obtain clinical history regarding helminth or parasitic infections in children whose tonsils we used for our experiment. This would have been very informative, given that these infections are known to produce protective IgE responses, and thus associations of total IgE to CD25<sup>+</sup> T<sub>F</sub> frequencies could be due to these types of infections rather than a predisposition to atopy and other IgE-related diseases. Nevertheless, our correlation analyses in the context of a demonstrated IgE suppression in our culture experiments provides an overall reinforcement for a role for CD25<sup>+</sup> T<sub>F</sub> cells in the repression of IgE.

Throughout this thesis we have shown descriptive comparisons between follicular CD25<sup>+</sup>Foxp3<sup>-</sup> (CD25<sup>+</sup> T<sub>F</sub>) cells along with CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in and around the GCs in tonsils. The GC CD25<sup>+</sup>Foxp3<sup>+</sup> cells (T<sub>FR</sub>) should have served as reference when assessing the suppressive/regulatory functions of CD25<sup>+</sup> T<sub>F</sub> cells in our functional experiments. Unfortunately, to date, it is impossible to sort FOXP3<sup>+</sup> T cells in humans, as live intracellular staining is impracticable and there are no fluorescent reporter gene tools available in humans.

Lastly, all of the experiments conducted in this thesis are *in vitro* studies. It is important to keep in mind that *in vitro* experiments may not always reflect *in vivo* conditions, as certain molecular and/or cellular components, as well as physical compartmentalization, may not be present in culture. Further, our co-culture experiments were performed using SEB as a T cell activator. SEB is a superantigen that promotes non-specific interactions between antigen presenting cells (APCs) and T cells. This results in a T cell polyclonal response and exaggerated cytokine release - causing toxic shock or chronic inflammation (Fraser and Proft, 2008). As such, differences in plasma cell differentiation or Ig secretion in our culture systems may not truly reflect output during a physiological antibody response, and the reported differences in our experimental settings could be a product of overactivated T cells sending aberrant differentiation signals to GC B cells in an uncontrolled manner.

## 7. Future directions

- Several lines of evidence have indicated that very different outcomes follow upon co-stimulation of CD4<sup>+</sup> T cells with CD28 *vs* ICOS. Whereas stimulation with the former produces high amounts of IL-2, which in turn diminishes T<sub>FH</sub> cell differentiation (Ballesteros-Tato et al., 2012), the latter has been long known to produce IL-10 (Witsch et al., 2002, Hutloff et al., 1999). It would be interesting to stimulate T<sub>FH</sub> cells with an ICOS agonist, and assess whether they convert into CD25<sup>+</sup> T<sub>F</sub> cells. This hypothesis would fit in a model where continuous ICOS signaling in T<sub>FH</sub> cells leads to their conversion into suppressor T cells, able to dampen by-stander T cell activity and pathogenic antibody isotypes, as well as a mechanism to resolve the GC reaction.
- We recognize that our CD25<sup>+</sup> T<sub>F</sub> cell gate is comprised of a heterogeneous cell population. Performing single cell RNA sequencing would provide a transcriptomic snapshot with such high resolution that would help resolve this heterogeneity. This

experiment could also lead to the discovery of additional T follicular cell subsets, which would aid in our understanding of T<sub>FH</sub> cell and germinal center biology.

- Both murine T<sub>FH</sub> and T<sub>FR</sub> cells produce IL-10. It would be important to explore whether this CD25<sup>+</sup> T<sub>F</sub> cells exist in mice, and perhaps in order to mimic a tonsillar environment we could make use of mouse models of oral tolerance (Mucida et al., 2005). Harvesting the mesenteric lymph nodes in these animals and performing an IL-10 capture would help us identify this cell subset. Subsequent investigations into their ontogeny, differentiation cues and mechanisms of suppression would allow us to gain a deeper understanding than what we were able to generate from our human *in vitro* studies. Additionally, fate mapping analysis using an *IL-10<sup>cre</sup>:Rosa26<sup>mT/mG</sup>* dual reporter system would allow us to ascertain whether these cells ever expressed Foxp3 at any stage in their life time.
- In humans, IL-10 has been reported to cooperate with IL-4 in inducing B cell expression of IgG4 (Jeannin et al., 1998), a poorly understood antibody class whose production correlates with allergy desensitization and tolerance (Platts-Mills et al., 2001). It would be interesting to assess whether CD25<sup>+</sup> T<sub>F</sub> cells induce IgG4 class switching and production, in addition to inhibiting IgE. This function would be consistent with the pro-tolerogenic role of CD25<sup>+</sup> T<sub>F</sub> cells that has been proposed in this thesis.
- We found a role for IL-10 to boost T<sub>reg</sub> conversion of T<sub>FH</sub> cells, as addition of exogenous IL-10 to T<sub>FH</sub> cells enhanced their FOXP3 expression. Whilst we did not explore how IL-10 regulates these events, one possibility could be nuclear translocation of FOXO1 as a result of IL-10 signaling, as this has already been documented in B cells (Laidlaw et al., 2017). FOXO1 is a transcription factor that has been shown to directly upregulate Foxp3 in mice (Kerdiles et al., 2010, Harada et al., 2010, Ouyang et al., 2010), as well as more than 300 other T<sub>reg</sub>-related genes that are not under the control of Foxp3 (Ouyang et al., 2012). Amongst these genes, CTLA4 appears to be a direct target of FOXO1, and selective ablation on FOXO1 in CD4<sup>+</sup> T cells severely reduced global CTLA4 expression in CD4<sup>+</sup> T cells (Kerdiles et al., 2010). Therefore, it is conceivable that IL-10 signaling acts on CD25<sup>+</sup> T<sub>F</sub> cells themselves, and endows regulatory properties via activation of the

FOXO1 transcriptional network. Given that we found very generous amounts of FOXO1 mRNA in all follicular T cell subsets in our RNA-seq data set, this hypothesis is not far-fetched and thus should be pursued further.

- Performing correlation analyses between allergen-specific IgE and CD25<sup>+</sup> T<sub>F</sub> cell frequencies in our serum/tonsil matched cohort will be performed. This experiment would further enhance the pro-tolerogenic role of CD25<sup>+</sup> T<sub>F</sub> cells proposed in this thesis in the context of allergy.

## 8. Concluding remarks

The mechanisms that control germinal center antibody output are of critical interest, given that aberrant B cell selection in GCs could lead to life-threatening autoimmune and allergic diseases. In this thesis we have uncovered a novel human follicular T cell subset that expresses abundant IL-10. By using flow cytometry protein profiling and a series of transcript detection and quantification techniques we showed that CD25<sup>+</sup> T<sub>F</sub> cells closely resemble T<sub>FR</sub> cells and T<sub>regs</sub>. A number of co-culture experiments, together with correlation analyses between tonsil and serum tissues, revealed that T cell-derived IL-10 in the GC is key to limit switching to IgE and IgE production. By demonstrating how CD25<sup>+</sup> T<sub>F</sub> cells controls pathogenic antibody responses, we hope our data contributes to the field that strives to understand the complex and fascinating regulation of the GC reaction.

The corollary from our work is that defective regulation of CD25<sup>+</sup> T<sub>F</sub> cell homeostasis or function is likely to underpin susceptibility to allergic diseases and other IgE-mediated conditions. Our findings may pose important clinical implications, as they provide a rationale for the use of biological therapies that boost CD25<sup>+</sup> T<sub>F</sub> cell development or function in these scenarios. Alternatively, therapeutic strategies aimed at lowering T<sub>FH</sub> cell numbers or function may be useful in cases of severe IgE-mediated disease, such as anaphylaxis. Lastly, we hope that this thesis may help us appreciate the importance of conducting human *in vitro* studies, despite being far more challenging due to limited resources and technical limitations.



## **Chapter 5: References**

- AKBARI, O., DEKRUYFF, R. H. & UMETSU, D. T. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol*, 2, 725-31.
- AKDIS, C. A. & BLASER, K. 2001. Mechanisms of interleukin-10-mediated immune suppression. *Immunology*, 103, 131-136.
- ALEGRE, M. L., FRAUWIRTH, K. A. & THOMPSON, C. B. 2001. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol*, 1, 220-8.
- ALLEN, C. D. C., OKADA, T. & CYSTER, J. G. 2007. Germinal-center organization and cellular dynamics. *Immunity*, 27, 190-202.
- ALMEIDA, A. R., LEGRAND, N., PAPIERNIK, M. & FREITAS, A. A. 2002. Homeostasis of peripheral CD4<sup>+</sup> T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4<sup>+</sup> T cell numbers. *J Immunol*, 169, 4850-60.
- ALOULO, M., CARR, E. J., GADOR, M., BIGNON, A., LIBLAU, R. S., FAZILLEAU, N. & LINTERMAN, M. A. 2016. Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun*, 7, 10579.
- ALTIN, J. A., GOODNOW, C. C. & COOK, M. C. 2012. IL-10<sup>+</sup> CTLA-4<sup>+</sup> Th2 inhibitory cells form in a Foxp3-independent, IL-2-dependent manner from Th2 effectors during chronic inflammation. *J Immunol*, 188, 5478-88.
- ANDERSON, M. S., VENANZI, E. S., KLEIN, L., CHEN, Z., BERZINS, S. P., TURLEY, S. J., VON BOEHMER, H., BRONSON, R., DIERICH, A., BENOIST, C. & MATHIS, D. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science*, 298, 1395-401.
- APOSTOLOU, I. & VON BOEHMER, H. 2004. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med*, 199, 1401-8.
- ARPIN, C., DECHANET, J., VAN KOOTEN, C., MERVILLE, P., GROUARD, G., BRIERE, F., BANCHEREAU, J. & LIU, Y. J. 1995. Generation of memory B cells and plasma cells in vitro. *Science*, 268, 720-2.
- AVERY, D. T., MA, C. S., BRYANT, V. L., SANTNER-NANAN, B., NANAN, R., WONG, M., FULCHER, D. A., COOK, M. C. & TANGYE, S. G. 2008. STAT3 is required for IL-21-induced secretion of IgE from human naive B cells. *Blood*, 112, 1784-93.
- BACHER, P., HEINRICH, F., STERVBO, U., NIENEN, M., VAHLDIECK, M., IWERT, C., VOGT, K., KOLLET, J., BABEL, N., SAWITZKI, B., SCHWARZ, C., BERESWILL, S., HEIMESAAT, M. M., HEINE, G., GADERMAIER, G., ASAM, C., ASSENMACHER, M., KNIEMEYER, O., BRAKHAGE, A. A., FERREIRA, F., WALLNER, M., WORM, M. & SCHEFFOLD, A. 2016. Regulatory T Cell Specificity Directs Tolerance versus Allergy against Aeroantigens in Humans. *Cell*, 167, 1067-1078.e16.
- BACHMANN, M. F., KOHLER, G., ECABERT, B., MAK, T. W. & KOPF, M. 1999. Cutting edge: lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous. *J Immunol*, 163, 1128-31.

- BALLESTEROS-TATO, A., LEÓN, B., GRAF, B. A., MOQUIN, A., ADAMS, P. S., LUND, F. E. & RANDALL, T. D. 2012. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity*, 36, 847-856.
- BALLESTEROS-TATO, A., RANDALL, T. D., LUND, F. E., SPOLSKI, R., LEONARD, W. J. & LEON, B. 2016a. T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite. *Immunity*.
- BALLESTEROS-TATO, A., RANDALL, T. D., LUND, F. E., SPOLSKI, R., LEONARD, W. J. & LEÓN, B. 2016b. T follicular helper cell plasticity shapes pathogenic T helper 2 cell-mediated immunity to inhaled house dust mite. *Immunity*, 44, 259-273.
- BATTAGLIA, M., GREGORI, S., BACCHETTA, R. & RONCAROLO, M. G. 2006. Tr1 cells: from discovery to their clinical application. *Semin Immunol*, 18, 120-7.
- BENNETT, C. L., CHRISTIE, J., RAMSDELL, F., BRUNKOW, M. E., FERGUSON, P. J., WHITESELL, L., KELLY, T. E., SAULSBURY, F. T., CHANCE, P. F. & OCHS, H. D. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*, 27, 20-1.
- BENOIST, C. & MATHIS, D. 2012. Treg cells, life history, and diversity. *Cold Spring Harbor perspectives in biology*, 4, a007021-a007021.
- BERGLUND, L. J., AVERY, D. T., MA, C. S., MOENS, L., DEENICK, E. K., BUSTAMANTE, J., BOISSON-DUPUIS, S., WONG, M., ADELSTEIN, S., ARKWRIGHT, P. D., BACCHETTA, R., BEZRODNIK, L., DADI, H., ROIFMAN, C. M., FULCHER, D. A., ZIEGLER, J. B., SMART, J. M., KOBAYASHI, M., PICARD, C., DURANDY, A., COOK, M. C., CASANOVA, J.-L., UZEL, G. & TANGYE, S. G. 2013. IL-21 signalling via STAT3 primes human naïve B cells to respond to IL-2 to enhance their differentiation into plasmablasts. *Blood*, 122, 3940-3950.
- BILLINGHAM, R. E., BRENT, L. & MEDAWAR, P. B. 1953. Actively acquired tolerance of foreign cells. *Nature*, 172, 603-6.
- BONILLA, F. A. & OETTGEN, H. C. 2010. Adaptive immunity. *J Allergy Clin Immunol*, 125, S33-40.
- BOTTA, D., FULLER, M. J., MARQUEZ-LAGO, T. T., BACHUS, H., BRADLEY, J. E., WEINMANN, A. S., ZAJAC, A. J., RANDALL, T. D., LUND, F. E., LEON, B. & BALLESTEROS-TATO, A. 2017. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol*, advance online publication.
- BRUNKOW, M. E., JEFFERY, E. W., HJERRILD, K. A., PAEPER, B., CLARK, L. B., YASAYKO, S. A., WILKINSON, J. E., GALAS, D., ZIEGLER, S. F. & RAMSDELL, F. 2001. Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*, 27, 68-73.
- BRYNJOLFSSON, S. F., PERSSON BERG, L., OLSEN EKERHULT, T., RIMKUTE, I., WICK, M.-J., MÅRTENSSON, I.-L. & GRIMSHOLM, O. 2018. Long-Lived Plasma Cells in Mice and Men. *Frontiers in immunology*, 9, 2673-2673.

- BURNET, F. M. 1957. A modification of Jerne's theory of antibody production using the concept of clonal selection. *The Australian Journal of Science*, 20, 67-69.
- BUTT, D., CHAN, T. D., BOURNE, K., HERMES, J. R., NGUYEN, A., STATHAM, A., O'REILLY, L. A., STRASSER, A., PRICE, S., SCHOFIELD, P., CHRIST, D., BASTEN, A., MA, C. S., TANGYE, S. G., PHAN, T. G., RAO, V. K. & BRINK, R. 2015. FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production. *Immunity*, 42, 890-902.
- CALZADA, D. & BAOS, S. 2018. Immunological Mechanisms in Allergic Diseases and Allergen Tolerance: The Role of Treg Cells. 2018, 6012053.
- CARRERAS, J., LOPEZ-GUILLERMO, A., FOX, B. C., COLOMO, L., MARTINEZ, A., RONCADOR, G., MONTSERRAT, E., CAMPO, E. & BANHAM, A. H. 2006. High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood*, 108, 2957-64.
- CEBULA, A., SEWERYN, M., REMPALA, G. A., PABLA, S. S., MCINDOE, R. A., DENNING, T. L., BRY, L., KRAJ, P., KISIELOW, P. & IGNATOWICZ, L. 2013. Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. *Nature*, 497, 258-62.
- CHAPLIN, D. D. 2010. Overview of the immune response. *The Journal of allergy and clinical immunology*, 125, S3-S23.
- CHATILA, T. A., BLAESER, F., HO, N., LEDERMAN, H. M., VOULGAROPOULOS, C., HELMS, C. & BOWCOCK, A. M. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *The Journal of clinical investigation*, 106, R75-R81.
- CHAUDHRY, A., RUDRA, D., TREUTING, P., SAMSTEIN, R. M., LIANG, Y., KAS, A. & RUDENSKY, A. Y. 2009. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science*, 326, 986-91.
- CHEN, L. & FLIES, D. B. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature reviews. Immunology*, 13, 227-242.
- CHEN, W., JIN, W., HARDEGEN, N., LEI, K. J., LI, L., MARINOS, N., MCGRADY, G. & WAHL, S. M. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*, 198, 1875-86.
- CHIKUMA, S. & BLUESTONE, J. A. 2007. Expression of CTLA-4 and FOXP3 in cis protects from lethal lymphoproliferative disease. *Eur J Immunol*, 37, 1285-9.
- CHOI, Y. S., GULLICKSRUD, J. A., XING, S., ZENG, Z., SHAN, Q., LI, F., LOVE, P. E., PENG, W., XUE, H. H. & CROTTY, S. 2015. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol*, 16, 980-90.
- CHOI, YOUNG S., KAGEYAMA, R., ETO, D., ESCOBAR, TANIA C., JOHNSTON, ROBERT J., MONTICELLI, L., LAO, C. & CROTTY, S. 2011. ICOS Receptor

Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. *Immunity*, 34, 932-946.

- CHOWDHURY, A., DEL RIO, P. M., THARP, G. K., TRIBLE, R. P., AMARA, R. R., CHAHROUDI, A., REYES-TERAN, G., BOSINGER, S. E. & SILVESTRI, G. 2015. Decreased T Follicular Regulatory Cell/T Follicular Helper Cell (TFH) in Simian Immunodeficiency Virus-Infected Rhesus Macaques May Contribute to Accumulation of TFH in Chronic Infection. *J Immunol*, 195, 3237-47.
- CHTANOVA, T., TANGYE, S. G., NEWTON, R., FRANK, N., HODGE, M. R., ROLPH, M. S. & MACKAY, C. R. 2004. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol*, 173, 68-78.
- CHUNG, Y., TANAKA, S., CHU, F., NURIEVA, R. I., MARTINEZ, G. J., RAWAL, S., WANG, Y. H., LIM, H., REYNOLDS, J. M., ZHOU, X. H., FAN, H. M., LIU, Z. M., NEELAPU, S. S. & DONG, C. 2011. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nature medicine*, 17, 983-8.
- COOPER, M. D. & ALDER, M. N. 2006. The evolution of adaptive immune systems. *Cell*, 124, 815-22.
- COQUET, J. M., SCHUIJS, M. J., SMYTH, M. J., DESWARTE, K., BEYAERT, R., BRAUN, H., BOON, L., KARLSSON HEDESTAM, G. B., NUTT, S. L., HAMMAD, H. & LAMBRECHT, B. N. 2015. Interleukin-21-Producing CD4(+) T Cells Promote Type 2 Immunity to House Dust Mites. *Immunity*, 43, 318-30.
- CUROTTO DE LAFAILLE, M. A., KUTCHUKHIDZE, N., SHEN, S., DING, Y., YEE, H. & LAFAILLE, J. J. 2008. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity*, 29, 114-26.
- DARCE, J., RUDRA, D., LI, L., NISHIO, J., CIPOLLETTA, D., RUDENSKY, A. Y., MATHIS, D. & BENOIST, C. 2012. An N-terminal mutation of the Foxp3 transcription factor alleviates arthritis but exacerbates diabetes. *Immunity*, 36, 731-741.
- DERBINSKI, J., SCHULTE, A., KYEWSKI, B. & KLEIN, L. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol*, 2, 1032-9.
- DUARTE, J. H., ZELENAY, S., BERGMAN, M. L., MARTINS, A. C. & DEMENGEOT, J. 2009. Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions. *Eur J Immunol*, 39, 948-55.
- ERAZO, A., KUTCHUKHIDZE, N., LEUNG, M., CHRIST, A. P., URBAN, J. F., JR., CUROTTO DE LAFAILLE, M. A. & LAFAILLE, J. J. 2007. Unique maturation program of the IgE response in vivo. *Immunity*, 26, 191-203.
- ETTINGER, R., SIMS, G. P., FAIRHURST, A.-M., ROBBINS, R., DA SILVA, Y. S., SPOLSKI, R., LEONARD, W. J. & LIPSKY, P. E. 2005. IL-21 Induces Differentiation of Human Naive and Memory B Cells into Antibody-Secreting Plasma Cells. *The Journal of Immunology*, 175, 7867-7879.

- FACCIOTTI, F., GAGLIANI, N., HARINGER, B., ALFEN, J. S., PENATTI, A., MAGLIE, S., PARONI, M., ISEPPON, A., MORO, M., CROSTI, M. C., STOLZEL, K., ROMAGNANI, C., MORONI, G., INGEGNOLI, F., TORRETTA, S., PIGNATARO, L., ANNONI, A., RUSSO, F., PAGANI, M., ABRIGNANI, S., MERONI, P., FLAVELL, R. & GEGINAT, J. 2016. IL-10-producing forkhead box protein 3-negative regulatory T cells inhibit B-cell responses and are involved in systemic lupus erythematosus. *J Allergy Clin Immunol*, 137, 318-321.e5.
- FATHMAN, C. G. & LINEBERRY, N. B. 2007. Molecular mechanisms of CD4+ T-cell anergy. *Nat Rev Immunol*, 7, 599-609.
- FEUERER, M., HILL, J. A., KRETSCHMER, K., VON BOEHMER, H., MATHIS, D. & BENOIST, C. 2010. Genomic definition of multiple ex vivo regulatory T cell subphenotypes. *Proc Natl Acad Sci U S A*, 107, 5919-24.
- FINNEY, C. A., TAYLOR, M. D., WILSON, M. S. & MAIZELS, R. M. 2007. Expansion and activation of CD4(+)CD25(+) regulatory T cells in Heligmosomoides polygyrus infection. *Eur J Immunol*, 37, 1874-86.
- FIorentino, D. F., BOND, M. W. & MOSMANN, T. R. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*, 170, 2081-95.
- FLAJNIK, M. F. 2002. Comparative analyses of immunoglobulin genes: surprises and portents. *Nat Rev Immunol*, 2, 688-98.
- FLOESS, S., FREYER, J., SIEWERT, C., BARON, U., OLEK, S., POLANSKY, J., SCHLAWKE, K., CHANG, H. D., BOPP, T., SCHMITT, E., KLEIN-HESSLING, S., SERFLING, E., HAMANN, A. & HUEHN, J. 2007. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*, 5, e38.
- FONSECA, V. R., AGUA-DOCE, A., MACEIRAS, A. R., PIERSON, W., RIBEIRO, F., ROMÃO, V. C., PIRES, A. R., DA SILVA, S. L., FONSECA, J. E., SOUSA, A. E., LINTERMAN, M. A. & GRACA, L. 2017. Human blood T<sub>fr</sub> cells are indicators of ongoing humoral activity not fully licensed with suppressive function. *Science Immunology*, 2.
- FONTENOT, J. D., GAVIN, M. A. & RUDENSKY, A. Y. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*, 4, 330-6.
- FONTENOT, J. D., RASMUSSEN, J. P., GAVIN, M. A. & RUDENSKY, A. Y. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*, 6, 1142-51.
- FRANCIS, J. N., TILL, S. J. & DURHAM, S. R. 2003. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol*, 111, 1255-61.
- FRASER, J. D. & PROFT, T. 2008. The bacterial superantigen and superantigen-like proteins. *Immunol Rev*, 225, 226-43.
- FRIEDLINE, R. H., BROWN, D. S., NGUYEN, H., KORNFELD, H., LEE, J., ZHANG, Y., APPLEBY, M., DER, S. D., KANG, J. & CHAMBERS, C. A. 2009. CD4+ regulatory

- T cells require CTLA-4 for the maintenance of systemic tolerance. *J Exp Med*, 206, 421-34.
- FU, W., ERGUN, A., LU, T., HILL, J. A., HAXHINASTO, S., FASSETT, M. S., GAZIT, R., ADORO, S., GLIMCHER, L., CHAN, S., KASTNER, P., ROSSI, D., COLLINS, J. J., MATHIS, D. & BENOIST, C. 2012. A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells. *Nat Immunol*, 13, 972-80.
- FU, W., LIU, X. & LIN, X. 2018. Deficiency in T follicular regulatory cells promotes autoimmunity. 215, 815-825.
- FUJIO, K., OKAMURA, T. & YAMAMOTO, K. 2010. The Family of IL-10-secreting CD4+ T cells. *Adv Immunol*, 105, 99-130.
- FURTADO, G. C., CUROTTO DE LAFAILLE, M. A., KUTCHUKHIDZE, N. & LAFAILLE, J. J. 2002. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med*, 196, 851-7.
- GAGLIANI, N., MAGNANI, C. F., HUBER, S., GIANOLINI, M. E., PALA, M., LICONA-LIMON, P., GUO, B., HERBERT, D. R., BULFONE, A., TRENTINI, F., DI SERIO, C., BACCHETTA, R., ANDREANI, M., BROCKMANN, L., GREGORI, S., FLAVELL, R. A. & RONCAROLO, M. G. 2013. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med*, 19, 739-46.
- GALLI, S. J., TSAI, M. & PILIPONSKY, A. M. 2008. The development of allergic inflammation. *Nature*, 454, 445-54.
- GAVIN, M. A., RASMUSSEN, J. P., FONTENOT, J. D., VASTA, V., MANGANIELLO, V. C., BEAVO, J. A. & RUDENSKY, A. Y. 2007. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*, 445, 771-5.
- GLATMAN ZARETSKY, A., TAYLOR, J. J., KING, I. L., MARSHALL, F. A., MOHRS, M. & PEARCE, E. J. 2009. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med*, 206, 991-9.
- GODFREY, V. L., WILKINSON, J. E., RINCHIK, E. M. & RUSSELL, L. B. 1991. Fatal lymphoreticular disease in the scurfy (sf) mouse requires T cells that mature in a sf thymic environment: potential model for thymic education. *Proc Natl Acad Sci U S A*, 88, 5528-32.
- GOTTSCHALK, R. A., CORSE, E. & ALLISON, J. P. 2012. Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *J Immunol*, 188, 976-80.
- GOULD H.J., B. R. 1998. Immunoglobulin E - an overview. *Encyclopedia of Immunology (Second Edition)*, 1202-1208.
- GRAINGER, J. R., SMITH, K. A., HEWITSON, J. P., MCSORLEY, H. J., HARCUS, Y., FILBEY, K. J., FINNEY, C. A., GREENWOOD, E. J., KNOX, D. P., WILSON, M. S., BELKAID, Y., RUDENSKY, A. Y. & MAIZELS, R. M. 2010. Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway. *J Exp Med*, 207, 2331-41.

- GROUX, H., O'GARRA, A., BIGLER, M., ROULEAU, M., ANTONENKO, S., DE VRIES, J. E. & RONCAROLO, M. G. 1997. A CD4<sup>+</sup>T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*, 389, 737-742.
- HAMMAD, H. & LAMBRECHT, B. N. 2015. Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity*, 43, 29-40.
- HANIUDA, K., FUKAO, S., KODAMA, T., HASEGAWA, H. & KITAMURA, D. 2016. Autonomous membrane IgE signaling prevents IgE-memory formation. *Nat Immunol*, 17, 1109-1117.
- HANSEN, J. D., DU PASQUIER, L., LEFRANC, M. P., LOPEZ, V., BENMANSOUR, A. & BOUDINOT, P. 2009. The B7 family of immunoregulatory receptors: a comparative and evolutionary perspective. *Mol Immunol*, 46, 457-72.
- HARADA, Y., HARADA, Y., ELLY, C., YING, G., PAIK, J. H., DEPINHO, R. A. & LIU, Y. C. 2010. Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells. *J Exp Med*, 207, 1381-91.
- HARIBHAI, D., LIN, W., EDWARDS, B., ZIEGELBAUER, J., SALZMAN, N. H., CARLSON, M. R., LI, S. H., SIMPSON, P. M., CHATILA, T. A. & WILLIAMS, C. B. 2009. A central role for induced regulatory T cells in tolerance induction in experimental colitis. *J Immunol*, 182, 3461-8.
- HARRIS, M. B., CHANG, C. C., BERTON, M. T., DANIAL, N. N., ZHANG, J., KUEHNER, D., YE, B. H., KVATYUK, M., PANDOLFI, P. P., CATTORETTI, G., DALLA-FAVERA, R. & ROTHMAN, P. B. 1999. Transcriptional repression of Stat6-dependent interleukin-4-induced genes by BCL-6: specific regulation of Iepsilon transcription and immunoglobulin E switching. *Mol Cell Biol*, 19, 7264-75.
- HAYNES, N. M., ALLEN, C. D., LESLEY, R., ANSEL, K. M., KILLEEN, N. & CYSTER, J. G. 2007. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1<sup>high</sup> germinal center-associated subpopulation. *J Immunol*, 179, 5099-108.
- HE, J.-S., MEYER-HERMANN, M., XIANGYING, D., ZUAN, L. Y., JONES, L. A., RAMAKRISHNA, L., DE VRIES, V. C., DOLPADY, J., AINA, H., JOSEPH, S., NARAYANAN, S., SUBRAMANIAM, S., PUTHIA, M., WONG, G., XIONG, H., POIDINGER, M., URBAN, J. F., LAFAILLE, J. J. & CUROTTO DE LAFAILLE, M. A. 2013a. The distinctive germinal center phase of IgE<sup>+</sup> B lymphocytes limits their contribution to the classical memory response. *The Journal of Experimental Medicine*, 210, 2755-2771.
- HE, J. S., MEYER-HERMANN, M., XIANGYING, D., ZUAN, L. Y., JONES, L. A., RAMAKRISHNA, L., DE VRIES, V. C., DOLPADY, J., AINA, H., JOSEPH, S., NARAYANAN, S., SUBRAMANIAM, S., PUTHIA, M., WONG, G., XIONG, H., POIDINGER, M., URBAN, J. F., LAFAILLE, J. J. & CUROTTO DE LAFAILLE, M. A. 2013b. The distinctive germinal center phase of IgE<sup>+</sup> B lymphocytes limits their contribution to the classical memory response. *J Exp Med*, 210, 2755-71.
- HEATH, W. R. & CARBONE, F. R. 2001. Cross-presentation in viral immunity and self-tolerance. *Nat Rev Immunol*, 1, 126-34.



- HOFMANN, J., GRETER, M., DU PASQUIER, L. & BECHER, B. 2010. B-cells need a proper house, whereas T-cells are happy in a cave: the dependence of lymphocytes on secondary lymphoid tissues during evolution. *Trends Immunol*, 31, 144-53.
- HORI, S., NOMURA, T. & SAKAGUCHI, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science*, 299, 1057-61.
- HSIEH, C. S., LEE, H. M. & LIO, C. W. 2012. Selection of regulatory T cells in the thymus. *Nat Rev Immunol*, 12, 157-67.
- HSIEH, C. S., ZHENG, Y., LIANG, Y., FONTENOT, J. D. & RUDENSKY, A. Y. 2006. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol*, 7, 401-10.
- HSU, P., SANTNER-NANAN, B., HU, M., SKARRATT, K., LEE, C. H., STORMON, M., WONG, M., FULLER, S. J. & NANAN, R. 2015. IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1. *J Immunol*, 195, 3665-74.
- HUEHN, J., POLANSKY, J. K. & HAMANN, A. 2009. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol*, 9, 83-9.
- HUTLOFF, A., DITTRICH, A. M., BEIER, K. C., ELJASCHEWITSCH, B., KRAFT, R., ANAGNOSTOPOULOS, I. & KROCZEK, R. A. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature*, 397, 263-6.
- IGEA, J. M. 2013. The history of the idea of allergy. *Allergy*, 68, 966-73.
- ISHIDA, Y., AGATA, Y., SHIBAHARA, K. & HONJO, T. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *Embo j*, 11, 3887-95.
- JACOB, J., KASSIR, R. & KELSOE, G. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J Exp Med*, 173, 1165-75.
- JANKOVIC, D., KULLBERG, M. C., FENG, C. G., GOLDSZMID, R. S., COLLAZO, C. M., WILSON, M., WYNN, T. A., KAMANAKA, M., FLAVELL, R. A. & SHER, A. 2007. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J Exp Med*, 204, 273-83.
- JEANNIN, P., LECOANET, S., DELNESTE, Y., GAUCHAT, J.-F. & BONNEFOY, J.-Y. 1998. IgE Versus IgG4 Production Can Be Differentially Regulated by IL-10. *The Journal of Immunology*, 160, 3555-3561.
- JINQUAN, T., QUAN, S., JACOBI, H. H., MADSEN, H. O., GLUE, C., SKOV, P. S., MALLING, H. J. & POULSEN, L. K. 2000. CXC chemokine receptor 4 expression and stromal cell-derived factor-1alpha-induced chemotaxis in CD4+ T lymphocytes are regulated by interleukin-4 and interleukin-10. *Immunology*, 99, 402-10.
- JOHNSTON, R. J., POHOLEK, A. C., DITORO, D., YUSUF, I., ETO, D., BARNETT, B., DENT, A. L., CRAFT, J. & CROTTY, S. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science*, 325, 1006-10.

- JOSEFOWICZ, S. Z., LU, L. F. & RUDENSKY, A. Y. 2012a. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol*, 30, 531-64.
- JOSEFOWICZ, S. Z., NIEC, R. E., KIM, H. Y., TREUTING, P., CHINEN, T., ZHENG, Y., UMETSU, D. T. & RUDENSKY, A. Y. 2012b. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature*, 482, 395-9.
- JUTEL, M., AKDIS, M., BUDAK, F., AEBISCHER-CASAULTA, C., WRZYSZCZ, M., BLASER, K. & AKDIS, C. A. 2003. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol*, 33, 1205-14.
- KERDILES, Y. M., STONE, E. L., BEISNER, D. R., MCGARGILL, M. A., CH'EN, I. L., STOCKMANN, C., KATAYAMA, C. D. & HEDRICK, S. M. 2010. Foxo transcription factors control regulatory T cell development and function. *Immunity*, 33, 890-904.
- KIM, C. H., LIM, H. W., KIM, J. R., ROTT, L., HILLSAMER, P. & BUTCHER, E. C. 2004. Unique gene expression program of human germinal center T helper cells. *Blood*, 104, 1952-60.
- KIM, K. S., HONG, S. W., HAN, D., YI, J., JUNG, J., YANG, B. G., LEE, J. Y., LEE, M. & SURH, C. D. 2016. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science*, 351, 858-63.
- KING, I. L. & MOHRS, M. 2009. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med*, 206, 1001-7.
- KLEIN, L., KYEWSKI, B., ALLEN, P. M. & HOGQUIST, K. A. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature reviews. Immunology*, 14, 377-391.
- KOBAYASHI, T., IJIMA, K., DENT, A. L. & KITA, H. 2016. Follicular helper T cells mediate IgE antibody response to airborne allergens. *J Allergy Clin Immunol*.
- KOBAYASHI, T., IJIMA, K., DENT, A. L. & KITA, H. 2017. Follicular helper T cells mediate IgE antibody response to airborne allergens. *J Allergy Clin Immunol*, 139, 300-313.e7.
- KOCH, M. A., TUCKER-HEARD, G., PERDUE, N. R., KILLEBREW, J. R., URDAHL, K. B. & CAMPBELL, D. J. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol*, 10, 595-602.
- KOCH, U. & RADTKE, F. 2011. Mechanisms of T cell development and transformation. *Annu Rev Cell Dev Biol*, 27, 539-62.
- KOJIMA, A. & PREHN, R. T. 1981. Genetic susceptibility to post-thymectomy autoimmune diseases in mice. *Immunogenetics*, 14, 15-27.
- KOMATSU, N., MARIOTTI-FERRANDIZ, M. E., WANG, Y., MALISSEN, B., WALDMANN, H. & HORI, S. 2009. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A*, 106, 1903-8.

- KRETSCHMER, K., APOSTOLOU, I., HAWIGER, D., KHAZAIE, K., NUSSENZWEIG, M. C. & VON BOEHMER, H. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*, 6, 1219-27.
- KRUMMEL, M. F. & ALLISON, J. P. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *The Journal of experimental medicine*, 182, 459-465.
- KRUMMEL, M. F. & ALLISON, J. P. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med*, 183, 2533-40.
- KUEHN, H. S., OUYANG, W., LO, B., DEENICK, E. K., NIEMELA, J. E., AVERY, D. T., SCHICKEL, J. N., TRAN, D. Q., STODDARD, J., ZHANG, Y., FRUCHT, D. M., DUMITRIU, B., SCHEINBERG, P., FOLIO, L. R., FREIN, C. A., PRICE, S., KOH, C., HELLER, T., SEROOGY, C. M., HUTTENLOCHER, A., RAO, V. K., SU, H. C., KLEINER, D., NOTARANGELO, L. D., RAMPERTAAP, Y., OLIVIER, K. N., MCELWEE, J., HUGHES, J., PITTALUGA, S., OLIVEIRA, J. B., MEFFRE, E., FLEISHER, T. A., HOLLAND, S. M., LENARDO, M. J., TANGYE, S. G. & UZEL, G. 2014. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science*, 345, 1623-7.
- KUROSAKI, T., SHINOHARA, H. & BABA, Y. 2010. B cell signaling and fate decision. *Annu Rev Immunol*, 28, 21-55.
- KYEWSKI, B. & KLEIN, L. 2006. A central role for central tolerance. *Annu Rev Immunol*, 24, 571-606.
- LA ROCCA, C., CARBONE, F., LONGOBARDI, S. & MATARESE, G. 2014. The immunology of pregnancy: regulatory T cells control maternal immune tolerance toward the fetus. *Immunol Lett*, 162, 41-8.
- LIDLAW, B. J., LU, Y., AMEZQUITA, R. A., WEINSTEIN, J. S., VANDER HEIDEN, J. A., GUPTA, N. T., KLEINSTEIN, S. H., KAECH, S. M. & CRAFT, J. 2017. Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. *Science immunology*, 2, eaan4767.
- LATHROP, S. K., BLOOM, S. M., RAO, S. M., NUTSCH, K., LIO, C.-W., SANTACRUZ, N., PETERSON, D. A., STAPPENBECK, T. S. & HSIEH, C.-S. 2011. Peripheral education of the immune system by colonic commensal microbiota. *Nature*, 478, 250-254.
- LEACH, D. R., KRUMMEL, M. F. & ALLISON, J. P. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*, 271, 1734-6.
- LEDFORD, H., ELSE, H. & WARREN, M. 2018. Cancer immunologists scoop medicine Nobel prize. *Nature*, 562, 20-21.
- LEE, S. K., RIGBY, R. J., ZOTOS, D., TSAI, L. M., KAWAMOTO, S., MARSHALL, J. L., RAMISCAL, R. R., CHAN, T. D., GATTO, D., BRINK, R., YU, D., FAGARASAN, S., TARLINTON, D. M., CUNNINGHAM, A. F. & VINUESA, C. G. 2011. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J Exp Med*, 208, 1377-88.

- LEON, B., BRADLEY, J. E., LUND, F. E., RANDALL, T. D. & BALLESTEROS-TATO, A. 2014. FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun*, 5, 3495.
- LI, H. & PAUZA, C. D. 2015. CD25(+) Bcl6(low) T follicular helper cells provide help to maturing B cells in germinal centers of human tonsil. *Eur J Immunol*, 45, 298-308.
- LI, Y., LI, W., YING, Z., TIAN, H., ZHU, X., LI, J. & LI, M. 2014. Metastatic heterogeneity of breast cancer cells is associated with expression of a heterogeneous TGFbeta-activating miR424-503 gene cluster. *Cancer Res*, 74, 6107-18.
- LICONA-LIMON, P., KIM, L. K., PALM, N. W. & FLAVELL, R. A. 2013. TH2, allergy and group 2 innate lymphoid cells. *Nat Immunol*, 14, 536-42.
- LIM, H. W., HILLSAMER, P. & KIM, C. H. 2004. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *The Journal of clinical investigation*, 114, 1640-9.
- LINTERMAN, M. A., PIERSON, W., LEE, S. K., KALLIES, A., KAWAMOTO, S., RAYNER, T. F., SRIVASTAVA, M., DIVEKAR, D. P., BEATON, L., HOGAN, J. J., FAGARASAN, S., LISTON, A., SMITH, K. G. & VINUESA, C. G. 2011. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med*, 17, 975-82.
- LISTON, A., ENDERS, A. & SIGGS, O. M. 2008. Unravelling the association of partial T-cell immunodeficiency and immune dysregulation. *Nat Rev Immunol*, 8, 545-558.
- LIU, W., PUTNAM, A. L., XU-YU, Z., SZOT, G. L., LEE, M. R., ZHU, S., GOTTLIEB, P. A., KAPRANOV, P., GINGERAS, T. R., FAZEKAS DE ST GROTH, B., CLAYBERGER, C., SOPER, D. M., ZIEGLER, S. F. & BLUESTONE, J. A. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *The Journal of experimental medicine*, 203, 1701-1711.
- LU, L., BARBI, J. & PAN, F. 2017. The regulation of immune tolerance by FOXP3. *Nat Rev Immunol*, 17, 703-717.
- LU, L. L., SUSCOVICH, T. J., FORTUNE, S. M. & ALTER, G. 2018. Beyond binding: antibody effector functions in infectious diseases. *Nat Rev Immunol*, 18, 46-61.
- LUND, J. M., HSING, L., PHAM, T. T. & RUDENSKY, A. Y. 2008. Coordination of early protective immunity to viral infection by regulatory T cells. *Science (New York, N.Y.)*, 320, 1220-1224.
- LUO, W., WEISEL, F. & SHLOMCHIK, M. J. 2018. B Cell Receptor and CD40 Signaling Are Rewired for Synergistic Induction of the c-Myc Transcription Factor in Germinal Center B Cells. *Immunity*, 48, 313-326.e5.
- MACEIRAS, A. R., ALMEIDA, S. C. P., MARIOTTI-FERRANDIZ, E., CHAARA, W., JEBBAWI, F., SIX, A., HORI, S., KLATZMANN, D., FARO, J. & GRACA, L. 2017. T follicular helper and T follicular regulatory cells have different TCR specificity. 8, 15067.

- MAITY, P. C., DATTA, M., NICOLO, A. & JUMAA, H. 2018. Isotype Specific Assembly of B Cell Antigen Receptors and Synergism With Chemokine Receptor CXCR4. *Front Immunol*, 9, 2988.
- MALEK, T. R., YU, A., VINCEK, V., SCIBELLI, P. & KONG, L. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity*, 17, 167-78.
- MANDELBROT, D. A., MCADAM, A. J. & SHARPE, A. H. 1999. B7-1 or B7-2 is required to produce the lymphoproliferative phenotype in mice lacking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). *J Exp Med*, 189, 435-40.
- MANZ, R. A., HAUSER, A. E., HIEPE, F. & RADBRUCH, A. 2005. Maintenance of serum antibody levels. *Annu Rev Immunol*, 23, 367-86.
- MARSON, A., KRETSCHMER, K., FRAMPTON, G. M., JACOBSEN, E. S., POLANSKY, J. K., MACISAAC, K. D., LEVINE, S. S., FRAENKEL, E., VON BOEHMER, H. & YOUNG, R. A. 2007. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature*, 445, 931-5.
- MARTINS, T. B., BANDHAUER, M. E., BUNKER, A. M., ROBERTS, W. L. & HILL, H. R. 2014. New childhood and adult reference intervals for total IgE. *J Allergy Clin Immunol*, 133, 589-91.
- MAY, C. D. 1985. The ancestry of allergy: being an account of the original experimental induction of hypersensitivity recognizing the contribution of Paul Portier. *J Allergy Clin Immunol*, 75, 485-95.
- MAYER, C. T. & GAZUMYAN, A. 2017. The microanatomic segregation of selection by apoptosis in the germinal center. 358.
- MIYAKE, K., SHIOZAWA, N., NAGAO, T., YOSHIKAWA, S., YAMANISHI, Y. & KARASUYAMA, H. 2017. Trogocytosis of peptide-MHC class II complexes from dendritic cells confers antigen-presenting ability on basophils. *Proc Natl Acad Sci U S A*, 114, 1111-1116.
- MIYAO, T., FLOESS, S., SETOGUCHI, R., LUCHE, H., FEHLING, H. J., WALDMANN, H., HUEHN, J. & HORI, S. 2012. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity*, 36, 262-75.
- MIYARA, M., YOSHIOKA, Y., KITO, A., SHIMA, T., WING, K., NIWA, A., PARIZOT, C., TAFLIN, C., HEIKE, T., VALEYRE, D., MATHIAN, A., NAKAHATA, T., YAMAGUCHI, T., NOMURA, T., ONO, M., AMOURA, Z., GOROCHOV, G. & SAKAGUCHI, S. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*, 30, 899-911.
- MUCIDA, D., KUTCHUKHIDZE, N., ERAZO, A., RUSSO, M., LAFAILLE, J. J. & CUROTTO DE LAFAILLE, M. A. 2005. Oral tolerance in the absence of naturally occurring Tregs. *The Journal of clinical investigation*, 115, 1923-1933.

- MURPHY, K., TRAVERS, P., WALPORT, M., & JANEWAY, C. 2016. Janeway's immunobiology (9th ed.). *New York: Garland Science.*
- NAKAYAMA, M., TAKEDA, K., KAWANO, M., TAKAI, T., ISHII, N. & OGASAWARA, K. 2011. Natural killer (NK)-dendritic cell interactions generate MHC class II-dressed NK cells that regulate CD4<sup>+</sup> T cells. *Proc Natl Acad Sci U S A*, 108, 18360-5.
- NIEUWENHUIS, P. & OPSTELTEN, D. 1984. Functional anatomy of germinal centers. *Am J Anat*, 170, 421-35.
- NISHIMURA, H., MINATO, N., NAKANO, T. & HONJO, T. 1998. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int Immunol*, 10, 1563-72.
- NISHIO, J., BABA, M., ATARASHI, K., TANOUE, T., NEGISHI, H., YANAI, H., HABU, S., HORI, S., HONDA, K. & TANIGUCHI, T. 2015. Requirement of full TCR repertoire for regulatory T cells to maintain intestinal homeostasis. *Proc Natl Acad Sci U S A*, 112, 12770-5.
- NISHIZUKA, Y. & SAKAKURA, T. 1969. Thymus and reproduction: sex-linked dysgenesis of the gonad after neonatal thymectomy in mice. *Science*, 166, 753-5.
- NURIEVA, R. I., CHUNG, Y., MARTINEZ, G. J., YANG, X. O., TANAKA, S., MATSKEVITCH, T. D., WANG, Y. H. & DONG, C. 2009. Bcl6 mediates the development of T follicular helper cells. *Science*, 325, 1001-5.
- OUYANG, W., BECKETT, O., MA, Q., PAIK, J. H., DEPINHO, R. A. & LI, M. O. 2010. Foxo proteins cooperatively control the differentiation of Foxp3<sup>+</sup> regulatory T cells. *Nat Immunol*, 11, 618-27.
- OUYANG, W., LIAO, W., LUO, C. T., YIN, N., HUSE, M., KIM, M. V., PENG, M., CHAN, P., MA, Q., MO, Y., MEIJER, D., ZHAO, K., RUDENSKY, A. Y., ATWAL, G., ZHANG, M. Q. & LI, M. O. 2012. Novel Foxo1-dependent transcriptional programs control T(reg) cell function. *Nature*, 491, 554-559.
- OWEN, R. D. 1945. IMMUNOGENETIC CONSEQUENCES OF VASCULAR ANASTOMOSES BETWEEN BOVINE TWINS. *Science*, 102, 400-1.
- OZAKI, K., SPOLSKI, R., FENG, C. G., QI, C. F., CHENG, J., SHER, A., MORSE, H. C., 3RD, LIU, C., SCHWARTZBERG, P. L. & LEONARD, W. J. 2002. A critical role for IL-21 in regulating immunoglobulin production. *Science*, 298, 1630-4.
- PACHOLCZYK, R., IGNATOWICZ, H., KRAJ, P. & IGNATOWICZ, L. 2006. Origin and T cell receptor diversity of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells. *Immunity*, 25, 249-59.
- PALOMARES, O. 2013. The role of regulatory T cells in IgE-mediated food allergy. *J Investig Allergol Clin Immunol*, 23, 371-82; quiz 2 p preceding 382.
- PANDIYAN, P., ZHENG, L., ISHIHARA, S., REED, J. & LENARDO, M. J. 2007. CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4<sup>+</sup> T cells. *Nat Immunol*, 8, 1353-62.

- PLATTS-MILLS, T., VAUGHAN, J., SQUILLACE, S., WOODFOLK, J. & SPORIK, R. 2001. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. *Lancet*, 357, 752-6.
- PLATTS-MILLS, T. A. E., HEYMANN, P. W., COMMINS, S. P. & WOODFOLK, J. A. 2016. The discovery of IgE 50 years later. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology*, 116, 179-182.
- PORTIER P., R. C. 1902. D l'action anaphylactique de certains venins. *Compt Rend Soc Biol*, 54, 170-172.
- PROTO, J. D., DORAN, A. C., GUSAROVA, G., YURDAGUL, A., JR., SOZEN, E., SUBRAMANIAN, M., ISLAM, M. N., RYMOND, C. C., DU, J., HOOK, J., KURIAKOSE, G., BHATTACHARYA, J. & TABAS, I. 2018. Regulatory T Cells Promote Macrophage Efferocytosis during Inflammation Resolution. *Immunity*, 49, 666-677.e6.
- QURESHI, O. S., ZHENG, Y., NAKAMURA, K., ATTRIDGE, K., MANZOTTI, C., SCHMIDT, E. M., BAKER, J., JEFFERY, L. E., KAUR, S., BRIGGS, Z., HOU, T. Z., FUTTER, C. E., ANDERSON, G., WALKER, L. S. & SANSOM, D. M. 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*, 332, 600-3.
- READ, S., GREENWALD, R., IZCUE, A., ROBINSON, N., MANDELBROT, D., FRANCISCO, L., SHARPE, A. H. & POWRIE, F. 2006. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. *J Immunol*, 177, 4376-83.
- REINHARDT, R. L., LIANG, H. E. & LOCKSLEY, R. M. 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol*, 10, 385-93.
- RILEY, J. L. 2009. PD-1 signaling in primary T cells. *Immunological reviews*, 229, 114-125.
- ROSSJOHN, J., GRAS, S., MILES, J. J., TURNER, S. J., GODFREY, D. I. & MCCLUSKEY, J. 2015. T cell antigen receptor recognition of antigen-presenting molecules. *Annu Rev Immunol*, 33, 169-200.
- RUBTSOV, Y. P., NIEC, R. E., JOSEFOWICZ, S., LI, L., DARCE, J., MATHIS, D., BENOIST, C. & RUDENSKY, A. Y. 2010. Stability of the regulatory T cell lineage in vivo. *Science*, 329, 1667-71.
- RUBTSOV, Y. P., RASMUSSEN, J. P., CHI, E. Y., FONTENOT, J., CASTELLI, L., YE, X., TREUTING, P., SIEWE, L., ROERS, A., HENDERSON, W. R., JR., MULLER, W. & RUDENSKY, A. Y. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*, 28, 546-58.
- RUDRA, D., DEROOS, P., CHAUDHRY, A., NIEC, R. E., ARVEY, A., SAMSTEIN, R. M., LESLIE, C., SHAFFER, S. A., GOODLETT, D. R. & RUDENSKY, A. Y. 2012. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol*, 13, 1010-9.

- SAGE, P. T., ALVAREZ, D., GODEC, J., VON ANDRIAN, U. H. & SHARPE, A. H. 2014a. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest*, 124, 5191-204.
- SAGE, P. T., FRANCISCO, L. M., CARMAN, C. V. & SHARPE, A. H. 2013. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol*, 14, 152-61.
- SAGE, P. T., PATERSON, A. M., LOVITCH, S. B. & SHARPE, A. H. 2014b. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity*, 41, 1026-39.
- SAGE, P. T., RON-HAREL, N., JUNEJA, V. R. & SEN, D. R. 2016. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol*, 17, 1436-1446.
- SAGE, P. T. & SHARPE, A. H. 2016. T follicular regulatory cells. *Immunol Rev*, 271, 246-59.
- SAITO, M., NAGASAWA, M., TAKADA, H., HARA, T., TSUCHIYA, S., AGEMATSU, K., YAMADA, M., KAWAMURA, N., ARIGA, T., TSUGE, I., NONOYAMA, S., KARASUYAMA, H. & MINEGISHI, Y. 2011. Defective IL-10 signaling in hyper-IgE syndrome results in impaired generation of tolerogenic dendritic cells and induced regulatory T cells. *The Journal of Experimental Medicine*, 208, 235-249.
- SAKAGUCHI, S., SAKAGUCHI, N., ASANO, M., ITOH, M. & TODA, M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*, 155, 1151-64.
- SAKAGUCHI, S., TAKAHASHI, T. & NISHIZUKA, Y. 1982. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *The Journal of Experimental Medicine*, 156, 1577-1586.
- SAKAGUCHI, S., VIGNALI, D. A., RUDENSKY, A. Y., NIEC, R. E. & WALDMANN, H. 2013. The plasticity and stability of regulatory T cells. *Nat Rev Immunol*, 13, 461-7.
- SATITSUKSANOVA, P., JANSEN, K., GŁOBIŃSKA, A., VAN DE VEEN, W. & AKDIS, M. 2018. Regulatory Immune Mechanisms in Tolerance to Food Allergy. *Frontiers in Immunology*, 9.
- SAYIN, I., RADTKE, A. J. & VELLA, L. A. 2018. Spatial distribution and function of T follicular regulatory cells in human lymph nodes. 215, 1531-1542.
- SCHATZ, D. G. & JI, Y. 2011. Recombination centres and the orchestration of V(D)J recombination. *Nat Rev Immunol*, 11, 251-63.
- SCHEFFOLD, A., MURPHY, K. M. & HOFER, T. 2007. Competition for cytokines: T(reg) cells take all. *Nat Immunol*, 8, 1285-7.
- SCHROEDER, H. W., JR. & CAVACINI, L. 2010. Structure and function of immunoglobulins. *The Journal of allergy and clinical immunology*, 125, S41-S52.



- SCHUBERT, D., BODE, C., KENEFECK, R., HOU, T. Z., WING, J. B., KENNEDY, A., BULASHEVSKA, A. & PETERSEN, B. S. 2014. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. *20*, 1410-6.
- SEDDIKI, N., SANTNER-NANAN, B., MARTINSON, J., ZAUNDERS, J., SASSON, S., LANDAY, A., SOLOMON, M., SELBY, W., ALEXANDER, S. I., NANAN, R., KELLEHER, A. & FAZEKAS DE ST GROTH, B. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *The Journal of experimental medicine*, *203*, 1693-700.
- SHEVACH, E. M. 2001. Certified professionals: CD4(+)CD25(+) suppressor T cells. *J Exp Med*, *193*, F41-6.
- SKY, N. T., BRITTON, G. J., HILL, E. V., VERHAGEN, J., BURTON, B. R. & WRAITH, D. C. 2013. Regulation of adaptive immunity; the role of interleukin-10. *Frontiers in Immunology*, *4*.
- SMITH-GARVIN, J. E., KORETZKY, G. A. & JORDAN, M. S. 2009. T cell activation. *Annu Rev Immunol*, *27*, 591-619.
- STAVNEZER, J., GUIKEMA, J. E. J. & SCHRADER, C. E. 2008. Mechanism and regulation of class switch recombination. *Annual review of immunology*, *26*, 261-292.
- SUNYER, J. O. 2012. Evolutionary and functional relationships of B cells from fish and mammals: insights into their novel roles in phagocytosis and presentation of particulate antigen. *Infectious disorders drug targets*, *12*, 200-212.
- SZUREK, E., CEBULA, A., WOJCIECH, L., PIETRZAK, M., REMPALA, G., KISIELOW, P. & IGNATOWICZ, L. 2015. Differences in Expression Level of Helios and Neuropilin-1 Do Not Distinguish Thymus-Derived from Extrathymically-Induced CD4+Foxp3+ Regulatory T Cells. *PLoS One*, *10*, e0141161.
- TAGA, K., MOSTOWSKI, H. & TOSATO, G. 1993. Human interleukin-10 can directly inhibit T-cell growth. *Blood*, *81*, 2964-71.
- TAI, X., VAN LAETHEM, F., POBEZINSKY, L., GUINTER, T., SHARROW, S. O., ADAMS, A., GRANGER, L., KRUHLAK, M., LINDSTEN, T., THOMPSON, C. B., FEIGENBAUM, L. & SINGER, A. 2012. Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells. *Blood*, *119*, 5155-63.
- TAKAHASHI, T., KUNIYASU, Y., TODA, M., SAKAGUCHI, N., ITOH, M., IWATA, M., SHIMIZU, J. & SAKAGUCHI, S. 1998. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol*, *10*, 1969-80.
- THORNTON, A. M., KORTY, P. E., TRAN, D. Q., WOHLFERT, E. A., MURRAY, P. E., BELKAID, Y. & SHEVACH, E. M. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *Journal of immunology*, *184*, 3433-41.

- THORNTON, A. M. & SHEVACH, E. M. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med*, 188, 287-96.
- TIVOL, E. A., BORRIELLO, F., SCHWEITZER, A. N., LYNCH, W. P., BLUESTONE, J. A. & SHARPE, A. H. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*, 3, 541-7.
- TOELLNER, K. M., GULBRANSON-JUDGE, A., TAYLOR, D. R., SZE, D. M. & MACLENNAN, I. C. 1996. Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B cell activation. *J Exp Med*, 183, 2303-12.
- TSUJI, M., KOMATSU, N., KAWAMOTO, S., SUZUKI, K., KANAGAWA, O., HONJO, T., HORI, S. & FAGARASAN, S. 2009. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science*, 323, 1488-92.
- VAETH, M., MULLER, G., STAUSS, D., DIETZ, L., KLEIN-HESSLING, S., SERFLING, E., LIPP, M., BERBERICH, I. & BERBERICH-SIEBELT, F. 2014. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med*, 211, 545-61.
- VENKATESH, B., LEE, A. P., RAVI, V., MAURYA, A. K., LIAN, M. M., SWANN, J. B., OHTA, Y., FLAJNIK, M. F., SUTOH, Y., KASAHARA, M., HOON, S., GANGU, V., ROY, S. W., IRIMIA, M., KORZH, V., KONDRYCHYN, I., LIM, Z. W., TAY, B.-H., TOHARI, S., KONG, K. W., HO, S., LORENTE-GALDOS, B., QUILEZ, J., MARQUES-BONET, T., RANEY, B. J., INGHAM, P. W., TAY, A., HILLIER, L. W., MINX, P., BOEHM, T., WILSON, R. K., BRENNER, S. & WARREN, W. C. 2014. Elephant shark genome provides unique insights into gnathostome evolution. *Nature*, 505, 174-179.
- VICTORA, G. D. & NUSSENZWEIG, M. C. 2012. Germinal centers. *Annu Rev Immunol*, 30, 429-57.
- VINUESA, C. G., LINTERMAN, M., YU, D. & MACLENNAN, I. C. 2016. Follicular Helper T cells. *Annu Rev Immunol.*, 34.
- VISSERS, J. L., VAN ESCH, B. C., HOFMAN, G. A., KAPSENBERG, M. L., WELLER, F. R. & VAN OOSTERHOUT, A. J. 2004. Allergen immunotherapy induces a suppressive memory response mediated by IL-10 in a mouse asthma model. *J Allergy Clin Immunol*, 113, 1204-10.
- VOEHRINGER, D., REESE, T. A., HUANG, X., SHINKAI, K. & LOCKSLEY, R. M. 2006. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J Exp Med*, 203, 1435-46.
- VON PIRQUET C, S. B. 1903. Zur theorie der inkubationszeit. *Wein Kin Wchnschr*, 16, 1244.
- WALKER, L. S. K. & SANSOM, D. M. 2015. Confusing signals: recent progress in CTLA-4 biology. *Trends in immunology*, 36, 63-70.

- WALUNAS, T. L., BAKKER, C. Y. & BLUESTONE, J. A. 1996. CTLA-4 ligation blocks CD28-dependent T cell activation. *J Exp Med*, 183, 2541-50.
- WANG, J., IOAN-FACSINAY, A., VAN DER VOORT, E. I., HUIZINGA, T. W. & TOES, R. E. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol*, 37, 129-38.
- WATERHOUSE, P., PENNINGER, J. M., TIMMS, E., WAKEHAM, A., SHAHINIYAN, A., LEE, K. P., THOMPSON, C. B., GRIESSER, H. & MAK, T. W. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*, 270, 985-8.
- WIECZOREK, G., ASEMISSEN, A., MODEL, F., TURBACHOVA, I., FLOESS, S., LIEBENBERG, V., BARON, U., STAUCH, D., KOTSCH, K., PRATSCHKE, J., HAMANN, A., LODDENKEMPER, C., STEIN, H., VOLK, H. D., HOFFMULLER, U., GRUTZKAU, A., MUSTEA, A., HUEHN, J., SCHEIBENBOGEN, C. & OLEK, S. 2009. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res*, 69, 599-608.
- WILDIN, R. S., RAMSDELL, F., PEAKE, J., FARAVELLI, F., CASANOVA, J. L., BUIST, N., LEVY-LAHAD, E., MAZZELLA, M., GOULET, O., PERRONI, L., BRICARELLI, F. D., BYRNE, G., MCEUEN, M., PROLL, S., APPLEBY, M. & BRUNKOW, M. E. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*, 27, 18-20.
- WING, J. B., ISE, W., KUROSAKI, T. & SAKAGUCHI, S. 2014. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity*, 41, 1013-25.
- WING, J. B., KITAGAWA, Y., LOCCI, M., HUME, H., TAY, C., MORITA, T., KIDANI, Y., MATSUDA, K., INOUE, T., KUROSAKI, T., CROTTY, S., COBAN, C., OHKURA, N. & SAKAGUCHI, S. 2017a. A distinct subpopulation of CD25(-) T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci U S A*, 114, E6400-e6409.
- WING, J. B., KITAGAWA, Y., LOCCI, M., HUME, H., TAY, C., MORITA, T., KIDANI, Y., MATSUDA, K., INOUE, T., KUROSAKI, T., CROTTY, S., COBAN, C., OHKURA, N. & SAKAGUCHI, S. 2017b. A distinct subpopulation of CD25- T-follicular regulatory cells localizes in the germinal centers. *Proceedings of the National Academy of Sciences*, 114, E6400-E6409.
- WING, K., ONISHI, Y., PRIETO-MARTIN, P., YAMAGUCHI, T., MIYARA, M., FEHERVARI, Z., NOMURA, T. & SAKAGUCHI, S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*, 322, 271-5.
- WITSCH, E. J., PEISER, M., HUTLOFF, A., BUCHNER, K., DORNER, B. G., JONULEIT, H., MAGES, H. W. & KROCZEK, R. A. 2002. ICOS and CD28 reversely regulate IL-10 on re-activation of human effector T cells with mature dendritic cells. *Eur J Immunol*, 32, 2680-6.

- WOLLENBERG, I., AGUA-DOCE, A., HERNANDEZ, A., ALMEIDA, C., OLIVEIRA, V. G., FARO, J. & GRACA, L. 2011a. Regulation of the germinal center reaction by Foxp3<sup>+</sup> follicular regulatory T cells. *Journal of immunology*, 187, 4553-60.
- WOLLENBERG, I., AGUA-DOCE, A., HERNANDEZ, A., ALMEIDA, C., OLIVEIRA, V. G., FARO, J. & GRACA, L. 2011b. Regulation of the germinal center reaction by Foxp3<sup>+</sup> follicular regulatory T cells. *J Immunol*, 187, 4553-60.
- WONG, J., OBST, R., CORREIA-NEVES, M., LOSYEV, G., MATHIS, D. & BENOIST, C. 2007. Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4<sup>+</sup> T cells. *J Immunol*, 178, 7032-41.
- WU, H., CHEN, Y., LIU, H., XU, L. L., TEUSCHER, P., WANG, S., LU, S. & DENT, A. L. 2016. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur J Immunol*, 46, 1152-61.
- XIONG, H., CUROTTO DE LAFAILLE, M. A. & LAFAILLE, J. J. 2012a. What is unique about the IgE response? *Adv Immunol*, 116, 113-41.
- XIONG, H., DOLPADY, J., WABL, M., CUROTTO DE LAFAILLE, M. A. & LAFAILLE, J. J. 2012b. Sequential class switching is required for the generation of high affinity IgE antibodies. *J Exp Med*, 209, 353-64.
- YAMAGIWA, S., GRAY, J. D., HASHIMOTO, S. & HORWITZ, D. A. 2001. A role for TGF- $\beta$  in the generation and expansion of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells from human peripheral blood. *J Immunol*, 166, 7282-9.
- YAMAGUCHI, T., KISHI, A., OSAKI, M., MORIKAWA, H., PRIETO-MARTIN, P., WING, K., SAITO, T. & SAKAGUCHI, S. 2013. Construction of self-recognizing regulatory T cells from conventional T cells by controlling CTLA-4 and IL-2 expression. *Proc Natl Acad Sci U S A*, 110, E2116-25.
- YANG, Z. & ROBINSON, M. J. 2016. Regulation of B cell fate by chronic activity of the IgE B cell receptor. 5.
- YANG, Z., ROBINSON, M. J., CHEN, X., SMITH, G. A., TAUNTON, J., LIU, W. & ALLEN, C. D. C. 2016. Regulation of B cell fate by chronic activity of the IgE B cell receptor. *eLife*, 5, e21238.
- YANG, Z., SULLIVAN, B. M. & ALLEN, C. D. 2012. Fluorescent in vivo detection reveals that IgE(+) B cells are restrained by an intrinsic cell fate predisposition. *Immunity*, 36, 857-72.
- YAO, Z., KANNO, Y., KERENYI, M., STEPHENS, G., DURANT, L., WATFORD, W. T., LAURENCE, A., ROBINSON, G. W., SHEVACH, E. M., MORIGGL, R., HENNIGHAUSEN, L., WU, C. & O'SHEA, J. J. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood*, 109, 4368-75.
- YU, D., RAO, S., TSAI, L. M., LEE, S. K., HE, Y., SUTCLIFFE, E. L., SRIVASTAVA, M., LINTERMAN, M., ZHENG, L., SIMPSON, N., ELLYARD, J. I., PARISH, I. A., MA, C. S., LI, Q. J., PARISH, C. R., MACKAY, C. R. & VINUESA, C. G. 2009. The

- transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity*, 31, 457-68.
- YU, F., SHARMA, S., EDWARDS, J., FEIGENBAUM, L. & ZHU, J. 2015. Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. *Nat Immunol*, 16, 197-206.
- ZHAO, H., LIAO, X. & KANG, Y. 2017. Tregs: Where We Are and What Comes Next? *Frontiers in Immunology*, 8.
- ZHENG, Y., CHAUDHRY, A., KAS, A., DEROOS, P., KIM, J. M., CHU, T. T., CORCORAN, L., TREUTING, P., KLEIN, U. & RUDENSKY, A. Y. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature*, 458, 351-6.
- ZHENG, Y., JOSEFOWICZ, S., CHAUDHRY, A., PENG, X. P., FORBUSH, K. & RUDENSKY, A. Y. 2010. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*, 463, 808-12.
- ZHENG, Y., JOSEFOWICZ, S. Z., KAS, A., CHU, T. T., GAVIN, M. A. & RUDENSKY, A. Y. 2007. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*, 445, 936-40.
- ZHOU, L., LOPES, J. E., CHONG, M. M. W., IVANOV, I. I., MIN, R., VICTORA, G. D., SHEN, Y., DU, J., RUBTSOV, Y. P., RUDENSKY, A. Y., ZIEGLER, S. F. & LITTMAN, D. R. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature*, 453, 236-240.
- ZHOU, X., BAILEY-BUCKTROUT, S. L., JEKER, L. T., PENARANDA, C., MARTINEZ-LLORELLA, M., ASHBY, M., NAKAYAMA, M., ROSENTHAL, W. & BLUESTONE, J. A. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol*, 10, 1000-7.
- ZHU, J., YAMANE, H. & PAUL, W. E. 2010. Differentiation of effector CD4 T cell populations (\*). *Annu Rev Immunol*, 28, 445-89.