UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA

THE REPERTOIRE AND SPECIFICITY OF FOLLICULAR REGULATORY T CELLS

Ana Raquel Maceiras de Oliveira

Orientador: Prof. Doutor Luís Ricardo Simões da Silva Graça

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas no ramo de Imunologia

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As opiniões expressas nesta publicação são da exclusiva responsablidade do seu autor.

Ao meu pai
que guardo com saudade

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Abbreviations

ADCC - antibody-dependent cell cytotoxicity

Ag – antigen

AHR – aryl hydrocarbon receptor

AICD – activation-induced cell death

AID – activation-induced cytidine deaminase

APC – antigen-presenting cell

ASC – antibody secreting cell

ASCL2 – achaete-scute homologue-2

AU – approximately unbiased

BATF – basic leucine zipper transcription factor

BCL-6 – B cell lymphoma 6

BCR – B cell receptor

BP – bootstrap probability

bp – base pairs

BTLA – B and T lymphocyte attenuator

cAMP – cyclic adenosine monophosphate

CD – cluster of differentiation

CDR – complementarity-determining region

CFA – Complete Freund's adjuvant

CSF – colony-stimulating factor

CSR – class switch recombination

cTfh – circulating Tfh cells

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4

CTV – CellTrace Violet

CXCL – C-X-C chemokine ligand

CXCR – C-X-C chemokine receptor

DAMP – damage-associated molecular patterns

DC – dendritic cell

DP – double positive

DZ – dark zone

EBI2 – Epstein-Barr virus-induced G-protein coupled receptor 2

EAE – experimental autoimmune encephalomyelitis

FACS – flow activated cell sorting

FASL – FAS ligand

FcR – Fc receptor

FcγR - IgG Fc receptor

FcεR – IgE Fc receptor

FDC – follicular dendritic cell

FOXP3 – forkhead box P3

FSC – forward scatter

GC – germinal center

GFI-1 – growth factor independent-1

GITR – glucocorticoid-induced TNF receptor-related protein

GM-CSF - granulocyte macrophage colony-stimulating factor

hCD2 – human CD2

H-chain – immunoglobulin heavy-chain

HVEM – herpesvirus entry mediator

IBD – inflammatory bowel disease

ICOS – inducible T cell co-stimulator

ICOSL – ICOS ligand

IFA – Incomplete Freund's adjuvant

IFN – interferon

IL – interleukin

ILC – innate lymphoid cell

IPEX – immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

IRF - interferon-regulatory factor

i.v. – intravenous

KLF2 – Krüppel-like factor 2

LAG-3 – lymphocyte-activation gene 3

L-chain – immunoglobulin light-chain

LN – lymph node

LZ – light zone

MAC - membrane attack complex

MCL1 – myeloid cell leukemia 1

MHC – major histocompatibility complex

MS – multiple sclerosis

MZ – marginal zone

NFAT2 - Nuclear factor of activated T-cells 2

NK cell – natural killer cell

NKT cell – natural killer T cell

NT-ES – nuclear transfer - embryonic stem cell

OVA – ovalbumin

PAMP – pathogen-associated molecular patterns

PD-1 – programmed cell death 1

PD-L – PD-1 ligand

PI3K – phosphoinositide 3-kinase

PRR – pattern recognition receptors

PSGL-1 – P-selectin glycoprotein ligand 1

pTreg – peripherally-derived Treg cells

RA – rheumatoid arthritis

RAG - recombination-activating gene

RORα – retinoic acid receptor-related orphan receptor alpha

RORγt – retinoic acid receptor-related orphan receptor gamma-T

RT – room temperature

SAP – SLAM-associated protein

s.c. – subcutaneous

SHM – somatic hypermutation

SLAM – signaling lymphocytic activation molecule

SLE – systemic lupus erythematosus

SP – single positive

SPF – specific pathogen free

SSC – side scatter

STAT – signal transducer and activator

S1PR1 – sphingosine-1-phosphate receptor 1

T-BET – T-box transcription factor

TCR – T cell receptor

TD – T cell dependent

TdT – terminal desoxyribonucleotidyl transferase

TI – T cell independent

TLR – Toll-like receptors

TNF – tumor necrosis factor

TGFβ – transforming growth factor beta

TRA/TCRα – T-cell receptor alpha chain

TRAF3 – TNF receptor-associated factor 3

TRAV – V region of TRA

TRB/TCRβ – T cell receptor beta chain

TRBV – V region of TRB

TRBVBJ – V and J regions of TRB

Tconv – conventional T cells

Tfh – follicular helper T cells

Tfr – follicular regulatory T cells

Treg – regulatory T cells

tTreg – thymic-derived Treg cells

WT – Wild-type

βLG – β-lactoglobulin

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Abstract

Germinal centers (GCs) are key structures where B cells are selected to produce high affinity immunoglobulins of the adequate class. This selection is dependent of B cell interactions with specialized follicular helper T (Tfh) cells that provide help for class switch recombination, somatic hypermutation, and selection signals to GC B cells. It was recently described that the GC reaction is controlled by specialized Foxp3^{+} follicular regulatory T (Tfr) cells. These cells regulate the size and magnitude of the GC reaction, and have been also implicated in the prevention of autoimmunity.

This thesis aimed to answer two questions regarding Tfr cells biology. First, it was investigated whether Tfr cells derive from thymic-derived regulatory T (tTreg) cells. The second objective was to establish whether Tfr cells are specific for the non-self antigen driving the GC response, as Tfh cells, or if Tfr cells have a repertoire closer to Treg cells with specificity towards self-antigens.

It was found that Tfr cells originate exclusively from tTreg cells. Also, Tfr cells were not specific for the immunizing antigen. Indeed, as antigen-specific TCR-transgenic Tfr cells were not specifically recruited into the GC, nor could antigen-specific Tfr cells be detected using class-II MHC tetramers. Moreover, Tfr cells did not specifically proliferate *in vitro* when stimulated with the immunizing antigen. Lastly, repertoire analysis of Tfr, Treg and Tfh cells demonstrated that Tfh cells and Tfr cells have different repertoires with the latter retaining a repertoire closer to Treg cells.

Taken together, the presented results show a clear difference in the specificity and TCR usage by Tfh and Tfr populations from the same GCs. This distinct specificity is in line with different putative functions of the two populations: while Tfh cells promote antigen-specific B cell responses, Tfr cells prevent autoimmunity.

Keywords: Germinal centers; Tfr cells; Tfh cells; FOXP3

Resumo

Os centros germinativos (GCs) são estruturas onde as células B sofrem hipermutação somática, seleção positiva e mudança de isotipo, de forma a produzirem anticorpos de alta afinidade e de isotipo adequeado à resposta imunitária em curso. No entanto, para que estes processos ocorram, as células B dos centros germinativos precisam de ajuda por parte de células T auxiliares foliculares (Tfh).

Recentemente, foi descrita uma nova população de células reguladoras FOXP3 positivas, as células T reguladoras foliculares (Tfr), que se encontram dentro dos GCs. Estas células estão envolvidas na regulação dos GCs, nomeadamente, na quantidade e na qualidade (afinidade) de anticorpos produzidos durante uma resposta imunitária. Para além disso, as células Tfr parecem também estar envolvidas na prevenção de doenças autoimunes.

As células Tfr partilham algumas características quer com as células T reguladoras (Treg) FOXP3 positivas, quer com as células Tfh. Tal como os linfócitos Treg, as células Tfr expressam FOXP3, CD25 e CTLA-4 e têm capacidade supressora. Por outro lado, assim como as células Tfh, as células Tfr expressam PD-1, ICOS, CXCR5 (que lhes permite migrar para o GC) e BCL-6, o principal factor de transcrição responsável pela diferenciação de células T CD4 foliculares. Tanto as células Tfr como as Tfh podem ser induzidas por imunização, já que esta induz GCs, num processo muito semelhante ao que é observado após vacinação.

O trabalho descrito nesta tese tinha dois objectivos principais. O primeiro era estabelecer qual a origem das células Tfr., isto é, se estas células se diferenciam a partir de células Treg originadas no timo ou a partir de células T CD4 convencionais (Tconv). O outro objectivo consistia em determinar a especificidade das células Tfr, ou seja, se estas células são específicas para o antigénio da imunização, ao qual as células B do GC estão a responder e a maturar a sua afinidade. Três linhas de trabalho foram estabelecidas para responder a estas questões.

Em primeiro lugar, efectuaram-se transferências de células Tconv específicas para um antigénio devido à expressão de um receptor de células T (TCR) transgénico. Foi verificado que estas células específicas, depois de uma imunização com o respectivo antigénio, não se diferenciavam em células Tfr. No entanto, quando uma população de células Treg específicas que se origina no timo está presente, estas dão origem a células Tfr. Assim, as células Tfr originam-se exclusivamente de células Treg que se originam no timo.

Para estabelecer se as células Tfr, como as células Tfh, são específicas para o antigénio que induz a resposta do GC, foram realizadas experiências *in vivo* e *in vitro*. Primeiro, realizaram-se transferências de células transgénicas específicas para um antigénio, entre as quais uma população de células Treg também específicas. Após imunização com o respectivo antigénio (ou um antigénio controlo), verificou-se um recrutamento específico de células transgénicas como Tfh para o centro germinativo. No entanto, as células Treg específicas não foram preferencialmente recrutadas como células Tfr já que a mesma percentagem de células foi observada após imunização com o antigénio específico e com o antigénio controlo. Para confirmar os resultados anteriores, mas sem a utilização de células transgénicas, estudou-se a existência de células Tfr específicas após imunização com dois péptidos, utilizando para isso dois tetrâmeros de MHC classe II. Detectou-se uma população de células Tfh específicas (tetrâmero positivas) em imunizações contendo o péptido correspondente. Porém, não foram detectadas células Tfr específicas (tetrâmero positivas) em nenhuma das imunizações efectuadas. Verificou-se ainda que as células Tfr não reconheciam especificamente o antigénio da imunização que lhe deu origem, já que a sua proliferação *in vitro* foi igual independentemente do antigénio fornecido durante a cultura.

Por último, estudou-se o reportório das células Tfr, Tfh e Treg. O estudo do reportório, inicialmente feito através da determinação da distribuição do comprimento de CDR3 para cada segmento V da cadeia β do TCR, mostrou que as células Tfh apresentavam diferenças em relação à distribuição verificada para células T CD4 *naïves*. Estas diferenças consistiam principalmente em aumentos de utilização de comprimentos CDR3 específicos. A existência destes aumentos indica a presença de expansões clonais dentro da população de células Tfh. Os mesmos aumentos não foram observados nas células Tfr que, apesar de também apresentarem algumas diferenças em comparação com as distribuições das células T CD4 *naïves*, tinham o seu reportório próximo das células Treg. Para confirmar os resultados da análise de utilização de comprimentos de CDR3 e obter uma maior informação sem as limitações da técnica anterior, o reportório da cadeia α do TCR de murganhos 1D2β foi também sequencido. Este modelo de murganho, para além de ser repórter para FOXP3, expressa uma cadeia β do TCR transgénica (não variável) e tem apenas um dos alelos da cadeia α do TCR disponível para recombinação. Apesar disso, estes animais são capazes de montar respostas específicas contra um antigénio, visto que após imunização há formação de células T CD4 foliculares e as células Tfh destes animais conseguem reconhecer especificamente o antigénio correspondente em ensaios *in vitro*. A utilização deste modelo

tem como vantagem obter a informação completa do reportório das populações em estudo sequenciando apenas a cadeia α do TCR. A análise do reportório das células Tfr, Treg e Tfh permitiu saber que, apesar de as células Tfr e Tfh serem oligoclonais, estas não têm clonótipos comuns. Por outro lado, a população celular que apresentava um reportório mais próximo das células Tfr era a população Treg, apesar desta última ser policlonal (sem expansões óbvias de clonótipos específicos). Estas observações permitem concluir que as células Tfr têm uma especificidade diferente das células Tfh, uma vez que o seu reportório é diferente, e que devem ser específicas para antigénios do próprio, tal como as células Treg de que se originam e com quem têm um reportório mais próximo.

Assim, os resultados permitem concluir que as células Tfr originam-se de células Treg e, não sendo específicas para o antigénio que induz a resposta do GC, têm um reportório próximo das células Treg, com especificidade para antigénios do próprio.

Palavras-chave: Centros germinativos; células Tfr; células Tfh; FOXP3

I. General Introduction

Immune system

The immune system is constituted by specialized cells and molecules which main role is to defend an organism from disease. Although immunity was first associated with protection against infectious disease, the immune system can also be activated by noninfectious foreign substances and damaged or altered cells of the self.

The immune system is divided into two fundamentally different parts, the innate and the adaptive immune systems, that interact and complement each other. The innate immune system consists of cellular and biochemical mechanisms that are already in place even before infection, and provides protection to the same extension independently of how many times it has encountered the same infectious agent. The adaptive immune system, on the other hand, takes longer to respond, but is characterized by the exquisite specificity for each infection and the ability to improve its response upon re-exposure to the same infectious agent.

Innate immune system

When the need for an immune response arises, the innate immune system is activated first to respond against the threat to the organism. In the case of an infection, the response is initiated within minutes through an inflammatory response.

The main activation mechanism of innate immune cells is through the recognition of a limited number of molecules called pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). PRRs include Toll-like receptors (TLRs)[1], C-type lectin receptors (like the mannose receptor[2]), scavenger receptors[3], among others. These receptors, which can be present on the surface, in the endosomal vesicles, or in the cytoplasm of innate immune cells, bind different molecules from distinct pathogens, and consequently trigger different immune responses. TLRs, the most extensively studied PPRs, are divided in two groups based on their cellular localization: TLRs 1, 2, 4, and 5 are found in the cell surface and recognize bacterial and viral products on the extracellular space, while TLRs 3, 7, 8, and 9 are expressed mainly in endosomal vesicles and detect the presence of bacterial and viral nucleic acids. Furthermore, some PRRs also recognize cell damage signals by dying cells, called DAMPs (damage-associated molecular patterns), which allow the system to identify candidate cells for phagocytosis and initiate an inflammatory and immune response with these signals. Examples of DAMP receptors are TLR2 and TLR4 that bind host heat shock proteins[4,5] and TLR9 that binds Chromatin-IgG complexes[6]. The activation

of PRRs cause cell activation, production of cytokines, and presentation of co-stimulatory signals that lead to activation of the immune system through an initial inflammatory response, recruitment of more cells to the local of the ongoing immune response, and further activation of specialized cell through cell-to-cell contact.

Cytokines are small molecules secreted by one or several cells to alter the behavior of itself (autocrine effect) or of other cells (paracrine effect if target cells in the vicinity or endocrine effect if the cytokine targets cells at long distances). Target cells receive the signal through receptors on their membrane that are specific for the cytokine which signaling will affect cell activation, division, apoptosis, or movement. While virtually all cells can produced cytokines, some immune cells, such as leukocytes, are specialized in that function. Cytokines are divided in subgroups depending on the function or target/producing cells: interleukins (IL) were initially described as being produced by, and affecting, white cells, but they have a very wide range of target cells and effects; chemokines induce cell migration by acting as chemoattractants; interferons (IFN) were initially associated with viral infections and their production mainly occurs in responses against intracellular pathogens; colony-stimulating factors (CSF) induce proliferation and differentiation of hematopoietic stem cells.

The innate immune system is composed by different types of cells with different functions, the complement system, and acute-phase proteins. Innate cells can be divided into three categories: cells that have high phagocytic capacity, like neutrophils, macrophages, and dendritic cells (DCs); basophils, mast cells, and eosinophils that act as producers of inflammatory mediators; and innate lymphoid cells (ILCs).

Macrophages and dendritic cells reside in different tissues and get activated upon onset of an immune response. Neutrophils, however, are the most abundant nucleated cells in the blood and are recruited into tissues due to local inflammatory signals. Macrophages and neutrophils are the main cells responsible for clearance of pathogens, infected cells, and immune complexes. Although DCs also have high phagocytic capacity, their main function is to connect the innate and adaptive immune system by, upon antigen uptake, migrating into the spleen and lymph nodes (LNs) where they act as antigen-presenting cells (APCs) to T cells. Other cell types can also act as APCs, as is the case of B cells and macrophages, but are not involved in the priming of T cells, a function mainly performed by DCs.

Eosinophils[7] protect against parasitic infections, particularly infections by nematodes. As these parasites are large, eosinophils action is not through direct phagocytosis, but rather the release of large granules containing cytotoxic mediators on the surface of the parasitic

organism. Tissue resident mast cells and blood basophils[7] release inflammatory meditators and induce inflammatory responses.

ILCs are divided in two main groups: cytotoxic ILCs and non-cytotoxic ILCs. The first comprises the long known natural killer (NK) cells, while the second is composed by group 1, 2, and 3 of ILCs (ILC1-3). All these cells have common characteristics with the adaptive immune T cells, however, they lack the capability of recognizing specific antigens[8]. NK cells recognize and kill microbial-infected or tumor cells and their surveillance is performed throughout the entire organism[9]. ILCs are common at sites of potential invasion or colonization by pathogens, such as barrier surfaces, and upon activation they rapidly initiate the release of cytokines. The type of cytokines release depends on the group of ILCs activated that in turn will depend on the kind of required response: while ILC1s produce IFNγ, ILC2s produce IL-4, IL-5, IL-9, and IL-13, and ILC3s produce IL-17, IL-22 (the same signature cytokines as Th1, Th2, and Th17 described later on the CD4 T cells subsection)[8].

Another important component of the innate immune system is the complement system[10]. This system is composed by a set of proteins (C1-C9) which bind to each other on a proteolytic cascade to generate pro-inflammatory mediators, pathogen opsonization, and lysis of the target cell through membrane-penetrating pores called membrane-attack complex (MAC). Although there are three activation pathways (the classical, the alternative, and the lectin pathways), all cascades converge in the generation of C3 convertase which cleaves C3 into the anaphylotoxin C3a, a peptide mediator of inflammation, and the opsonin C3b, the main effector molecule of the complement system.

The innate immune system has a major impact on the adaptive immune system, since the latter is dependent on antigen presentation by innate immune cells for full activation. Also, the ability to determine the necessary type of response by the innate immune system has an important role in directing the effector responses by the adaptive immune system (namely, Th1, Th2, or Th17). However, adaptive immune responses also have an extensive impact on innate capacity to fight infections: Fc receptors are present in most of innate immune cells and either facilitate phagocytose or lead to release of inflammatory molecules. Also, the classical activation pathway of the complement system is dependent on antibodies for activation, contrary to the other two pathways (alternative and lectin pathways) that rely on PAMP recognition.

Adaptive Immune system

The two main characteristics of the adaptive immunity that differentiates it from the innate immunity are the use of antigen-specific receptors and its ability to improve its response upon re-exposure to the same infectious agent.

T and B cells are the cell types that constitute the adaptive immune system. While B cells are associated with antibody production and consequently humoral immunity, T cells are responsible for cellular immunity through the action of CD8 T cells. However, both types of adaptive immunity require help from CD4 T cells.

One of the key points of these cells is their receptors, the T-cell receptor (TCR) in the case of T cells and the B-cell receptor (BCR) in the case of B cells. These receptors originate from the random recombination and mutation of multiple DNA segments that code for the antigen recognition area of the receptors, which in turn leads to the production of a very wide range of receptors: the T and B cell repertoires comprise over 10^8 different TCRs and over 10^{10} BCRs to guarantee the recognition of all pathogens that can ever be encountered.

The other key point of T and B cells is their capability to acquire a memory phenotype during a primary immune response that allows more robust and rapid responses upon subsequent exposure to the same pathogen.

Both cell types derive from pluripotent stem cells, but, while B cells develop in the bone-marrow, T cell progenitors migrate into the thymus during their initial stages of development. As T and B development occurs in the thymus and the bone marrow, these organs are considered primary lymphoid organs. Both T and B cell development is dictated by the rearrangement steps of their antigen receptors and the ability to recognize antigens. However, while the BCR can recognize free antigens, T cells need another cell to process and present antigen peptides in major histocompatibility complex (MHC) molecules for TCR recognition. Once their development is complete, these cells migrate from the primary lymphoid organs into secondary lymphoid tissues, such as the spleen, LNs, and mucosa associated lymphoid tissues, where they wait the direct recognition or the presentation (in the case of B or T cells, respectively) of an antigen that their unique receptor can recognize in order to start an adaptive immune response.

B cells

The main function of B cells is the production of antibodies, which are key for the immune protection of the host against invading pathogens and are the basis for the vast majority of prophylactic vaccination strategies.

Although antibodies immune action is in their secreted form, they can also be produced as a membrane-bound receptor – the BCR. They are constituted by two heavy chains (H-chains) and two light chains (L-chains) bound by disulfide bonds (see Figure 1). The two recognition zones (bivalent), formed by N-terminal of each H-chain with an L-chain, bind the antigen through three hypervariable complementarity-determining regions (CDR1-3). B cells only express a functional immunoglobulin gene after successfully perform gene segments rearrangement, a process called V(D)J recombination[11]. There are four kind of gene segments (or regions): V (variable), D (diversity), J (joining), and C (constant) segments (see Figure 1). While the H-chain comprises the four segment types, the L-chain does not have D segments. On the other hand, there are two sets of V_L, J_L, and C_L segments, κ and λ, that can be rearranged and originate a functional L-chain. During V(D)J recombination, segments are cut and spliced together by the action of several enzymes, such as recombination-activating gene 1 (RAG-1) and RAG-2. To further increase the diversity on the antigen specificity of these molecules, the splicing action of the enzymes is inaccurate, leading to frameshifts in the encoded base pairs, while the enzyme terminal desoxyribonucleotidyl transferase (TdT) can also insert nucleotides to change the coding sequence. As the CDR3 comprises the segment junctions, the same combination of V(D)J segments may have different CDR3 lengths (see Figure 1). As result, these processes remarkably increase the antibodies variability and can produce more than 10^{10} different immunoglobulins. There are also several C regions for the heavy chain that encode for different classes and subclasses of the antibody. In mouse there are five immunoglobulins classes and one of them with four subclasses: IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA (coded by eight C segments: C_μ, C_δ, $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_ε, and C_α, respectively, and ordered by gene order on the genome).

The recombination process takes place during B-cell development in the bone marrow or in the splanchnopleural region and fetal liver of the embryo prior to the bone marrow development. Upon BCR recombination, when a B cell successfully expresses an IgM molecule as BCR on its cell surface, auto-reactivity is tested in order to prevent the generation of harmful B cells that recognize and react against the self[12]. B cells are positively

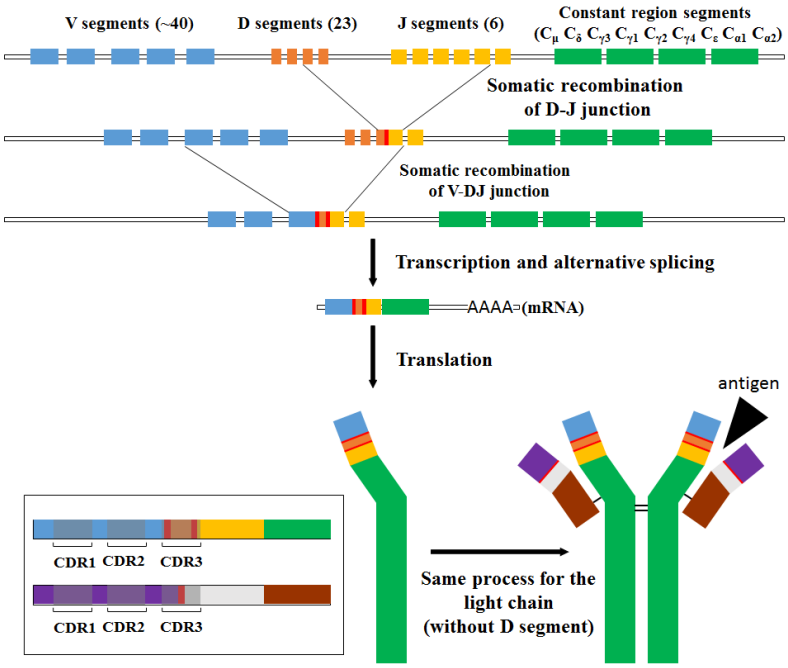

Figure 1 – VDJ recombination process of BCR heavy chain.
The BCR is the result of a process denominated V(D)J recombination, where different gene segments are combined to obtain the final gene sequence. In the case of the heavy chain, a D segment is initially recombined with a J and the result is then recombined with a V segment. In the case of the light chain, there is only recombination of a J segment with a V segment (the light chain does not possess D segments). The recombination involves cut and splice in order to remove the unwanted gene sequences. Within the V(D)J rearranged sequences there are three complementarity-determining regions (CDR1-3) that are responsible for the direct recognition and binding of the antigen. These regions are hyper variable, especially in the case of the CDR3 that comprises the V(D)J segments junctions, which may suffer frameshifts in the encoded base pairs and insertion of nucleotides.

selected and leave the bone marrow as immature B cells when BCRs mildly recognize and respond to self-antigens; if not, tolerance is achieved by clonal deletion of non-autoreactive or highly autoreactive B cells [13-18]. When still available, secondary rearrangements of the non-rearranged L-chains can still rescue BCR specificity in a process denominated receptor editing[19-21]. Nevertheless, some self-reactive cells escape into the periphery[22].

Only the H-chain C region recombination does not occur during the initial B cell development. B cells that have completed the initial development will only express IgM and IgD antibodies isotypes. For the other isotypes to be expressed by a B cell, class-switch recombination (CSR) has to occur. This process, mediated by the enzyme activation-induced cytidine deaminase (AID)[23], involves the removal from the chromosome of portions of the antibody heavy chain locus, and the gene segments surrounding the deleted portion are rejoined to retain a functional antibody gene that produces antibody of a different isotype (see Figure 2). CSR occurs in secondary lymphoid organs upon activation and in the presence of specific cytokine signals.

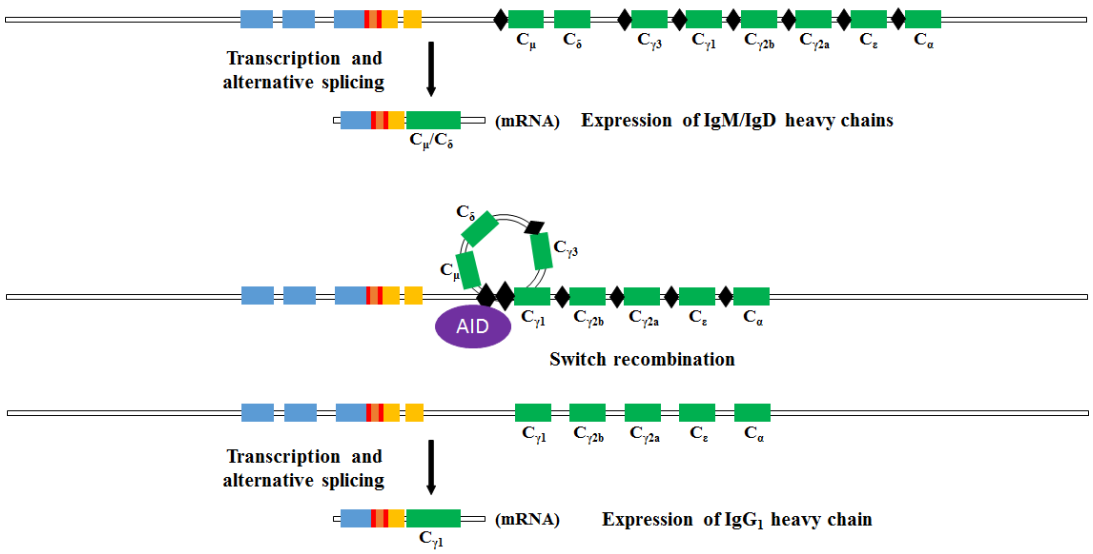

Figure 2 – Class Switch Recombination process.
In order for B cells to express antibodies of different classes, CSR needs to occur. This process involves somatic recombination through the action of the AID enzyme. The recombination involves the removal of a DNA fragment from the Cμ segment (if no recombination has happened yet) until the sequence before the wanted C segment. Specific sequence motifs on the DNA (black lozenges) indicate sites where recombination can occur. At the end of development in the bone marrow, and prior to CSR, B cells will only express IgM and IgD. CSR mainly occurs on germinal centers after B cell activation. The type of isotype to be expressed by the B cell is determined by the cytokines produced by CD4 helper T cells.

There are two subsets of B cells: B-1 and B-2 B cells. B-1 cells locate in peritoneal and pleural cavities and mucosal sites and are responsible for the production of natural IgM antibodies[24].

B-2 cells develop in the bone-marrow, but their early differentiation is only terminated upon migration into B cell follicles in the spleen, where they differentiate into naïve follicular or marginal zone (MZ) B cells[25]. When their early development is completed, naïve follicular B cells can migrate into other secondary lymphoid organs such as LNs and Payer patches.

MZ B cells, as B-1 cells, are also part of the first line of defense against blood-borne pathogens through T cell independent (TI) humoral responses[26]. On the first couple of days after the onset of an immune response, these cells rapidly differentiate into extrafollicular IgM-producing plasma cells, which secrete IgM as a decavalent pentamer that forms immune complexes with the pathogen[27]. However, since these cells differentiate independently of T cell help, they are short-lived and of low specificity as there is no affinity maturation.

Follicular B cells are also able to respond to TI antigens; however they, seem to be more specialized in responding to antigens that also activate CD4 T cells, thus gaining the help of these cells and consequently mounting T cell dependent (TD) humoral responses. Follicular B cells get initially activated by recognition of antigen brought to lymphoid tissues. The recognized antigen is internalized and processed by the B cell to be presented on class-II

MHC (MHC-II) molecules to primed antigen-specific and specialized T cells. Thus, upon antigen receptor-dependent activation, B cells migrate to the border of the T cell zone and B cell follicle (T-B border), where they encounter pre-activated antigen-specific T cells (pre-follicular helper T cells or pre-Tfh cells). At this location, T and B cells form long-lived interactions, allowing B cells to proliferate and become fully activated (as well as leading to complete maturation of pre-Tfh cells into Tfh cells). Once a B cell becomes fully activated, it will reach a bifurcation between two fates: either join the extrafollicular response or the germinal center (GC) response.

In the extrafollicular response, B cells differentiate into unswitched IgM memory B cells[28] or, after some rounds of division and in some cases CSR, into short-lived plasmablasts[29]. From the initial pool of activated B cells, cells with higher affinity to the antigen are the ones selected to become plasmablasts[30,31]; however, as they do not undergo affinity maturation, their antigen-specificity is rather low and unchanging.

B cells that commit to the GC fate migrate back to the center of the follicle where, with the help of Tfh cells and follicular dendritic cells (FDCs), establish a GC[32,33]. Within GCs, B cells undergo proliferation, affinity maturation, and CSR, leaving them as plasma or memory B cells with high affinity to the antigen.

Some published studies seem to indicate that the GC output is not plasma cells but rather plasmablasts not fully differentiated[34,35]. Then, these cells home mainly to the bone marrow, where they finally differentiate into long-lived plasma cells which have the capacity of sustaining a high level of antibody secretion for long periods of time[36-38].

Memory B cells are characterized by their longevity and the capacity to rapidly and robustly respond to antigen re-exposure. These properties are one of the basis of vaccine success. IgM and IgG memory B cells, from the extrafollicular and GC responses, respectively, are the most studied populations within the memory B cell compartment. These two populations seem to have different properties and roles on secondary responses. IgM memory B cells, not having undergone affinity maturation and CSR, have a more diverse specificity, are able to persist longer in the organism, and are more prone to proliferate and join the new GC response. IgG^+ cells memory B cells, with high affinity to the antigen, readily differentiate into antibody secreting plasmablasts, allowing a rapid response upon antigen rechallenge[39,40].

Germinal centers

GCs are specialized structures where antibody affinity maturation and CSR occur[41-45]. The affinity maturation is the process that allows the improvement of antibody affinity over time during an immune response. This is accomplished through rounds of somatic hypermutation (SHM) of the V gene segment and selection of B cell clones with mutations that successfully improve their affinity to the antigen[41,42,45].

As described before, follicular B cells migrate into the T-B border upon activation, where they engage in cognate interactions with pre-Tfh cells[46,47] (Figure 3). These long-lived interactions ultimately lead to commitment to the GC pathway of the B cells with higher affinity to the antigen, and consequent changes in their transcriptional profile[48-51]. Genes like interferon-regulatory factor 4 (IRF4), MYC, B cell lymphoma 6 (BCL-6), and myeloid cell leukemia 1 (MCL1) are critical for GC B cells generation, and their upregulation occurs during the early initiation phase until day 3 of response. While some genes are key during early activation and/or late differentiation and their expression is only observed during that time (as is the case of IRF4[52,53]), other genes need to be expressed throughout the whole GC reaction. One of them is BCL-6 that acts as a transcription repressor and is a key regulator of the GC B cell phenotype[54-56]: it is responsible for enabling the migration of B cells back to the follicle after commitment to the GC pathway by downregulation of Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2) and sphingosine-1-phosphate receptor 1 (S1PR1) that are responsible for localization at the T-B border[32,33,57,58]; BCL-6 induces an pro-apoptotic state, through the silencing of BCL-2, that will lead to the deletion of low affinity or autoreactive clones[59,60]; BCL-6 induces cell tolerance to DNA damage originated from AID activity and rapid proliferation by downregulating p53 and ATR[61,62]; BCL-6 regulates the expression of positive signaling mediators to allow a fine tune selection of high affinity BCRs[63,64]; and BCL-6 is involved in regulating plasma cell differentiation through the downregulation of BLIMP-1[63].

Thus, three to four days after initial activation by antigen encounter, GC B cell precursors, upon expression of BCL-6, are able to reverse their migratory properties and migrate back into the center of the B cell follicle. There, B cells form an early GC (already observable by microscopy) in-between a pre-existing network of C-X-C chemokine ligand 13 (CXCL13) secreting FDCs[65,66]. The GC B cell population has high expansion rates, and consequently a rapid increase in size of the GC occurs and leads to the formation of the B cell mantle compartment. This mantle is formed by the naïve B cells of the follicle that are pushed away by the GC[67]. Seven days after primary immunization, GCs have substantially increased in

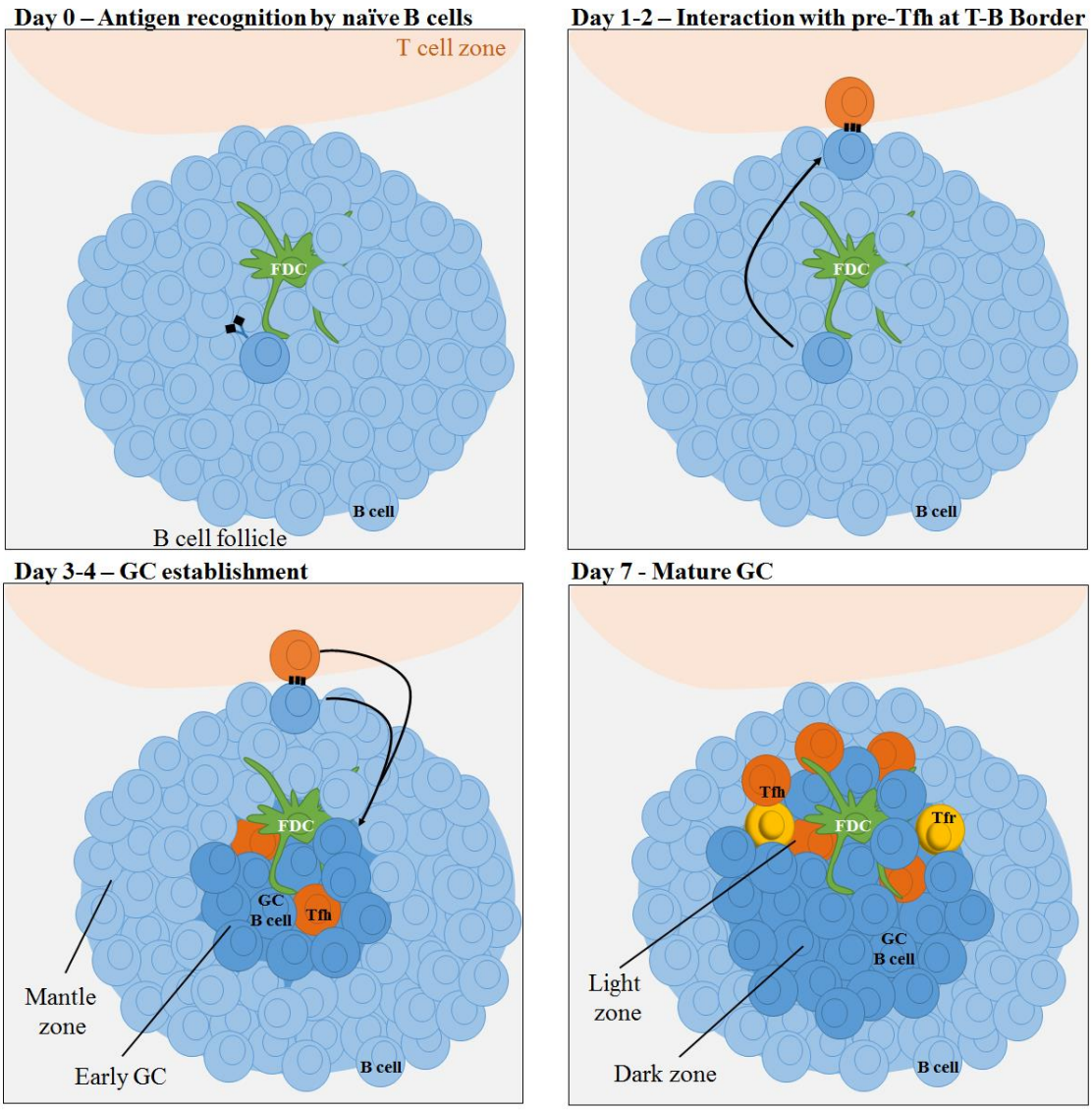

Figure 3 – GC initiation and development.
GC responses are initiated by antigen recognition by B cells within the follicle. Within 1-2 days after activation, B cells migrate into the T-B border where they engage in cognate interactions with pre-Tfh cells. Upon commitment to the GC program, Tfh and GC B cells migrate into the center of the follicle where an early GC establishes in between a network of FDCs. The formation of the GC pushes away pre-exiting naïve B cells which form the mantle zone. At day 7, the now mature GC has increased in size due to fast cell proliferation and it can be divided in two zones: the light zone composed by GC B cells, Tfh, Tfr, FDCs and other cell types, while the dark zone is mainly composed by rapidly dividing B cells.

size and are now denominated mature GCs[67-69]. Moreover, they can be easily divided in two regions: the light and dark zones (LZ and DZ, respectively)[70]. The light zone is populated by B cells, FDCs, macrophages, Tfh cells, follicular regulatory T (Tfr) cells, and other cell types[68]. FDCs are found not only on GCs, but also on primary follicles, and their main functions during a GC reaction are to present unprocessed antigen on their surface and secrete cytokines important for GC maintenance like IL-6 and the CXCL13 chemokine[65,71-

[73]. Tingible-body macrophages are responsible for eliminating apoptotic B cells, thus preventing the accumulation of self-antigens which can lead to the generation and selection of autoreactive clones[74-77]. The dark zone is only densely populated with B cells among a network of CXCL-12 producing reticular cells (morphologically similar to FDCs)[70].

During the maturation process undergone on the GC, B cells repeatedly migrate between dark and light zones. This migration is enabled by the chemokine receptors C-X-C chemokine receptor 4 (CXCR4) and CXCR5: centrocytes express CXCR5 and are found in the CXCL13 rich LZ, while centroblasts that also express CXCR4 are localized in the DZ where CXCL12 is abundant[78]. Within the DZ, GC B cells proliferate and undergo SHM that introduces point mutations in the rearranged V gene[41,42,67,79]. As for CSR, SHM is also mediated by the action of AID[23]. As result, a high number of cells with a vast range of affinities for the antigen arise. B cells then migrate into the LZ for selection by Tfh cells[68]. B cells that have successfully gained high affinity to the antigen receive positive signals, perform CSR, and differentiate into memory B cells or plasma cells before leaving the GC. B cells that have only moderately increased the affinity of their BCR to the antigen also receive positive signals, may also undergo CSR, and are able to migrate back into the DZ to undergo a new round of SHM to increase their affinity to the antigen. B cells that have lost their capability to recognize the antigen, and therefore are not able receive positive signals, are eliminated by apoptosis[69].

The signals that trigger CSR are still poorly understood[69]. Nevertheless, the type of ongoing response and the signature cytokines of Th1, Th2, and Th17 responses determine the class of Ig to be produced[80-82]. IL-4 leads to the secretion of IgG1 and IgE, while IFNγ promotes IgG2a production. Published data so far favor the hypothesis that these cytokines are produced in situ by Tfh which can produce low levels of IL-4 or IFNγ[83].

The affinity maturation process is dependent on Tfh signals[79,84]. High affinity B cells in the LZ capture more antigen from FDCs (compared to cells with low affinity BCRs), and consequently perform more MHC-TCR interactions with Tfh cells that ultimately lead to positive selection signals[79,85]. Moreover, from the pool of B cells that are selected to re-enter the DZ, the ones with higher affinities receive stronger signals from Tfh and present higher proliferation rates after migrating back into the DZ[86]. Additionally, the changes of antigen availability on FDCs during a GC reaction also ensure the increase in affinity of the B cells leaving the GC overtime[87]. Antibodies produced by plasma cells originated early from the GC will coat the antigen deposited in FDC. Thus, only GC B cells with higher affinity to the antigen will be able to overcome this competition and acquire antigen to present to Tfh cells.

Since antibodies with increasing affinities are gradually produced, the level affinity required also increases over time[87]. Although SHM allows the generation of high affinity antibodies, it may also give rise to GC B cells with self-reactive BCRs that must be deleted to prevent the onset of auto-antibody mediate autoimmunity[88,89].

The signals that lead GC B cells to differentiate into memory B cells or plasma cells are still not very well understood. Nevertheless, differentiation of GC B cells into plasma cells or memory B cells seems to be time dependent[90]. Memory B cells, that present lower affinities and number of somatic mutation, are mostly generated first while differentiation into plasma only occurs at later stages, long after GCs peak in size.

T cells

T cells play a central role in adaptive immune responses since they are not only responsible for cytotoxic function, but are responsible for orchestrating the response by providing help to other cells. This functional duality is accomplished by the existence of two T cells populations: CD8 T cells and CD4 T cells, respectively. The designation "T cell" originated from the fact that its precursors leave the bone marrow and their differentiation occurs in the thymus.

As for B cells, a key feature of T cells is their specialized receptor that can recognize a wide range of antigens. However, the TCR is not able to bind and recognize free antigens in their natural structure, but rather small peptides presented on MHC molecules[91]. The TCR is constituted by two chains which are also product of somatic V(D)J recombination (see Figure 4)[91]. The majority of T cells expresses the αβ TCR that is composed by α and β chains. Nevertheless, there are also γδ T cells, which TCR is composed by γ and δ chains and have a more restrict repertoire, and natural killer T (NKT) cells that, although express an αβ TCR receptor, their TCR is considered invariant (Vα24Jα18 combines with a limited TCRβ repertoire in the case of type-1 NKTs) and recognizes hydrophobic antigens such as glycolipids[91-93].

The TCR recombination process is performed in the same manner as the one described for the BCR recombination: a V, a D, and a J segments, in the case of the β chain, are cut and spliced together by the action of the same enzymes (RAG-1, RAG-2, and TDT); while in the case of the α chain, as there are no D segments, the recombination consists only in the junction of a V and a J segments (Figure 4). Again, besides the inaccuracy of the recombination process, the action of the enzyme desoxyribonucleotidyltransferase can insert

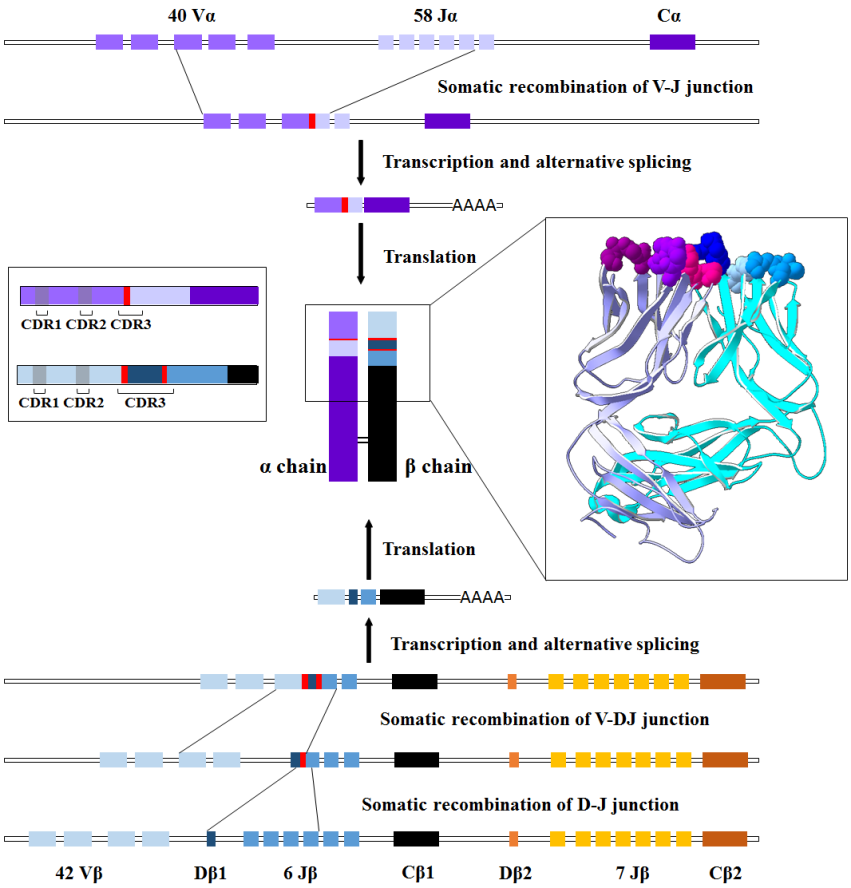

Figure 4 – V(D)J recombination of the TCR.
As for the BCR, the TCR is also the result of V(D)J recombination. In the case of the α chain, there is the recombination of a J segment with a V segment, while for the β chain a D segment is recombined with a J followed by the recombination with a V. The three CDRs are the binding region of the TCR that not only recognize the peptide presented by a MHC molecule but also the MHC molecule itself. The three CDR3 regions are represented as spheres in violet (α chain CDRs) and blue (β chain CDRs) in the three dimensional representation in the box on the right.

nucleotides in the coding sequence and further contributes for the high variability of the TCR repertoire.

Consequently, the CDR3, that comprises the segment junctions, may have different CDR3 lengths due to frameshift in the coding sequence. TCR specificity cannot be altered once the T cell finishes its receptor recombination, contrary to what is observed for the BCR that can improve its affinity to the antigen after the initial development by somatic hypermutation in GCs. Also, the TCR complex is not only composed by the αβ chains module (that recognize the peptides presented on MHC molecules) but also by invariant polypeptide chains responsible for intracellular signal-transmission, the CD3 module: CD3ε, CD3γ, CD3δ, and ζ.

There are several fate decision steps important in T cell development. The first occurs when committed lymphoid progenitors, which arise in the bone marrow, migrate into the thymus, lose the potential to develop as B cells or NK cells, and become committed T cells precursors. A second fate decision occurs when thymocytes start expressing a rearranged TCRβ chain and lose the potential to differentiate into γδ T cells.

Upon successful rearrangement of the α and β chains, thymocytes start co-expressing CD4 and CD8, thus becoming double positive (DP) thymocytes. As DP thymocytes already express a mature αβ TCR, the next steps of differentiation and fate decision involve the selection of the TCRs that, based on their affinity, can leave the thymus and are capable of participating in immune responses without triggering responses against the self. For that, the selection comprises 3 distinguishable processes: death by neglect, negative selection, and positive selection[94]. Thymocytes bearing TCRs that interact poorly with self-antigens-MHC complexes, and consequently are unable to induce intracellular signaling required for survival, suffer death by neglect. On the contrary, cells with TCRs that present very high affinity to self-antigens-MHC complexes are instructed to commit apoptotic cell death. This negative selection process prevents potential T cells that could cause autoimmune pathology from leaving the thymus, although some self-reactive T cells escape deletion[95]. DP thymocytes that generate intercellular signaling levels between death by neglect and negative selection initiate the multi-step positive selection.

The last important fate decision involves the CD4- or CD8-lineage commitment. This differentiation step comprises the passage from DP to single-positive (SP) state ($CD4^+CD8^-$ or $CD8^+CD4^-$) by silencing the transcription of one co-receptor locus[96,97]. CD8 and CD4 co-receptors are specific to class-I and class-II MHC molecules (MHC-I and MHC-II), respectively. Therefore, their expression restricts the interaction of the TCR to only one of the MHC molecules, and consequently establish the function of the cells when they leave the thymus. The selection of either CD4 or CD8 as co-receptor is determined by TCR affinity to self-peptides presented by either MHC-I or MHC-II molecules, as well as for the MHC molecule itself. This dichotomy on CD4 or CD8 expression, which results on T cell ability to recognize peptides presented by either MHC-I or MHC-II molecules and consequent associated function, is of high importance, since the two MHC molecules present peptides of different origins. MHC-I molecules, which are expressed by almost all nucleated cells, present peptides of intracellular (cytosolic and nuclear) origin to alert CD8 T cells for intracellular alterations and target cells for destruction. On the other hand, MHC-II molecules are only constitutively expressed on professional APCs (although their expression

can be induced in other cell types by IFNγ[98-100]) and present peptides derived from exogenous proteins degraded in the endocytic pathway. These peptides are then presented to CD4 T cells which in turn orchestrate intercellular immune responses. Nonetheless, there is a link between the two pathways (named cross-presentation) that allows exogenous peptides to be presented on MHC-I molecules and endogenous molecules on MHC-II molecules[101,102]. Taken all together, the commitment to CD4- or CD8-lineage depends on the capability of TCR to interact with MHC-II or MHC-I molecules in the thymus. This commitment then determines the function to be performed by the cell in the periphery.
In the end of the development, mature CD4 and CD8 T cells, as well as γδ T cells and NKT cells, that have been positively selected, leave the thymus and migrate into secondary lymphoid organs.

CD8 T cells

CD8 T cells mediate cellular immunity by detecting infected or altered cells and inducing cell-mediated lysis, and these functions are primarily dependent on TCR recognition of antigens presented on MHC-I molecules.
Naïve CD8 T cells are initially primed on secondary lymphoid organs such as draining LNs and spleen. Priming is preferentially performed by DCs and occurs on peripheral regions enriched for antigens during early immune responses[97,103]. Upon activation, CD8 T cells with antigen-specific TCRs start producing IFNγ and rapidly expand[104-106]. This initial activation and expansion is not only dependent on TCR affinity but also on co-stimulatory signals (e.g., OX40 and CD27) and the presence of inflammatory cytokines like IL-12 and IFNα[107,108]. However, upon activation, antigen recognition by the TCR is sufficient to trigger CD8 T cells to kill altered cells. After activation in central lymphoid organs, CD8 T cells upregulate CXCR3 and migrate into site of ongoing response, where they continue to be stimulated by interacting with altered cells, resulting in cytolysis of those cells, and further inducing CD8 T cells proliferation[109]. Of note, in most types of responses, CD8 T cells initial activation by DCs and recruitment into infection site requires help from CD4 T cells[110]. Another important cytokine for CD8 T cells biology is IL-2. This cytokine, mainly produced by CD4 T cells, has not only an impact in the proliferation and cytolytic capacity of CD8 T cells but also regulates the formation of memory CD8 T cells[111].
CD8 T cells use two different mechanisms to induce cell death on target cells, namely the perforin/granzyme-mediated apoptosis[112] and activation-induced cell death (AICD)[113], and

both are initiated upon antigen recognition and consequent TCR signaling. The first mechanism involves the secretion close to the target cell of granzymes and perforin that are stored in lysosomes within CD8 T cells. Perforin can either form pores on the plasma membrane of target cells or mediate endocytosis to allow granzyme entrance inside the cells and induce apoptosis. The AICD is dependent on the FAS ligand (FASL)-FAS interaction: the binding of the FASL on the CD8 T cell to the FAS receptor induces dead on the target cell by initiating a caspase dependent apoptosis process through the intracellular death domain of FAS.

Besides inducing cell death to target cells, CD8 T cells also produce cytokines like IFNγ and IL-10. IFNγ further induces CD8 T cells proliferation and function, acting a feed forward loop. On the contrary, the immune suppressive cytokine IL-10 has a regulatory function and is essential to avoid excessive tissue injury and consequently prevent autoimmune pathology during a viral infection[114,115].

CD4 T cells

CD4 T cells are responsible to support immune responses by activating innate immune cells, B cells, CD8 T cells as well as non-immune cells. Moreover, CD4 T cells are also responsible for regulating and suppressing immune reactions. To be able to perform all these functions, naïve CD4 T cells can differentiate into different effector subpopulations depending on the signals provided by cells of the innate immune system at the time of T cell activation (See Table 1). These signals will in turn depend on the PRRs triggered on the DCs, and consequently the type of immune response that needs to be mounted. The duality of responses against intracellular and extracellular pathogens lead to the early discovery of type 1 T helper (Th1) and type 2 T helper (Th2) responses[116]. Since then, more differentiation subsets of CD4 T cells have been identified: Th17, Tfh, Th9, and Th22 cells that are involved in inflammatory responses, and forkhead box P3 positive ($FOXP3^{+}$) regulatory T (Treg) and class 1 regulatory T (Tr1) cells that are engaged in immune suppression.

For a naïve CD4 T cell to differentiate into any of these subsets it is necessary an initial activation step that includes TCR recognition and co-stimulation signals. TCR recognition occurs when a naïve CD4 T cell is able to specifically recognize a peptide presented by an activated APC on a MHC-II molecule. The strength of the TCR stimulation depends on the affinity of the TCR to the molecule, and may also have an impact on the lineage commitment[117,118]. For a CD4 T cell to get fully activated, co-stimulation signals are also

Table 1 – CD4 T cell subsets and its characteristics.

CD4 Subset	Differentiation signals	Key regulators	Cytokines produced	Surface markers
Th1	IL-12, IFNγ	T-BET, STAT1, STAT4	IFNγ, Lfα, IL-2	$CXCR3^+IFN\gamma R^+$
Th2	IL-4, IL-2	GATA3, STAT6, STAT5, STAT3, GFI-1, IRF4	IL-4, IL-5, IL-13, IL-25	$CCR4^+CCR6^-ST2^+$
Th17	IL-6, TGFβ, IL-21, IL-23	RORγt, RORα, STAT3	IL-17A, IL-17F, IL-21, IL-22	$CCR4^+CCR6^+$
Th9	IL-4, TGFβ	IRF4, PU.1	IL-9	$CCR4^-$ $CCR6^+$
Th22	IL-6, TNFα, IL-1β	AHR	IL-22, IL-13, TNFα	$CCR4^+CCR6^+CCR10^+$
Tfh	IL-6, IL-21	BCL-6, ASCL2, c-MAF, BATF, IRF4, STAT3, STAT1	IL-21, IL-4	$CXCR5^+PD1^+ICOS^+$
pTreg	TGFβ, IL-2	FOXP3, Smad2, Smad3, STAT5	IL-10, TGFβ	$CD25^+CTLA\text{-}4^+$ $GITR^+$
Tr1	IL-27, IL-10, IL-6	c-MAF, AHR	IL-10	$CD49b^+LAG\text{-}3^+$

required. These signals are provided by the DC that expresses CD80/CD86, inducible T cell co-stimulatory ligand (ICOSL), and OX40L, and provides positive signals through interaction with the CD4 T cells co-stimulatory receptors CD28, ICOS, and OX40, respectively. The last factors that determine the differentiation into a specific lineage are the cytokines produced by the DCs, which in turn depend on the PRRs triggered on the DC (see Table 1).

Th1 cells

Th1 cells are important for the protection against obligate intracellular pathogens, such as intracellular bacteria and viruses, as well as in immune response against tumors. Th1 differentiation is promoted by IL-12 and IFNγ, together with TCR signaling. While IL-12 is highly secreted by APCs upon activation through their PRR, IFNγ is produced by NK cells when exposed to IL-12[119,120].

The master regulator of Th1 differentiation is the T-box transcription factor (T-BET), which activates genes necessary for the differentiation and is also capable of suppressing the differentiation into other cell lineages. T-BET expression is dependent on another transcription factor, the signal transducer and activator 1 (STAT1) which expression is upregulated by IFNγ[121,122]. Since T-BET induces high levels of IFNγ expression, a feed forward loop is established to ensure selective expansion and differentiation of Th1 cells[123]. Another important axis of Th1 differentiation is the IL-12-STAT4 pathway. IL-12 induces STAT4 which in turn activates IFNγ expression[124]. Since high levels of T-BET lead to the upregulation of IL-12 receptor β (IL-12Rβ) expression, Th1 differentiation is further

enhanced[122]. On the other hand, T-BET suppresses Th2 and Th17 lineages by impairing the function of their master regulators GATA3 and retinoic acid receptor-related orphan receptor gamma-T (RORγt), respectively[125,126]. Th2 development is further suppressed by inhibiting IL-4 expression[127].

Upon full differentiation, Th1 cells main function is to produce cytokines that mount and boost an intracellular immune response. The two main cytokines produced are IFNγ that activates phagocytes, like macrophages, to increase their phagocytic activity, and IL-2 that, besides inducing proliferation and acquisition of cytolytic phenotype by $CD8^+$ T cells, is required for the development of memory $CD8^+$ T cells[128].

Although the pro-inflammatory action of Th1 cells is of maximum importance for clearance of intracellular pathogens and tumors, their unregulated action can cause unwanted inflammatory diseases and self-reactivity. Th1 cells and their IFNγ production have been implicated in inflammatory diseases like inflammatory bowel disease (IBD), transplant rejection, graft-versus-host disease, and autoimmune diseases as type-1 diabetes and rheumatoid arthritis (RA)[129-133].

Th2 cells

Th2 cells are involved in responses to extracellular parasites, including helminthes and nematodes, as well as in mucosal immunity of the lung, through the production of IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, and amphiregulin.

Besides TCR recognition and initial activation, IL-4 and IL-2 are critical for a naïve CD4 T cell to follow the differentiation pathway to a Th2 cell. IL-4, the positive feedback cytokine for Th2 differentiation, induces STAT6 expression which in turn upregulates GATA3, the Th2 master regulator[134-137]. GATA3 is indispensable for Th2 response and it has been postulated that its action is mediated through three different mechanisms: (1) enhances Th2 cytokines production, (2) induces Th2 cells proliferation, and (3) suppresses Th1 differentiation by interacting with T-BET and suppressing STAT4 expression[137,138]. STAT6 is also important for the full Th2 differentiation as GATA3 does not directly regulate all Th2-realted genes[139]. On the other hand, STAT6 interaction with several of those genes loci is in turn dependent on the presence of STAT3[140]. STAT5 is another key transcription factor for this differentiation pathway. STAT5 is activated by IL-2 signaling and in coordinated activity with GATA-3 induces IL-4 expression by Th2 cells[141,142]. Growth factor independent-1 (GFI-1), a transcriptional repressor which expression is induced by TCR signaling alone or the IL-4-STAT6 pathway, selectively leads to Th2 expansion since it

induces proliferation on GATA3 high expressing cells[143]. IRF4 is another transcription factor that also has an impact on Th2 differentiation by activating the IL-4 promoter and upregulating GATA3 expression[144,145].

During an ongoing immune response against extracellular pathogens, Th2 key effector cytokines act mainly upon innate cells. IL-4 induces the expression of IgE receptors: $Fc_{\varepsilon}RI$ on macrophages and B cells, while $Fc_{\varepsilon}RII$ expression is induced on basophils and mast cells[146]. Also, IL-4 induces lung mucus hypersecretion and secretion of inflammatory cytokines by lung fibroblasts[147,148]. Eosinophils and its precursors depend on IL-5 signaling for activation, recruitment, and apoptosis inhibition[149,150]. IL-9, firstly described as produced by Th2 cells, functionally activates several immune cell types such as B cells, neutrophils, eosinophils, and mast cells, while inducing mucin and chemoattractant factors production by airway epithelial cells[151]. IL-13 is involved in the elimination of gastrointestinal helminthes through the induction of mucus secretion and the increase of intestinal fluid content and intestinal contractility, and lung inflammatory responses by eosinophils activation and enhanced mucus secretion, as IL-4. Indeed, IL-4 and IL-13 seem to have redundant effects during Th2 responses. IL-25, a member of the IL-17 cytokines family, promotes Th2 responses by inducing IL-4, IL-5, and IL-13 production by a non-lymphocyte population[152]. Amphiregulin induces epithelial cell proliferation and is also involved in nematode expulsion[153]. Th2 cells also produce the anti-inflammatory cytokine IL-10 that, in this case, suppress Th1 proliferation and DC function[154,155].

Moreover, and contrary to what is observed in Th1, Th2 responses are characterized by the production of high levels of specific immunoglobulins that neutralize foreign organisms. IL-4 and IL-13 production during this type of immune response have been implicated in inducing CSR and secretion of IgM, IgG_1, IgE, and IgA antibodies.

Dysregulation of Th2 responses are responsible for allergic diseases, especially airway allergic diseases such as persistent asthma. IL-4, IL-13, and IL-9 have been implicated and play major roles in the induction and the immuno-pathogenesis of asthma.

Th17 cells

Th17 cells were the first CD4 T cells independent subset described after Th1 and Th2 discovery[156]. Th17 cells are involved in immune responses against fungi and extracellular bacteria and its action is mediated through the secretion of IL-17a, IL-17f, IL21, and IL-22. However, even before the discovery of Th17 cells, IL-17 production had already been

implicated in the generation of several autoimmune diseases like multiple sclerosis (MS), RA, psoriasis, and IBD[157-159].

Th17 differentiation is divided in three steps which in turn depend on different cytokines. Th17 differentiation is initiated in the presence of IL-6 and low levels of transforming growth factor beta (TGFβ) that induce the expression of RORγt[160], the master regulator of this subset, as well as IL-21 and IL-23R[161-163]. In turn, the expression of RORγt induces the production of IL-17a and IL-17f[164]. However, RORγt is not able to fully commit into Th17 differentiation, and several other transcription factors need to be present. That is the case of STAT3, which is activated in the presence of IL-6, IL-21, and IL-23, and that induces RORγt, IL-17a, and IL-17f expression[165,166]. Another transcription factor critical in this lineage is the retinoic acid receptor-related orphan receptor alpha (RORα), that also belongs to the ROR family of transcription factors, and Th17 development is aborted in its absence[164]. The basic leucine zipper transcription factor (BATF) and IRF4 have also been implicated in Th17 differentiation, since knockout mice for each of the transcription factors have impaired Th17 responses[167,168]. The second step, the self-amplification phase is dependent of TGFβ and IL-21. Contrary to what occurs in Th1 and Th2 differentiations, the Th17 main cytokines IL-17a and IL-17f do not act as amplifying signals. Therefore, the Th17 amplification occurs by the action of IL-21, produced by Th17 cells[169,170]. The last phase that consists on the expansion and maintenance of the lineage is dependent on IL-23 that is mainly produced by APCs[161,171].

The use of IL-17RA chain for signaling by IL-17a and IL-17f indicates similar function by both cytokines, although IL-17a presents a higher affinity for the receptor[172,173]. Both IL-17a and IL-17f activate and recruit neutrophils. IL-17a can also induce inflammatory responses by inducing inflammatory cytokines, such as IL-6 and IL-1, and chemokines, such as CXCL8, that ensure chemotaxis of inflammatory cells to inflammation sites[174]. IL-21 has a large specter of target cells, activating CD8 T cells, NK cells, and DCs, and inducing B cells differentiation[175]. IL-22 targets non-immune cells and induces the production of anti-bacterial peptides and proteins that differ depending on the target cell. In the mucosa, where IL-17 producing cells have critical roles in host defense against pathogens, IL-22 has been described as a mediator of mucosal host defense against bacterial pathogens[176,177]. Furthermore, intestinal Th17 cells have the capacity to differentiate into Tfh cells and play a major role in the development of host-protective IgA responses[178].

Th9 cells

Th9 cells are an IL-9 and IL-10 producing CD4 effector T cell subset that seem to have protective roles in melanoma tumors and intestinal parasitic infections, while having a pathogenic effect in some diseases like allergy, colitis, and experimental autoimmune encephalomyelitis (EAE)[179,180]. Th9 differentiation is dependent on IL-2, TGFβ, and IL-4[179-183]. IL-2R signaling induces STAT5 which in turn binds *Il9* gene locus[184,185]. TGFβ inhibits Th1 transcription factor T-BET and induces PU.1 expression that promotes Th9 differentiation[183,186-188]. As for Th2 differentiation, IL-4R signaling leads to STAT6 activation, which induces *Gata3* and *Irf4* expression that are essential for both Th2 and Th9 differentiation[134-137,145,180,189]. Moreover, IRF4 directly binds the *Il9* promoter and seems to play a role on Th9 differentiation, even though it is also involved in Th2 and Th17 differentiation[145,168,189]. The contribution of Th9 cells to IL-9 production and the fact that Th9 cells are discrete Th cells subset are still matter of debate. For once, Th9 cells seem to be very plastic and easily change their differentiation profiles[190,191]. Also, even though IL-4 and IL-9 seems not to be produced by the same cell, other cell types, namely Th17, peripherally-derived Treg (pTreg) cells, and innate lymphoid cells (ILCs) are also able to produce IL-9[179,189,192-195]. Indeed, a recent report showed that, in a model of mouse lung inflammation, ILCs and not Th9 cells were responsible for IL-9 production[195]. Furthermore, a specific master transcription factor of this lineage as not been found yet.

Th22 cells

Th22 cells were identified in humans as an IL-22 producing CD4 T cell lineage, independent of Th17 cells, and implicated in epithermal immunity[196-198]. Th22 cells also produce low levels of TNFα and IL-13, while no production of IFN, IL-4, and IL-17 could be observed[196-198]. Their differentiation occurs in the presence of IL-6 and tumor necrosis factor-α (TNFα) and is further promoted by IL-1β[197]. The transcription factor aryl hydrocarbon receptor (AHR) has been associated with this lineage, but whether it is the master regulator of this lineage is still controversial[196,197]. Th22 cells express not only the chemokine receptor CCR6 but also CCR10 and CCR4 that allow them to locate in the skin[196-198]. Therefore, these cells not only seem to play a role in host defense against some pathogens but also have been implicated in skin inflammation diseases, such as psoriasis, atopic dermatitis, and allergic contact dermatitis[199,200]. Their action is mediated by IL-22 which targets non-immune cells and induces the production of different anti-bacterial peptides and proteins depending on the area of the body[176,177,201]. In keratinocytes, IL-22 promotes the expression of β-defensin 2,

β-defensin 3, and lipocalin 2 among other anti-bacterial molecules[201]. Nevertheless, further studies are necessary to confirm if Th22 cells are indeed an independent CD4 T cell subset: the signaling pathways involved in this differentiation are not very clear and, though Th22 have been implicated on several diseases, the contribution of IL-22 produced by this subset still needs to be evaluated.

Follicular helper T cells

Although the first studies showing the requirement of T cell help for antibody mediated B cell responses date back to the 1960s, only in 1999-2000 the first reports were published showing that Tfh cells were an independent CD4 T cells subset that expressed $CXCR5^+$ and were able to migrate into the GC and give help to B cells to produce antibodies. Moreover, CXCR5 was described as the chemokine receptor that allowed the migration of those CD4 T cells into B cell follicles where its ligand CXCL13 is produced[202-204]. The confirmation that Tfh cells were an independent subset of CD4 cells came in 2009 with identification of its master regulator BCL-6[205-210]. Since then, further studies have shown that Tfh differentiation is a complex multi-step process, tightly controlled, with many transcriptional and post-transcriptional mechanisms involved[211] (Figure 5).

Initial priming by dendritic cells

The initial priming of naïve CD4 T cell to become a Tfh is given by DCs, while B cells are involved in the second differentiation step to fully induce the Tfh differentiation[212-215]. This initial activation by DCs is dependent on TCR recognition of antigen presented by MHC-II, co-stimulation through CD28-CD80/CD86 and ICOS-ICOSL, and secretion of cytokines such as IL-6[212-214,216-218]. Co-stimulation through OX-40-OX40L also seems to further induce Tfh differentiation but its signals are not mandatory[219,220]. After the first antigen encounter, activated CD4 T cells undergo a few rounds of division and undertake a cell-fate decision to become pre-Tfh upon expression of BCL-6[211].

The transcription repressor BCL-6 is the master regulator of Tfh differentiation by not only regulating genes required for Tfh differentiation, migration, and function, but also by directly repressing the Tfh differentiation antagonist BLIMP1 as well as promoters and enhancers involved in the differentiation into other CD4 subsets (*Tbx21*, *Gata3*, *Rorα*, etc.)[208-210,221]. BCL-6 directly or indirectly regulates the expression of receptors involved in T cell migration: it upregulates CXCR5 which expression allows migration towards the B cell follicle, while it downregulates CCR7 and P-selectin glycoprotein ligand 1 (PSGL-1) that

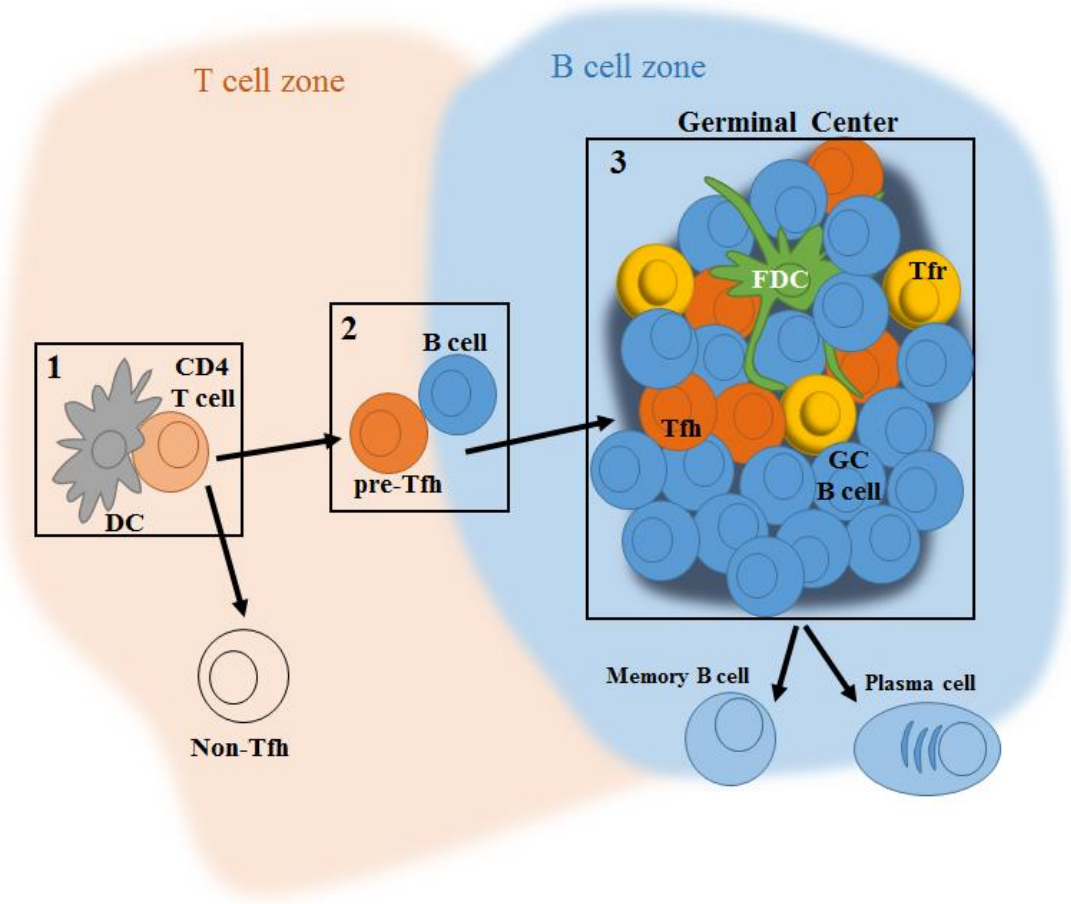

Figure 5 – Tfh differentiation steps.
The Tfh differentiation is a multi-step process. The first step involves CD4 T cell priming by DCs in the T cell zone where some cells pre-commit to the Tfh program and migrate into the T-B border, while other differentiate into other Th subsets (1). During the second step, pre-Tfh engage in cognate interactions with pre-activated B cells and full commit to the Tfh differentiation (2). In the last step, committed Tfh cells migrate into the GC within the B cell zone where they give help to GC B cells and in turn continue to receive signals to survive and maintain the differentiation program.

bind CCL19 and CCL21 produced in the T cell zone and lead T cells to stay in the T cell zone[222]. Furthermore, BCL-6 also downregulates S1PR1, expressed on activated T cells that binds S1P and leads cells to migrate into the lymph and blood, and EBI2 that reduces CXCR5 affinity to CXCL13 by dimerization with CXCR5 while its ligand is abundant in outer follicular regions thus preventing migration into B cell follicles[222]. BCL-6 expression is triggered upon CD28 signaling and cytokines signaling, mainly IL-6 and IL-21 which induce STAT1 and STAT3 activation[208,218,223,224]. Indeed, IL-6 is able to induce an early expression of BCL-6[218]. However, BCL-6 is also expressed in its absence which indicates that IL-6 has an important, but not essential, role in the initial differentiation of Tfh cells, probably due to signaling overlap between IL-6 and IL-21[225,226].

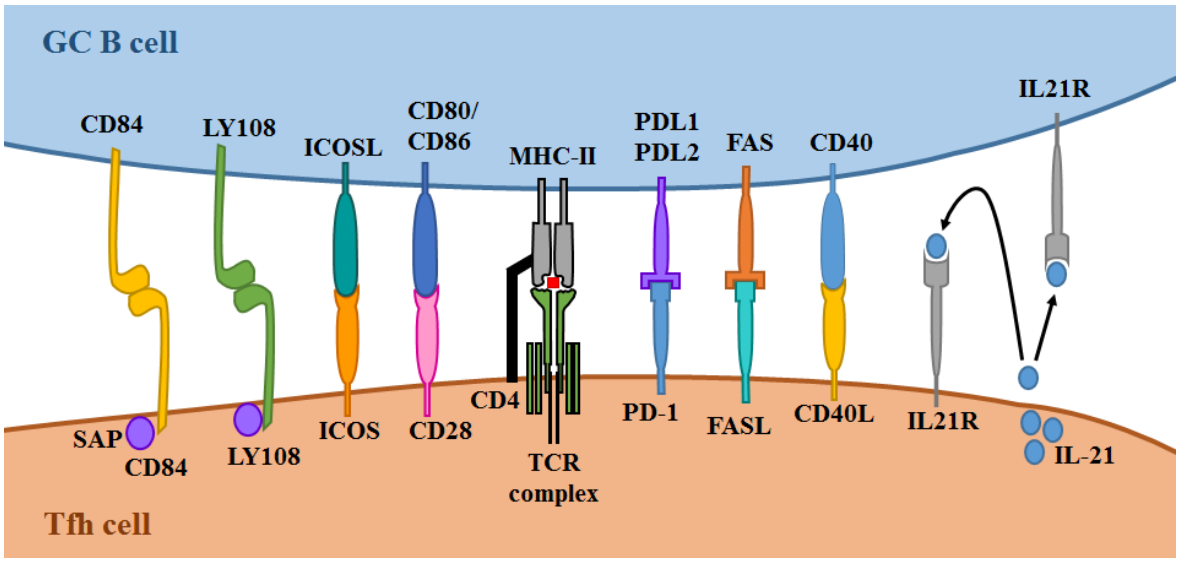

Figure 6 – Tfh-B cell signals.
Tfh and B cells engage in long-lived interactions at the T-B border and within the GC. Besides antigen presentation and recognition by TCR and MHC-II interactions, Tfh cells receive positive signals through CD28-CD80/CD86, ICOS-ICOSL, and CD84-CD84. On the contrary, Tfh receive negative signals from LY108-LY108 and PD-1-PDL1/PDL2 interactions. B cells receive positive signaling through CD40-CD40L and apoptotic signals through FAS-FASL. Lastly, Tfh cells produce IL-21 that stimulates both Tfh and B cells.

Besides BCL-6, which expression is imperative for Tfh differentiation, ASCL2 is another transcription factor which expression is also important at early stages of differentiation[227]. ASCL2 expression occurs before BCL-6 and is key to initially regulate genes involved in cell migration: it upregulates CXCR5 and downregulates CCR7 and PSGL-1[227]. Moreover, ASCL2 also represses expression of Th1-associated genes, such as *Ifng* and *Tbx21*[227].
More recently, two other transcription factors have been involved in the early steps of Tfh differentiation: LEF-1 and TCF-1[228-230]. As for ASCL2, their expression occur prior to BCL-6 and they regulate expression of molecules important for Tfh differentiation such as BCL-6, IL-6R, ICOS, BLIMP1, and IL-2R[228-230].
Ultimately, expression of BCL-6 leads $CXCR5^{+}$ pre-Tfh cells to migrate into the T-B border to receive further stimulation from B cells.

Full commitment to the Tfh program at the T-B border

After migrating into the T-B border, pre-Tfh cells encounter pre-activated B cells and engage in long-lived interactions that ultimately lead pre-Tfh and some B cells to definitely commit to the GC pathway and to become Tfh cells and GC B cells[211] (Figure 6). These interactions between pre-Tfh and pre-activated B cells include TCR recognition of antigen presented by MHC-II and co-stimulation through ICOS-ICOSL and CD28-CD80/CD86 in a manner similar to the ones observed between T cells and DCs[212].

ICOS-ICOSL interactions are very important for Tfh commitment[231]. ICOS signaling, mediated by phosphoinositide 3-kinase (PI3K), induces c-MAF and consequently IL-21 production[232,233]. In turn, IL-21 has an autocrine effect and, by signaling through the IL-21R, further activates STAT3 and STAT1, creating a positive loop that stabilizes the Tfh differentiation[224,234]. Additionally, ICOS signaling also targets FOXO1 for degradation which in turn no longer represses *Bcl-6* expression or activates Krüppel-like factor 2 (*Klf2*) expression[223,235]. Consequently, KLF2, which is highly expressed on naïve CD4 T cells, no longer promotes expression of receptors against migration into the B cell zone (CCR7, PSGL-1, S1PR1) or genes involved in other CD4 subsets differentiation (*Prdm1*, *Tbx21*, and *Gata3*), or represses CXCR5 expression, that would inhibit Tfh differentiation[223,236]. ICOS is also involved in Tfh migration into follicles by interaction with bystanders B cells[237].

Besides TCR and co-stimulation receptor binding, T:B cell conjugates are further stabilized by homophilic binding of signaling lymphocytic activation molecule (SLAM) family members 5 (SLAMF5 or CD84) and 6 (SLAMF6 or LY108) that provide positive and negative signals, respectively[238,239]. The link to the intracellular signaling cascades is then performed by SLAM-associated proteins (SAP) that balance the positive and negative signals required for long-lived interactions, and consequently GC Tfh and B cell differentiation[47,238,239].

A few days after the migration into the T-B border, pre-Tfh cells commit to the Tfh subset, by stabilizing BCL-6 expression, further upregulate CXCR5, downregulate CCR7, PSGL-1, and EBI2, and migrate into developing GCs where they will provide help to B cells[211].

Tfh function and maintenance at germinal centers

In the GC, Tfh cells are responsible for providing help to GC B cells undergoing the processes of affinity maturation, selection, and CSR.

As described previously, GC B cells in the LZ establish cognate interaction with Tfh cells. The duration and strength of these interactions are determined by the capability of GC B cells to uptake antigen and present it on MHC-II molecules to Tfh cells[79]. Thus, B cells that have higher affinity to the antigen will be able to receive further stimulation by Tfh cells: high number of TCR-MHC interactions lead to a rapid externalization by Tfh cells of CD40L and establishment of CD40L-CD40 interactions[240]. In turn, CD40-CD40L interactions promote increased expression of ICOSL that permits longer T:B interactions[240]. Moreover, CD40 together with BCR signaling also provide positive signals of survival, proliferation, and differentiation[241-244]. Furthermore, IL-21 produced by Tfh cells also targets GC B cells

and stimulates BCL-6 expression maintenance, the key regulator of GC B cell program, thus controlling the affinity maturation process[245,246]. In opposition, IL-21 also induces BLIMP-1 expression that is involved in plasma cell differentiation[247]. Hence, B cells with higher affinity to the antigen receive more Tfh stimulation that leads them either to migrate back into the DZ, where they undergo higher rates of expansion and mutation (compared to cells that received less stimulation from Tfh cells), or to differentiate into memory B cells or plasma cells[86]. On the other hand, B cells that have lost their affinity to the antigens and, consequently, do not successfully engage in cognate interactions with Tfh cells receive signals to die: GC B cells express high levels of FAS and, in the absence of positive signals, Tfh cells induce AICD through FASL-FAS interactions[248,249]. Besides IL-21, Tfh cells also produce IL-4 which provides survival signals to B cells and enhances plasma cell differentiation[83,250,251].

Tfh cells also require surviving signals from B cells in the GC. GC B cells stimulate Tfh cells by providing TCR and ICOS stimulation signals that induce Tfh proliferation and survival[212,231]. Furthermore, IL-21 produced by Tfh cells not only targets GC B cells but also has an autocrine effect that stabilizes the Tfh differentiation[224,234].

Tfh cells express high levels of program cells death 1 (PD-1), which is already expressed by pre-Tfh cells, but, as for other markers such as CXCR5, ICOS, and BCL-6, its maximum expression is observed on Tfh cells[252,253]. Thus, Tfh cells can be identified by the double expression of CXCR5 and PD-1, or CXCR5 and ICOS. PD-1 is an inhibitory receptor induced by extended TCR signaling and its ligands, PD-1 ligand 1 (PD-L1) and PD-L2, are expressed by GC B cells[254,255]. Thus, PD-1 signaling is involved in preventing excessive Tfh proliferation and may also dampen the duration of T:B interactions, thus leading to higher affinity requirements[255,256]. Another inhibitory receptor expressed by Tfh cells is the B and T lymphocyte attenuator (BTLA)[257]. BTLA inhibits T cell proliferation by binding of the herpesvirus entry mediator (HVEM)[258]. In the case of Tfh cells, BTLA does not impact on differentiation but decreases Tfh functional capacity, especially IL-21 production. In agreement, in the absence of BTLA, GC B cell number, as well as antibody titers, increase after immunization[257].

Additional transcription factors involved in Tfh differentiation

There are other transcription factors known to have a role in Tfh differentiation. However, their function, and/or the differentiation step when their action is necessary, are still poorly understood. This it is the case of BATF and IRF4. BATF is required for Tfh and Th17

differentiation; in the case of Tfh, it induces expression of BCL-6 and c-MAF[259]. Besides its involvement in Th2, Th17, and Th9 differentiation and in the repressive ability of Treg cells, IRF4 also plays a major role in Tfh differentiation, since in its absence Tfh differentiation is stalled[260].

Tfh cells memory

Besides the formation of memory B cells during primary responses, the capacity for rapid responses upon antigen rechallenge also depends on the formation of memory Tfh cells[261,262]. There are $CXCR5^+CCR7^+$ and $CXCR5^+CCR7^-$ memory Tfh cells that can arise prior or after migration into the GC[263,264]. $CXCR5^+CCR7^+$ memory Tfh cells are abundant in the blood and upon antigen encounter can rapidly differentiate into Tfh cells and migrate into GC to support GC B cells[263]. The presence of these cells in the blood allows a faster response in subsequent reinfections at different locations or a better control of pathogen systemic disseminations.

Tfh cells in human beings

Tfh cells have been studied not only in mice but also on human samples. In mice most of the studies are conducted in tissue samples (draining LNs and spleen). However, the difficulty in obtaining these kind of samples from human subjects led to the majority of human studies to be performed on $CXCR5^+$ CD4 T cells from peripheral blood. These $CXCR5^+$ CD4 T cells have been denominated circulating Tfh (cTfh) cells. The first studies on cTfh cells were performed in samples from HIV-infected patients (and healthy donors), since Tfh cells from these individuals are greatly dysregulated in function and number[265,266]. Moreover, several reports have also compared cTfh cells from healthy donors to cTfh cells from patients with autoimmune pathologies associated with auto-antibody production or patients with immune deficiencies[267-274].

Several differences have been detected between mouse and human Tfh cells. For once, while Tfh cell differentiation in mice is dependent of IL-6 and IL-21, in humans IL-12, IL-23, and TGFβ are the key cytokines for Tfh differentiation[226,275]. These differences are even more surprising since in mice TGFβ has the contrary effect and inhibits Tfh differentiation[276].

Another difference is the capacity to identify subsets within cTfh cells. Three subsets have been described based on CXCR3 and CCR6 expression: $CXCR3^+CCR6^-$ cells have Th1-like properties, $CXCR3^-CCR6^-$ share characteristics with Th2 cells, and $CXCR3^-CCR6^+$ have common characteristics with Th17 cells[277]. Furthermore, although $CXCR3^+CCR6^-$ Th1-like Tfh cells are not capable to provide help to naïve or memory B cells, $CXCR3^-CCR6^-$ Th2-

like Tfh cells and $CXCR3^{-}CCR6^{+}$ Th17-like Tfh cells give help to B cells and have different capacities to induce CSR: the first induce IgG and IgE production, while the second induce IgG and IgA secretion[277]. Nevertheless, the role of these cell subsets on subsequent responses still needs to be elucidated and, in particular, the relationship between time and cTfh cells.

Regulatory T cells

Antigen receptors of the adaptive immune cells originate from the random recombination of multiple DNA segments that leads to the production of a very wide range of receptors and receptor affinities. This characteristic allows the specific recognition and response against virtually any pathogen. On the downside, T and B cells with receptors that recognize the self (or environmental antigens) can also arise. To avoid detrimental responses against the self, the adaptive immune system employ several mechanisms to induce tolerance. During T and B cell development, self-reactive cells are deleted in the thymus and bone marrow, respectively. Also, in the periphery, two other mechanisms are used to control T cell responses. First, T cells that receive chronic TCR stimulation are rendered anergic. Second, in order for T cells to be activated, DCs have to provide TCR signaling stimuli and co-stimulation signals through CD80/CD86; since DCs only upregulate CD80/CD86 upon PPRs activation, this two-signal requirement guarantees that T cells only get activated during inflammatory responses. Nevertheless, another mechanism that involves a specialized T cell subset is also required to maintain immune tolerance throughout life[278-280].

The most studied regulatory T (Treg) cells are $CD4^{+}$ T cells with high suppressive capacity that were initially identified by their high expression of the IL-2 receptor α chain (CD25)[278]. Furthermore, SP $CD4^{+}CD25^{+}$ T cells could be identified in the thymus indicating that Treg cells originated in that organ[278]. Although it is now known that Treg cells from the periphery can recirculate into the thymus, the differentiation of Treg cells in this organ has been fully elucidated[281,282]. However, activated CD4 T cells also express high levels of CD25 and that common characteristic hampered for some time further studies on the mechanistic action of these cells.

Mutations in the *Foxp3* gene were first identified as the cause of fatal and early-onset, T cell dependent, lymphoproliferative, immune-mediated disorder in scurfy mice and human patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome[283-286]. The impact and characteristics of the *Foxp3* mutations then lead to the identification of FOXP3 as the key transcriptional factor for Treg cells[279,287,288]. FOXP3 is

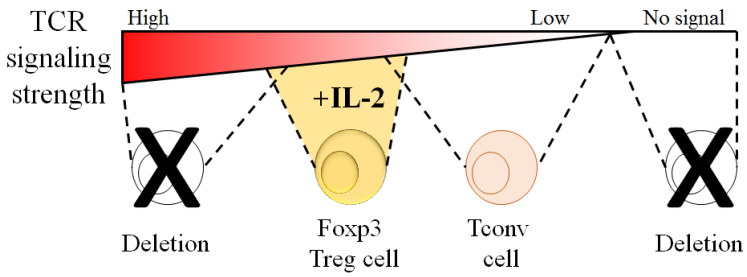

Figure 7 – TCR signaling strength determines Treg differentiation in the thymus.
During selection in the thymus, the avidity that T cells demonstrates to the self-antigen and MHC molecule determines the TCR signaling strength obtained by the cell and consequently the T cell fate. Most of the cells present either very high or no avidity leading to their deletion (negative selection and death by neglect, respectively). SP $CD4^+$ T cells undergoing positive selection that receive TCR signals of intermediate-low strength become Tconv. SP $CD4^+$ T cells that present intermediate-high TCR signaling strength differentiate into $Foxp3^+$ Treg cells in the presence of IL-2. Some cells present TCR signaling strengths that, in the absence of IL-2, may suffer deletion or may differentiate into a Tconv cell.

not only required for Treg differentiation but it is also critical for its suppressor function and phenotype maintenance[279,289,290]. Treg cells are usually characterized by the double expression of FOXP3 and CD25. However, other markers like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced TNF receptor-related protein (GITR) are also characteristic of this population[287].

Although the majority of $FOXP3^+$ Treg cells originate in the thymus (tTreg), conventional CD4 T (Tconv) cells can also differentiate into Treg cells in the periphery (pTreg). These two populations have different characteristics, such as antigen-specificity, but whether both populations perform the same functions is still a matter of debate.

Thymic derived Treg cells

The main characteristic of tTreg cells is their TCR capability to recognize self-antigen. Thus, for a T cell to differentiate into a Treg cell, its TCR avidity for self-antigens has to be in between the affinities that lead to positive selection of Tconv cells and negative selection[291,292] (see Figure 7). Therefore, even though other signals are needed, TCR signaling is crucial for FOXP3 induction and commitment to the Treg lineage. Treg differentiation is also influenced by intraclonal precursor competition that restricts the differentiation of numerous Treg cells expressing the same TCR while facilitates the generation of Treg cells with a broad TCR repertoire[293,294]. In turn, this competition for Treg differentiation signals may explain the small, but existent, overlap of TCR repertoire between Treg and Tconv cells[295,296]. Besides TCR signaling, CD28 co-stimulatory signals and cytokine signaling (mainly IL-2 and, to a lesser extent, IL-7 and IL-15, all of which

signal through the common γ chain) are also essential for Treg development[297-299]. The critical role of IL-2 led to the proposal of a two-step differentiation model, where the first step depends on TCR signaling and leads to the upregulation of CD25, while the second step depends on IL-2 signals, which in turn depends on the previous CD25 upregulation, that are required for FOXP3 induction[300].

Peripherally induced Treg cells

While tTreg cells specificity is biased towards self-antigens, pTreg cells are induced by recognition of non-self antigens like food, allergens, and commensal microbiota. These antigens are continuously present in the organism in homeostatic and non-inflammatory conditions and support differentiation of pTreg cells. Accordingly, pTreg differentiation requires high-affinity TCR signaling in combination with low co-stimulation (low CD28, but increased CTLA-4 signaling) to induce FOXP3 expression[301-303]. Moreover, cytokine signaling is also necessary: induction of pTreg cells is enabled in the presence of high amounts of TGFβ[304]. Furthermore, TCR sub-optimal activation also seems to favor Treg conversion in a TGFβ-dependent way[305]. Taking into account the necessary conditions, such as the requirement for antigen exposure without triggering an immune response, as well as the antigens that usually induce pTreg, one would expect that the mucosal tissues are environment favorable for pTreg differentiation. Indeed, mesenteric LNs, Peyer's patches, and gut-associated lymphoid organs are environments of excellence for pTreg induction. Furthermore, the capability to generate pTreg cells in these organs is further favored due to the presence of $CD103^+$ DCs which present the antigen and produce TGFβ and retinoic acid in homeostatic conditions[306,307].

Treg cells suppression mechanisms

Treg cells rely on several suppression mechanisms to induce tolerance and control on going immune responses: cytokine deprivation, release of inhibitory cytokines, induce cell death to target cells, and cell-to-cell suppression of APCs.

The cytokine deprivation mechanism depends on the fact that Treg cells express high levels of CD25, which is indispensable for Treg cells homeostasis[298,308]. Since IL-2 is an effector cytokine required for survival and expansion of effector T cells, the expression of high levels of CD25 by Treg cells consumes the IL-2 present in the environment, thus diminishing the proliferation and survival of effector T cell[309]. On the other side, Treg cells produce the immune suppressive cytokines IL-10, IL-35, and TGFβ. IL-10 and IL-35 have important roles in maintaining tolerance: the first is important in environments like the lung and colon,

while the second is required to keep immune cells of the gut in check[310,311]. TGFβ produced by Treg cells suppresses Th1 and Th17 responses[312]. Another mechanism employed by Treg cells is the induction of cell death to APCs and activated T cells by granzyme B-mediated apoptosis[313,314]. Moreover, Treg cells suppress APC by cell-to-cell interactions mediated by receptors CTLA-4 and lymphocyte-activation gene 3 (LAG-3). The principal mechanism through which CTLA-4 acts is still a matter of debate. CTLA-4 binds the co-stimulatory molecules CD80 and CD86 with higher affinity than CD28. Thus, the proposed mechanisms involve regulation of co-stimulatory signals that APCs provide to effector T cells by competition or removal of CD80 and CD86 from APC cell surface by transendocytosis[315]. LAG-3 is a CD4 homologue that suppresses DC maturation and co-stimulatory capacity by binding MHC-II molecules with high affinity[316,317]. Two other highly expressed receptors involved in Treg suppression are CD39 and CD73. These two ectoenzymes release cyclic adenosine monophosphate (cAMP) to the extracellular matrix which directly inhibits proliferation and function of T cells and DCs, respectively[318-321].

Treg cell subsets

Treg cells control different types of immune responses and effector cells. Therefore, Treg cells need to acquire specific features depending on the environmental properties of the response and where it takes place. In order to acquire these features, Treg cells sense the ongoing responses by the cytokines present in the environment and accordingly upregulate transcription factors associated with ongoing Th response[322]. Thus, during Th1 responses, Treg cells also upregulate T-BET, which in the case of Treg cells allows the expression of CXCR3 and consequent migration, proliferation, and accumulation where Th1 responses take place[323]. Moreover, Treg cells are able to obtain Th2 features and regulate Th2 responses by expression of IRF4 and GATA3, while upregulation of RORγt grants Th17-like properties important for intestinal inflammation control[324-327]. Another Treg cell subset recently described is the Tfr cell subset. This Treg subset is responsible for controlling Tfh cells and GC responses by expressing the Tfh master transcription regulator BCL-6 and migrating into B cell follicles[328-330].

Foxp3$^-$ regulatory T cells

Besides FOXP3$^+$ CD4 T cells, two other regulatory CD4 T cells populations that do not express FOXP3, Tr1 and Th3 cells, have been described as being also involved in suppressing and regulating the immune system[331,332]. Tr1 cells are immune suppressive CD4 T cells that exert their function through the production of high amounts of IL-10, and can

suppress antigen-specific T cell responses and prevent colitis[332]. Tr1 differentiation is induced by IL-27 or IL-6[333]. Although many characteristic receptors and transcription factors have been associated to these cells and their functions, no unique transcription factor or master regulator for this subset has been described yet. Besides IL-10 production, Tr1 cells are also characterized by expression of the receptors LAG-3 and CD49b, and expression of transcription factors like c-MAF and AHR[334-336]. Th3 cells are also immune suppressive CD4 T cells, but this subset is characterized by the secretion of high amounts of TGFβ[337]. Th3 cells develop upon oral tolerance induction in a TGFβ-dependent fashion and are specific to the antigen provided by oral administration. Moreover, Th3 cells can suppress both Th1 and Th2 responses in an antigen independent way, and also provide help for IgA CSR and production[331]. Once again, no master transcription factor is known for the Th3 subset, however, they can be identified by the expression of TGFβ, CTLA-4, and low CD25[337,338].

Follicular regulatory T cells

In order for Treg cells to control and suppress immune responses, they have to acquire phenotypic characteristics of effector Th cells and migrate into the local of immune reaction. That is the case of the recently described Tfr cells, a subset of Treg cells that migrates into GC to regulate the GC responses.

The importance of Treg cells on the control of antibody responses has been observed since the discovery of these cells. Indeed, one of the consequences of their absence is the increased level of circulating antibodies (both IgG and IgE that originate in GCs)[339,340]. Further studies showed that Treg cells are capable of controlling antibody responses by inhibiting AID expression and CSR, and by directly killing B cells[314,341]. Also, $CXCR5^+$ Treg cells could be found within GCs of immunized mice. However, the confirmation of the existence of a specialized subset of Treg cells that migrated into GC and controlled antibody responses came with the identification of a population with mixed characteristics of Tfh and Treg cells[328-330]. These Tfr cells express not only the Treg master regulator FOXP3 but also CD25, CTLA-4, GITR, and granzyme B. On the other hand, Tfr cells express Tfh cell markers like CXCR5, ICOS, PD-1, SAP, and the master transcription factor of the Tfh subset BCL-6. Thus, Tfr cells present Tfh characteristics, but have a suppressive function as Treg cells.

Tfr cells were initially described in mouse and also humans. Indeed, one of the first reports that described Tfr cells demonstrated the presence of these cells within GC of human tonsils[328]. However, as for Tfh cells, the difficulty in obtaining human samples led to the

majority of the work to be performed in mice. Nevertheless, recent studies have investigated a population of circulating $CXCR5^{+}FOXP3^{+}$ CD4 T cells from human blood, considered circulating Tfr cells, and that has been correlated with autoimmune diseases[342-345].

There is some controversy regarding the origin and specificity of Tfr cells. These cells were initially described as deriving from tTreg cells[328-330]. However, a recent report suggests that these cells can originate from Tconv cells by initially conversion into pTreg cells followed by differentiation of those pTreg cells into Tfr cells. Consequently, these cells would be specific to the immunizing antigen[346].

Tfr cell differentiation

Although less studied than Tfh cell differentiation, Tfr cells also seem to undergo a multi-step differentiation process. In this process, the initial priming is dependent on DCs, while B cells are required for full development and expansion[264,329,347]. Nevertheless, circulating Tfr cells with memory characteristics can differentiate in the absence of B cells, indicating that, as for Tfh cells, these cells can originate upon the first commitment with DCs before fully commitment[264]. Another similarity between the differentiation processes of Tfh and Tfr cells is the requirement of TCR stimulation and co-stimulatory signals through CD28 and ICOS[329,348]. However, Tfr cells differentiation also seems to be affected by co-inhibitory signals. PD-1 that is expressed by Tfr and Tfh cells has a detrimental effect on Tfr cells differentiation which is dependent on the binding of PD-L1 (but not PD-L2)[348]. CTLA-4 is expressed by Treg and Tfr cells (but not by Tfh cells) and, as PD-1, also has negative impact on Tfr differentiation[349,350].

There are also similarities and differences in the transcription factors involved in Tfr and Tfh cell differentiation. For once, BCL-6 is a master regulator for both subsets and both require STAT3 signaling[351,352]. Surprisingly, Tfr cells express the mutual antagonists and repressors BCL-6 and BLIMP-1: while the first is important to acquire a Tfh-like phenotype, the second may be necessary for the Treg-like suppressive function of Tfr cells[329]. Another difference is that, while ASCL2 is important for initial CXCR5 expression in Tfh differentiation, Tfr cells do not express ASCL2, and CXCR5 expression requires the transcription factor nuclear factor of activated T cells 2 (NFAT2)[227,353]. The TNF receptor-associated factor 3 (TRAF3) is also important for Tfr differentiation and function, and is involved in the regulation of ICOS in Treg cells[354].

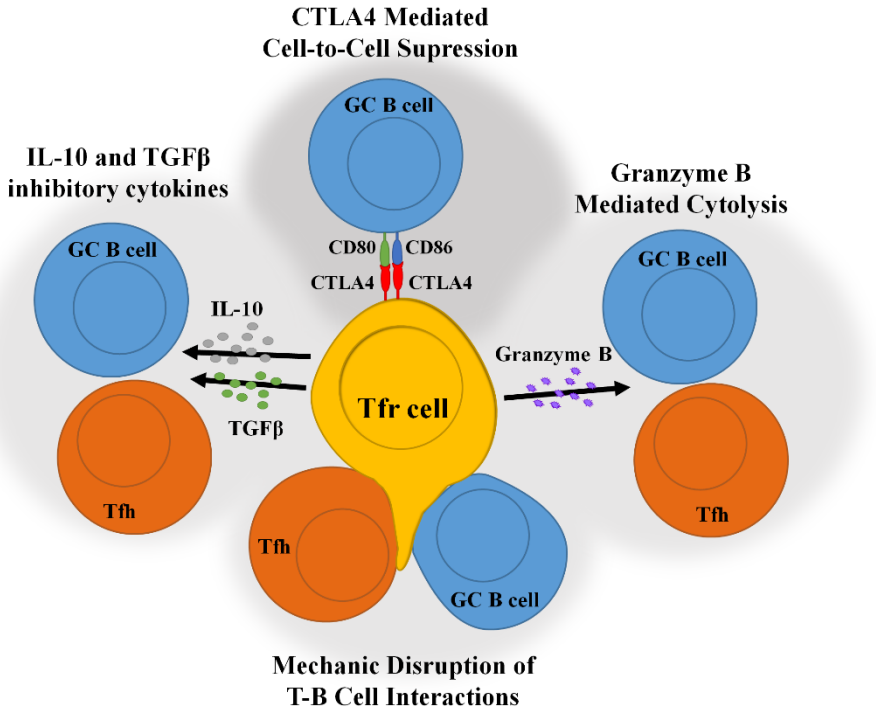

Figure 8 – Tfr cell suppression mechanisms.
Tfr cells have a suppressive effect on GC. However, only the suppressive mechanism mediated by CTLA-4 action has been described so far. Nevertheless, three other have been suggested: induction of cytolysis by release of granzyme B, mechanic disruption of interactions between Tfh and GC B cells, and production of the inhibitory cytokines IL-10 and TGFβ.

GC regulation by Tfr cells

Tfr cells are involved in GC regulation. The first studies that described these cells already showed that Tfr cells controlled the GC size and the amount of antibodies produced[328-330]. However, the impact on the antibody affinity generated has been controversial. One of the first studies claimed that Tfr cells did not have an impact on antibody affinity, while recent studies demonstrated that, in the absence of this regulatory population, the amount of antibodies produced is higher, but the affinity generated is lower[328,350,355]. This possible effect on antibody affinity, could be explained by facilitated help from Tfh cells to B cells in the absence of Tfr cells, and consequent generation of low affinity plasma cells[356]. Indeed, Tfr cells not only suppress Tfh proliferation and the production of IL-21 and IL-4, but also have been implicated in the regulation of non-antigen-specific B cell clones in the GC[264,329]. Moreover, Tfr cells seem to regulate Tfh and GC B cells by affecting their metabolism: Tfr cells induce a suppressive state on Tfh and GC B cells that persists in the absence of Tfr cells and is associated with epigenetic changes[357].

Even though several mechanisms have been suggested to explain Tfr cells suppressive action, only one has been confirmed so far[358](see Figure 8). Two recent reports have shown

that, as Treg cells, Tfr cells express CTLA-4 and this receptor is involved in the suppressive capacity of these cells[349,350]. One of the mechanisms suggested for Tfr action is through the production of the suppressive cytokines IL-10 and TGFβ. However, although Tfr cells produce IL-10, this cytokine was detected in higher amounts in the supernatants of Tfh and GC B cells cultures when Tfr cells were not added to the culture[264]. Even though Tfr cells seem to inhibit IL-10, this effect may still be in agreement with their functions, since IL-10 is important for B cell survival and proliferation[359]. Nevertheless, the effect of IL-10 produced by Tfr cells requires further study *in vivo*. On the other hand, since Tfh cells are suppressed by TGFβ, the production of this cytokine may also be a mechanism of Tfr suppression[276]. Tfr cells also express granzyme B, though in lower levels than Treg cells, and granzyme B-mediated cytolysis may be another regulatory mechanism employed by these cells[329]. Lastly, Tfr cells may exert a suppressive effect by mechanical disruption of T:B interactions that are required by Tfh and GC B cells[358].

Tfr cells memory

As observed in Tfh cells, there is a circulating Tfr cells population that develops upon immunization. These memory Tfr cells can originate prior to the migration into the GC as only stimulation from DCs is required. These cells can persist in the system for long periods of time and be recruited into the GC to suppress further responses[264].

GCs and autoimmunity

Several autoimmune diseases are caused by the production of autoantibodies. It is the case of systemic lupus erythematosus (SLE), RA, Sjögren's syndrome, and myasthenia gravis. Besides the increased antibodies titers, these diseases are also associated with an increased number of circulating Tfh cells, and both may be associated with GC dysregulation[267-270]. Since Tfr cells functions involve the regulation and suppression of GC, it is likely that a diminished function of these cells leads to the onset and development of auto-antibody-mediated autoimmune diseases. In agreement, several studies have so far associated altered circulating Tfr:Tfh ratios to humoral autoimmune diseases in humans, such as ankylosing spondylitis, myasthenia gravis, MS, and IPEX-like disease[342-345]. Moreover, alterations on the Tfr population were also observed in murine models of spontaneous and induced lupus-like disease as well as in murine arthritis models[353,360-364]. Nevertheless, further studies are needed to determine if Tfr cells are directly implicated in humoral autoimmunity, and a better

knowledge of Tfr cells biology may provide strategies to modulate humoral immune responses.

II. Objectives

A key outstanding question regarding Tfr cell biology is its ontogeny. Tfr cells were initially described as originating from tTreg cells, but a recent report claimed that these cells can also derive from Tconv cells when favorable conditions to pTreg differentiation are present.
Another open question, perhaps with greater implications, resides on the specificity of Tfr cells. Tfr cells present themselves as perfect candidates for the detection and elimination of auto-reactive B cells that can arise during affinity maturation in the GC, namely because of (1) their regulatory and suppressive functions as regulators of the GC, (2) the necessity of a mechanism to prevent the arise of auto-reactive B cells from the GC, and (3) the reports associating the reduction of this population to the onset and development of autoimmune diseases in both human and mouse. In turn, that capacity of preventing the generation of auto-reactive B cells would be in agreement with Tfh and Tfr having different specificities: Tfh cells specific to the immunizing antigen, while Tfr cells, originating from tTreg cells, with specificity towards self-antigens.

Therefore, this dissertation aimed to answer those two questions. In brief:

1. What is the cellular ontogeny of Tfr cells?
2. What is the specificity of Tfr cells?

Three lines of experiments were conducted to answer those questions. Accordingly, the results obtained are presented in three chapters.
For the first question, several *in vivo* TCR-transgenic model were used to verify if Tfr cells could originate from Tconv cells.
The second line of experiments involved *in vivo* and *in vitro* experiments to demonstrate whether Tfr cells were preferentially specific to an immunizing antigen. These experiments involved TCR-transgenic models to show that antigen-specific TCR-transgenic tTreg cells were not preferentially recruited into the GC, the use of tetramers to demonstrate the absence of antigen-specific tetramer^{+} Tfr cells, and *in vitro* cultures of Tfr cells to show that the presence of the immunizing antigen did not give any advantage in terms of proliferation or survival.
Lastly, the TCR repertoire of Tfr, Tfh, and Treg cells was investigated and used to show that Tfr cells had a repertoire closer to the one of Treg cells than to Tfh cells.

III. Methods

Mice

C57BL/6, C57BL/6.Thy1.1, Foxp3^{GFP}, Foxp3^{hCD2}, Balb/c, TCRβ$^{-/-}$, DO11.10.Rag$^{-/-}$, OT-II.Rag$^{-/-}$, OT-II.Rag^{+}.Thy1.1^{+}.Thy1.2^{+}, P25, and 1D2β mice were bred and maintained at Instituto de Medicina Molecular and Instituto Gulbenkian de Ciência in specific pathogen-free facilities. Animals of both sexes (same sex per experiment) and with age ranging from 2 to 6 months were used. Procedures were conducted in accordance with guidelines from the Animal User and Institutional Ethical Committees.

Immunizations and cell transfers

Animals were immunized subcutaneously in the footpad with six different antigens in combination with two adjuvants listed on Table 2. Antigens were prepared by mixing the antigen solution 1:1 (v:v) with the adjuvants. 50 μl of emulsion were injected in each footpad.

For adoptive cell transfers, purified CD4 T cells were injected i.v. in saline solution. In all experiments, the immunization occurred 1 day after the adoptive cell transfers, and collection of popliteal LN at day 11 following immunization.

Flow cytometry and cell sorting

For flow cytometry analysis and cell sorting, single cell suspensions were obtained and stained with the monoclonal antibodies listed on Table 3. Intracellular FOXP3 staining was performed using the FOXP3 Staining Set (eBioscience) according to the manufacturer's instructions. $OVA_{323-339}$ or $Ag85B_{280-294}$ specific T cells were detected with a PE-(OVA) or APC-(Ag85B) conjugated MHC-II I-A^b tetramer containing an $OVA_{329-337}$ (AAHAEINEA) or $Ag85B_{280-294}$ (FQDAYNAAGGHNAVF) peptide, respectively (both offered by the NIH Tetramer Core Facility). Staining was performed for 1h at RT as described before[365]. Enrichment of tetramer^{+} cells was performed using MACS cell separation system and anti-PE and anti-APC magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions.

All samples were acquired on a BD LSRFortessa flow cytometer and acquisition data were analyzed with the FlowJo software (Tree Star). For flow cytometry analysis of cultured cells and tetramer enriched cell samples, 10 μm latex counting beads were added to cell suspensions to obtain total cell counts (Counter Beckman).

Table 2 – List of antigens and adjuvants used for immunization

Antigen	*Adjuvant*	*Brand (Ag/Adjuvant)*	*Ag Concentration on emulsion*
Ovalbumin (OVA)	IFA	Sigma-Aldrich/Sigma-Aldrich	1.6 mg/ml
β-lactoglobulin (βLG)	IFA	Sigma-Aldrich/Sigma-Aldrich	1.6 mg/ml
$OVA_{323-339}$-BSA	IFA	Thermo Fisher Scientific or Schafer-N/Sigma-Aldrich	1.6 mg/ml
$Ag85B_{240-254}$-BSA	IFA	Thermo Fisher Scientific or Schafer-N/Sigma-Aldrich	1.6 mg/ml
$OV_{323-339}$ peptide (ISQAVHAAHAEINEAGR)	IFA/CFA	Schafer-N/both Sigma-Aldrich	2 mg/ml
$Ag85B_{240-254}$ peptide (FQDAYNAAGGHNAVF)	IFA/CFA	Schafer-N/both Sigma-Aldrich	2 mg/ml

Table 3 – Antibodies used for flow cytometry analysis

Antibody	*Clone*	*Brand*	*Conjugation*
CD4	RM4-5	Biolegend or eBioscience	BV510, PE, APC, APC-e Fluor 780
CD19	ebio1D3	eBioscience	FITC, PE, PerCP-Cy5.5, APC, APC-e Fluor 780
CD25	PC61.5	eBioscience	Alexa Fluor 488, PE-Cy7, APC-e Fluor 780
CXCR5	2G8	BD Pharmigen	Biotin
FOXP3	FJK-16s	eBioscience	eFluor 450, Alexa Fluor 488, APC
GITR	DTA.1	BD Pharmigen	PE
hCD2	RPA-2.10	eBioscience	APC
PD-1	J43	eBioscience	FITC, PE, PE-Cy7
Thy1.1	HIS51	eBioscience	PerCP-Cy5.5, APC-eFluor 780
Thy1.2	53-2.1	eBioscience	PE-Cy7, APC-e Fluor 780
TCRβ	H57-597	eBioscience	FITC, APC-eFluor 780
Vα2	B20.1	eBioscience	eFluor 450
Vβ5	MR9-4	BD Pharmigen	FITC

For cell sorting of CD4 T cells for adoptive cell transfers, cells were purified from spleen and mesenteric LNs using MACS cell separation system and anti-CD4 (L3T4) magnetic beads (Miltenyi Biotec). FACS-sorting of cells for *in vitro* assays and RNA extraction with monoclonal antibodies was performed on a BD FACSAria cell sorter.

In vitro cultures

Bone marrow derived DCs were generated *in vitro*. Bone marrow progenitors were cultured for 7 days in presence of 20 ng/ml GM-CSF (PeproTech); medium was changed on days 3

and 6 of culture. DCs specific antigen loading was performed for 3h at 37ºC in presence of 1 mg/ml of protein.

In OT-II Treg/WT Treg and Tfr/Tfh/Treg cultures, CD4 T cells were pre-incubated with CellTrace Violet (Life Technologies) for tracking cell proliferation according to manufacturer's instructions. A 3:2 ratio of CD4 T cells to DCs was used in these experiments to a final number of $5x10^4$ cells per well in the case of OT-II Treg/WT Treg cultures and $2.5x10^4$ cells per well in the case of Tfr/Tfh/Treg cultures. Cells were co-cultured in the presence of 2 ng/ml IL-2 (eBioscience) and, in some conditions, 3 μg/ml anti-CD3 (145-2C11, eBioscience) were also added to the culture. After 3 days of culture, cells were stained with monoclonal antibodies and analyzed by flow cytometry.

For the Tfh radioactive proliferation assay, $2.0x10^4$ Tfh cells were cultured with the same number of DCs. In the wells where unloaded DCs were cultured, 3 μg/ml anti-CD3 were added to the culture. After 3 days of culture, cells were incubated with 1.0 μCi/well of 3H-thymidine (Perkin Elmer) at 37ºC for 6 hours and stored at –20°C until harvesting. Cells were harvested on a Tomtec Harvester (Tomtec) into a Filtermat, covered with Meltilex, and scintillation counted on a Microbeta Trilux (all PerkinElmer).

All cultures were performed in complete RPMI (RPMI-1640 with glutamine and 25mM HEPES [Lonza] supplemented with 10% FBS (v:v) [Gibco], 100U/ml penicillin/streptomycin [Gibco], 1mM sodium pyruvate [Gibco] and 50μM β-mercaptoethanol [Gibco]) and kept at 37ºC with 5% CO_2 level.

RNA extraction, cDNA synthesis and CDR3 length analysis

RNA extraction from cell-sorted populations ($2x10^5$ - $5x10^6$ cells) was performed using TRIzol (Life Technologies). cDNA was amplified using Random Primers and SuperScript III Reverse Transcriptase (both Invitrogen). Both RNA extraction and cDNA synthesis were performed following the manufacturer's instructions. CDR3 spectratyping was performed as described previously[366]. Briefly, each obtained cDNA was used to perform 23 PCR reactions in parallel with a common Cβ reverse primer and 23 Vβ-specific forward primers (GoTaq DNA Polymerase from Promega and primers from Life Technologies). Run-off reactions were done using dye-labelled Cβ primer. All used primers are listed on Table 4. Run-off products were run on ABI 3130XL Automatic Sequencer (Applied Biosystems) together with GeneScan 500 ROX dye Size Standard (Applied Biosystems), and consequently

Table 4 – Primers used for CDR3 spectratyping/immunoscope analysis

Sequence	*Direction*	*Target gene/region*
TCACTGATACGGAGCTGAGGC	Forward	TRBV1
GCCTCAAGTCGCTTCCAACCTC	Forward	TRBV2
CACTCTGAAAATCCAACCCAC	Forward	TRBV3
ATCAAGTCTGTAGAGCCGGAGGA	Forward	TRBV4
CTGAATGCCCAGACAGCTCCAAGC	Forward	TRBV5
AAGGTGGAGAGAGACAAAGGATTC	Forward	TRBV12-1
CATTATGATAAAATGGAGAGAGAT	Forward	TRBV12-2
TGCTGGCAACCTTCGAATAGGA	Forward	TRBV13-1
CATTATTCATATGGTGCTGGC	Forward	TRBV13-2
CATTACTCATATGTCGCTGAC	Forward	TRBV13-3
AGGCCTAAAGGAACTAACTCCAC	Forward	TRBV14
GATGGTGGGGCTTTCAAGGATC	Forward	TRBV15
GCACTCAACTCTGAAGATCCAGAGC	Forward	TRBV16
TCTCTCTACATTGGCTCTGCAGGC	Forward	TRBV17
CTCTCACTGTGACATCTGCCC	Forward	TRBV19
CCCATCAGTCATCCCAACTTATCC	Forward	TRBV20
CTGCTAAGAAACCATGTACCA	Forward	TRBV21
TCTGCAGCCTGGGAATCAGAA	Forward	TRBV23
AGTGTTCCTCGAACTCACAG	Forward	TRBV24
CCTTGCAGCCTAGAAATTCAGT	Forward	TRBV26
TACAGGGTCTCACGGAAGAAGC	Forward	TRBV29
CAGCCGGCCAAACCTAACATTCTC	Forward	TRBV30
ACGACCAATTCATCCTAAGCAC	Forward	TRBV31
GCCCATGGAACTGCACTTGGC	Reverse	TRBC(1)
FAM-CTTGGGTGGAGTCACATTTCTC	Reverse	TRBC(2)-FAM

separated based on their nucleotide size. Gene Mapper software (Applied Biosystems) was used to obtain nucleotide length and area of each peak.

RNA extraction, cDNA synthesis, TRA gene amplification, and deep sequencing

RNA from 1D2β mice sorted cell populations ($1.1x10^4$ – $1.0x10^6$ cells) was extracted using RNeasy Midi kit (Qiagen) following manufacturer's instructions. Full-cDNA library was prepared using Mint-2 kit (evrogen) which introduces 5-prime adapters to cDNA fragments. TRA was then specifically amplified using Pfx DNA polymerase (Invitrogen) and a primer pair specific for the 5'-adapter and the C region of the TRA gene. Primers used for full-cDNA library preparation and TRA amplification can be found on Table 5. The sequencing library was prepared using the Nextera kit, in which each sample was barcoded and sequenced using 250bp paired-end illumina MiSeq technology (both illumina).

Table 5 – Primers used for TCRα sequencing

Sequence	*Primer direction*	*Target gene/region*
AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGGGG	Forward	5' PlugOligo adapter
AAGCAGTGGTATCAACGCAGAGTACTGGAG(T)$_{20}$VN	Reverse	3' CDS adapters
CAACGCAGAGTGGCCATTAC	Forward	5' adapter primer
GCAGGTGAAGCTTGTCTGGT	Reverse	TRBC(3)

CDR3 data analysis

In order to have adequate representation of a complete TRBV repertoire, it is necessary to analyze at least $2x10^5$ cells. To achieve this number of cells, it was necessary to pool draining LNs from 15 mice for each biological replicate. In our experiments, at least three biological replicates were used for each T cell subset. The ISEApeaks® software was used to extract and analyze the data obtained for CDR3 fragment size[367,368]. Briefly, this software quantifies the percentage of use of each CDR3 length, obtained by dividing the area of CDR3 peaks by the total area of all peaks within the profile. On C57BL/6 mice, TRBV21 and 24 are pseudogenes and thus ignored on the analysis. TRBV12-2 was also discarded since it could not be detected on three of the samples. To facilitate the comparison between samples and populations, a perturbation score[369] was computed to obtain the overall differences between TRBV CDR3 spectratypes of each sample and the average profiles of naïve CD4 T cells or Tfr cells as control group for each TRBV. Calculated scores were used to perform hierarchical clustering (using Euclidean distance and average linkage).

Deep sequencing data analysis

Paired-end 250bp illumina sequencing data were initially trimmed and subsequently merged using PEAR[370]. clonotypeR[371] toolkit was then used to perform TCR sequence annotation. Two samples had to be discarded due to low sequencing quality (mouse 3 Tfh sample and mouse 4 Tfr sample). Out of the 7,547,998 raw reads obtained for the 18 remaining samples, 25,099 TCR clonotypes were identified from 949,729 productive TCR sequences. For the samples to be comparable, the analyses were performed on 9,000 randomly selected TCR sequences for each dataset as it was the lowest number of TCR sequences found in a dataset. The presented clonality metric is 1- Pielou's evenness index[372], and can vary from 0 to 1 (more diverse to less diverse). The Pielou's evenness corresponds to the Shannon's entropy[373,374] (using log 2) for each sample divided by the number of unique clonotypes (in log 2) of the same sample. For the histogram of cumulative frequency, the 20 most

predominant clonotypes were determined for each sample, and gathered across all samples to plot the cumulative frequency of those clonotypes for each sample. The 20 most predominant Tfr clonotypes were selected from each of the three Tfr samples and gathered into a list used to perform hierarchical clustering in all samples (using Euclidean distance and average linkage). The same was performed for the 20 most predominant clonotypes of each Tfh sample. Dendrogram of overall relation between all samples was obtained using Horn-Morisita index[375,376] as distance and average linkage. This index assesses the similarity between samples taking into account the abundance of each clonotype in the sample. Approximately unbiased (AU) p-values and bootstrap probability (BP) values were calculated for each cluster through 1000 bootstrap resampling iterations[377].

Statistical analysis

Scatter plots and column bar graphs were obtained using GraphPad PRISM. Unless otherwise stated, n represents the number of individual mice analyzed per experiment. To determine statistical significance, two-tailed nonparametric Mann–Whitney U test were performed, and $p<0.05$ was deemed significant (in figures: * $p<0.05$; ** $p<0.01$). Clustering analysis, Venn diagrams, ANOVA, pairwise multiple comparison analysis with Holm-Bonferroni correction for multiple sampling, and multivariate analysis were performed using R software (http://www.r-project.org/).

IV. Results

1. Tfr cells originate from thymic Treg cells

1.1. Introduction

Tfr cells are a recently described population of FOXP3$^+$ Treg cells found within the GCs resembling Treg and Tfh cells[328-330]. Several studies have so far demonstrated that these cells have not only a direct impact on the GC size, but are also involved in the regulation of the quantity and quality of the antibodies produced[330,350,355]. In spite of the advances in the understanding of Tfr biology, their ontogeny is still a matter of debate. Initial experiments, mostly performed in lymphopenic conditions, demonstrated that Tfr cells derive from tTreg cells. However, it was recently reported that Tfr cells can also originate from Tconv cells: upon immunization, and if favorable conditions for pTreg differentiation are present, Tconv cells initially convert into pTreg cells which then are able to differentiate into Tfr cells and migrate into the GC[346].

To clarify whether Tfr cells originate from tTreg or Tconv cells, the precursors of Tfr cells were investigated by determining if FOXP3$^-$ antigen-specific TCR-transgenic CD4 T cells were able to differentiate into Tfr cells upon immunization with OVA in IFA. These experiments were performed in non-lymphopenic mice in order to avoid potential non-physiological effects. Also, two distinct genetic backgrounds were used to account for possible biological heterogeneity. It was found that, under all conditions tested, virtually all Tfr cells derived from tTreg cells.

1.2. Results

In order to avoid the analysis of Tfh and Tfr cells unrelated to the immunization, mice were immunized in the footpad. As shown in Figure 9a, popliteal LNs, the draining LNs of this location of immunization, are virtually free of T follicular cells prior to immunization, but these populations greatly increase upon immunization with OVA-IFA, a process similar to what is observed following vaccination. This conclusion is well illustrated on Figure 9b, where the numbers of Tfh and Tfr cells between non-immunized C57BL/6 and OVA immunized mice are compared.

To determine if Tfr cells originate from Tconv cells, OVA-specific TCR-transgenic CD4 T cells from DO11.10.Rag$^{-/-}$ or OT-II.Rag$^{-/-}$ mice, devoid of thymic FOXP3$^+$ Treg cells (Figure 10a), were adoptively transferred into naïve wild-type (WT) Balb/c.Thy1.1 or C57BL/6.Thy1.1 hosts, respectively (Figure 10b). These cells, besides being specific for OVA, are also trackable after transfer by identification of the congenic markers Thy1.1 and

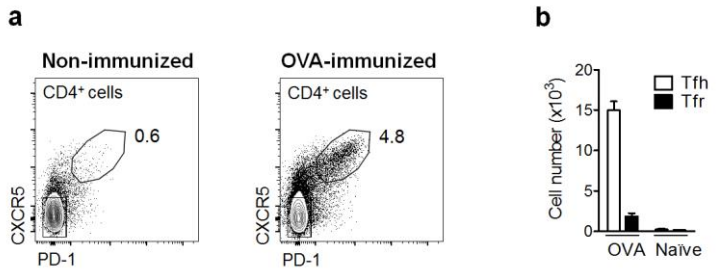

Figure 9 – T follicular cells are virtually inexistent on popliteal LNs prior to immunization.
(**a**) Relative frequency of T follicular cells in popliteal LNs of non-immunized (left) and OVA-immunized (right) C57BL/6 mice. (**b**) Absolute number of Tfh and Tfr cells within popliteal LNs from non-immunized C57BL/6 mice compared to OVA-immunized mice. Popliteal LNs were analyzed 11 days after immunization. Mean ± SEM is presented for n=3.

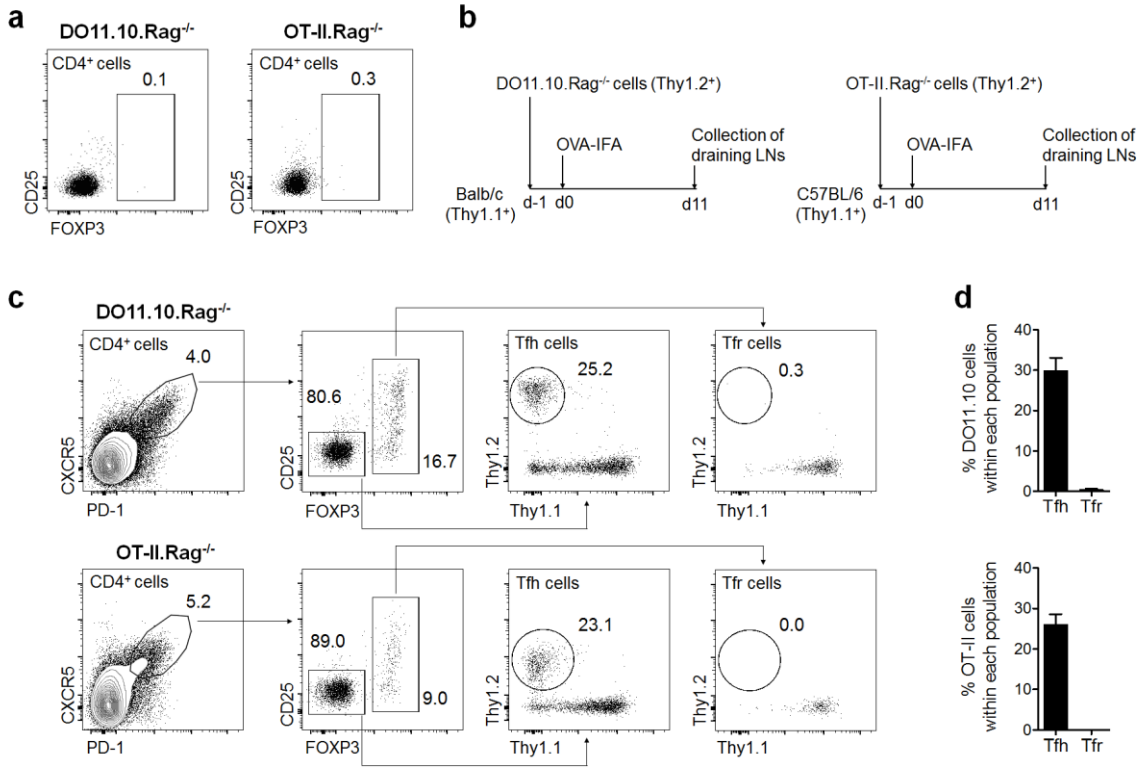

Figure 10 – Tfr cells do not differentiate from FOXP3$^-$ T cell precursors.
(**a**) CD4 T cells from OVA-specific OT-II.Rag$^{-/-}$ or DO11.10.Rag$^{-/-}$ mice are devoid of FOXP3$^+$ Treg cells. (**b**) 10^6 CD4 T cells from OT-II.Rag$^{-/-}$ or DO11.10.Rag$^{-/-}$ mice were adoptively transferred into, respectively, C57BL/6 or BALB/c hosts subsequently immunized with OVA-IFA in the footpad. At day 11, popliteal LNs were analyzed by flow cytometry. (**c**) Gating strategy for detection of DO11.10.Rag$^{-/-}$ (upper panel) or OT-II.Rag$^{-/-}$ (bottom panel) within Tfh and Tfr cell populations. (**d**) Percentages of TCR-transgenic DO11.10 (top) or OT-II cells (bottom) within Tfh and Tfr cells from the analysis in (**c**). While Tfh (CD4$^+$CXCR5^{high}PD-1^{high}FOXP3$^-$) cells contained ~25-30% TCR-transgenic cells, those adoptively transferred cells could not be detected among the Tfr (CD4$^+$CXCR5^{high}PD-1^{high}FOXP3$^+$) population in any of the two genetic backgrounds. Mean ± SEM are presented for n=5.

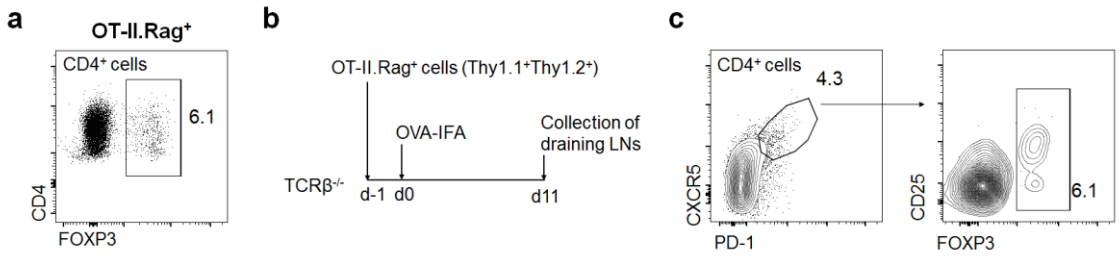

Figure 11 – Antigen-specific FOXP3$^+$ tTreg cells can differentiate into Tfr cells.
(a) OT-II.Rag$^+$ mice have FOXP3$^+$ Treg cells. (**b**) Adoptive transfer of CD4 T cells from OT-II.Rag$^+$ mice into TCRβ$^{-/-}$ hosts subsequently immunized with OVA-IFA in the footpad. Draining LNs were analyzed 11 days after immunization. (**c**) Contour plots showing FOXP3$^+$ Tfr and FOXP3$^-$ Tfh cells within CD4 T follicular cells. Under these conditions, the transferred TCR-transgenic cells originated both Tfh and Tfr cells.

Thy1.2 (transgenic cells were Thy1.2 while recipient mice were Thy1.1). Recipient mice were subsequently immunized with OVA-IFA in the footpad and 11 days later, at the peak of GC response, draining LNs were analyzed by flow cytometry. Day 11 was considered the peak of the response since it has been previously described as when higher numbers of Tfh and Tfr cells can be obtained[330,378]. Popliteal LNs were found to contain Tfh populations derived to a great extent from transferred TCR-transgenic cells, while Tfr cells derived exclusively from endogenous T cells (Figure 10c,d).

On the contrary, TCR-transgenic CD4 T cells from OT-II.Rag$^+$ mice that, unlike RAG-deficient counterparts harbor a population of TCR-transgenic FOXP3$^+$ tTreg cells (Figure 11a), can support the differentiation of Tfr cells, following adoptive transfer into T cell deficient mice (Figure 11b). Indeed, and as shown on Figure 11c, when OT-II.Rag$^+$ CD4 T cells were transferred into TCRβ$^{-/-}$ mice, they readily gave rise to a population of Tfr cells upon immunization with OVA-IFA.

These results show that, while adoptive transfer of tTreg cells can differentiate into Tfr cells, FOXP3$^-$ Tconv cells only differentiate into Tfh.

1.3. Discussion

In order to avoid the presence of pre-existent Tfh and Tfr cells unrelated to the response against the immunizing antigen, mice were immunized in the footpad and the popliteal LNs analyzed at the peak of the response. In that anatomic location, the numbers of CD4 T follicular cells prior to immunization are very low; thus, nearly all Tfh and Tfr cells are derived from GCs induced by the immunizing antigen (with negligible contaminants).

Tfr cells were initially described as originating from tTreg cells. Indeed, experiments from initial reports, using lymphopenic conditions, demonstrated that, upon immunization, Tfr

cells derived exclusively from CD4 T cells that harbored a population of WT tTreg cells[328,330]. Moreover, it was demonstrated that antigen-specific TCR-transgenic cells from RAG knockout background (that do not contain a tTreg cell population) were not capable of differentiating into Tfr cells[329]. However, a recent report suggested that Tfr cells could also originate from Tconv cells, by initial conversion into pTreg followed by differentiation into Tfr cells[346]. This double differentiation occurred in the presence of favorable conditions for pTreg induction, which had not been used in the previous experiment. Such favorable conditions had been previously described and involved the use of IFA as adjuvant[379].

The presented results show that TCR-transgenic cells, upon immunization with the antigen to which they are specific to, did not differentiate into Tfr cells, even though in these experiments IFA was used as adjuvant. In contrast, Tfr cells were detected in mice containing TCR-transgenic cells with a population of tTreg cells. These results suggest that, on the tested conditions, only tTreg cells were capable of differentiating into Tfr cells. Thus, Tfr cells originate from tTreg cells, but not Tconv cells.

It is possible that, under certain conditions, Tfr cells may be specific for the immunizing antigen and differentiate from peripherally induced Treg cells as reported. However, besides constituting a minority within the population, these Tfr cells of pTreg origin do not seem capable to substitute Tfr cells that originate from tTreg cells: Tfr cells seem to influence the affinity maturation process[350,355] and it has been shown that mice lacking tTreg cells able to migrate into the GC have impaired affinity maturation[355].

2. Tfr cells are not specific for the immunizing antigen

2.1. Introduction

TCR recognition and specificity to the external antigen that drives the GC response is very important for Tfh differentiation and function. On one hand, Tfh differentiation depends on the TCR recognition of the antigen presented first by DCs and later by B cells, and there seems to exist a relation between TCR affinity to the antigen and the capacity of a CD4 T cell to differentiate into a Tfh cell[117,118]. On the other hand, the TCR specificity to the antigen driving the GC response is important for Tfh function in the GC as it allows these cells to distinguish between GC B cells that have improved their affinity to the antigen from the ones that suffered detrimental mutations to their affinity[79].

The specificity of Tfr cells, however, is still mostly unknown. For once, based on published results and the results presented on the previous chapter, the vast majority, if not all in physiological conditions, of these cells seems to originate from tTreg cells. Consequently, one would expect Tfr cells to share specificity towards self-antigens with the cells they originate from. Moreover, Tfr cells are involved in controlling the quantity and affinity of the antibodies produced as well as in preventing the development of auto-reactive B cells, and these functions are also in line with a different TCR usage between Tfr and Tfh cells.

The only report that so far addressed Tfr cells specificity stated that a small percentage of Tfr cells could be specific for the immunizing antigen by originating not from tTreg, but from Tconv cells[346]. Nonetheless, the report only addressed the specificity of a small portion of the Tfr population, leaving the specificity of the overall Tfr population still unknown.

In order to determine if Tfr cells are specific for the antigen driving the GC response, three lines of experiments were performed. First, it was investigated if TCR-transgenic $FOXP3^+$ tTreg cells, specific for the immunizing antigen, are preferentially recruited into the GC upon immunization with the corresponding antigen, as occurs in the case of TCR-transgenic $FOXP3^-$ Tconv that are recruited as Tfh cells. It was also assessed if antigen-specific Tfr cells could be detected using MHC-II $I\text{-}A^b$ tetramers upon immunization. Lastly, it was studied the ability of Tfr cells to survive and proliferate *in vitro* when cultured in the presence of antigen signals.

2.2. Results

OVA-specific TCR-transgenic CD4 T cells from OT-II.Rag^+ mice that, as previously described, harbor a population of TCR-transgenic $FOXP3^+$ tTreg cells, capable of differentiation into Tfr cells, were used to assess the specificity requirements of Tfr cells. As

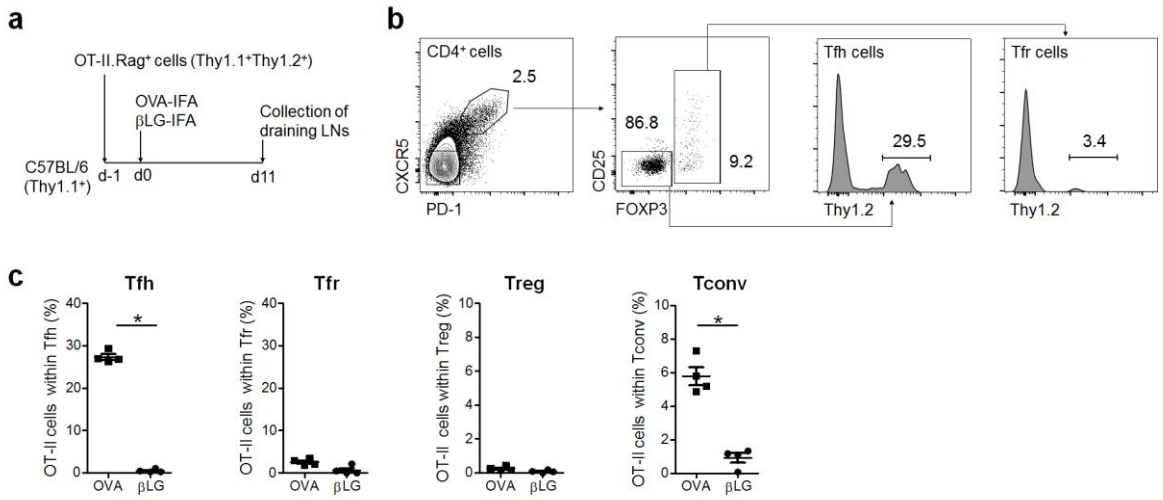

Figure 12– Preferential accumulation of OVA-specific cells within Tfh, but not Tfr cells.
(**a**) 10^7 CD4 T cells from OT-II.Rag$^+$ mice were transferred into C57BL/6 hosts, subsequently immunized with OVA-IFA or βLG-IFA in the footpad. (**b**) Popliteal LNs were analyzed for the presence of OT-II.Rag$^+$ TCR-transgenic cells within Tfh and Tfr populations based on Thy1.2 staining. Tfh cells were defined as CD4$^+$CXCR5^{high}PD-1^{high}FOXP3$^-$ and Tfr cells as CD4$^+$CXCR5^{high}PD-1^{high}FOXP3$^+$. The same gating strategy was applied on Treg cells (CD4$^+$CXCR5$^-$PD-1$^-$FOXP3$^+$) and Tconv cells (CD4$^+$CXCR5$^-$PD-1$^-$FOXP3$^-$) to determine the percentage of Thy1.2$^+$ OT-II.Rag$^+$ TCR-transgenic cells within these two populations. (**c**) OVA-specific TCR-transgenic cells were over-represented within Tfh and Tconv populations in mice immunized with OVA (* $p<0.05$). Within Tfr and Treg cells there was no significant increase in OVA-specific cells in mice immunized with OVA, compared to βLG immunized animals. Similar results were obtained in two additional independent experiments, with n=5.

schematized in Figure 12a, OVA-specific TCR-transgenic CD4 T cells from these mice were transferred into congenic C57BL/6 mice, subsequently immunized with OVA or a control antigen (βLG) in IFA. It was possible to quantify the frequency of TCR-transgenic cells within the Tfh and Tfr populations using congenic markers (Figure 12a,b). It was found that a large fraction of Tfh cells in mice immunized with OVA were derived from OVA-specific precursors, while those TCR-transgenic precursors were almost excluded from Tfr cells even in OVA-immunized mice (Figure 12b,c).

To exclude a potential incapacity of TCR-transgenic FOXP3$^+$ tTreg cells to differentiate into Tfr cells due to over-representation of host FOXP3$^+$ tTreg cells, adoptive cell transfers were performed into TCRβ$^{-/-}$ mice, allowing competition of the same number of WT and TCR-transgenic cells (Figure 13a). Once again, recipient mice were immunized 1 day after transfer and 11 days later draining LNs were analyzed by flow cytometry. Under those conditions, TCR- transgenic cells became vastly over-represented among Tfh cells in mice immunized with OVA (Figure 13b,c). In addition, TCR-transgenic cells differentiated into Tfr in significant numbers, but their frequency (approximately 1/5 of Tfr cells) did not change regardless of the immunizing antigen. Additionally, the percentage of OT-II.Rag$^+$ Treg cells

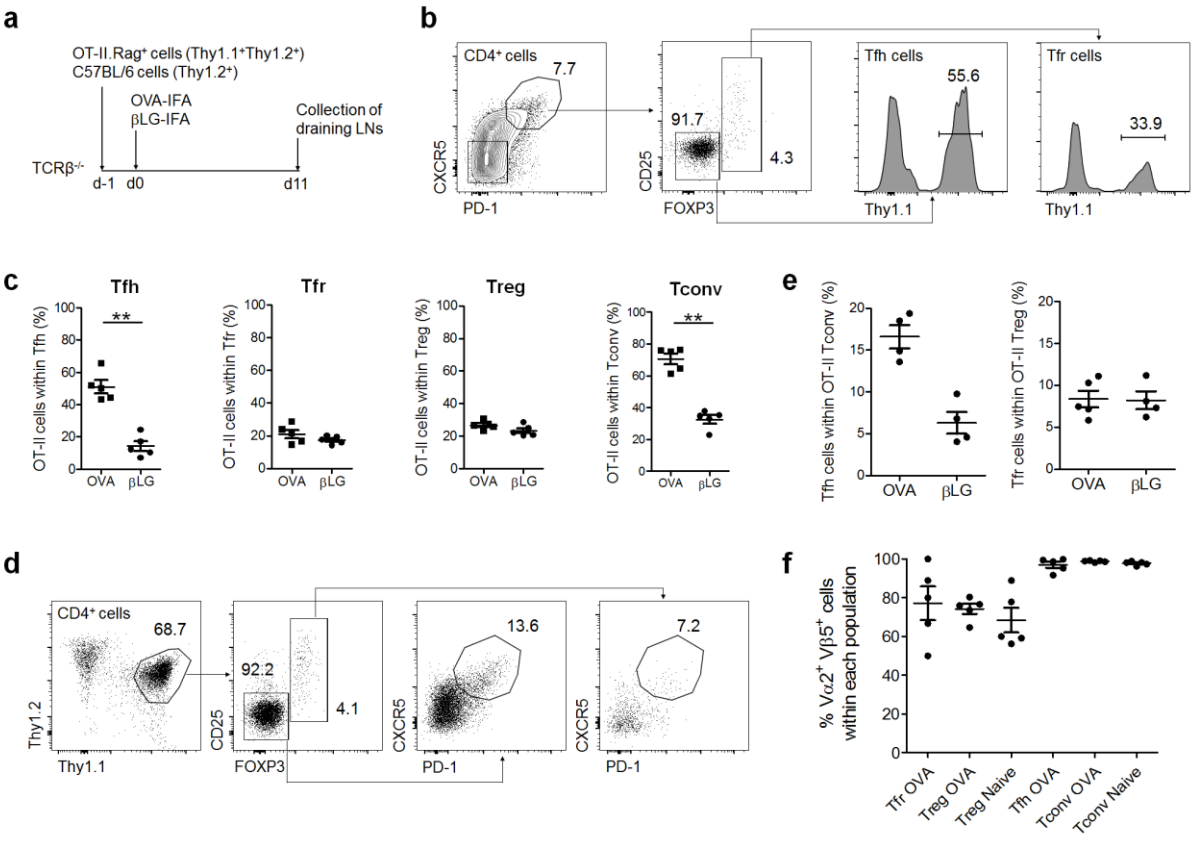

Figure 13– Recruitment of OVA-specific cells as Tfr cells is independent of the immunizing antigen. (**a**) An equal number (10^7) of CD4 T cells from OT-II.Rag$^+$ and C57BL/6 mice were transferred into T cell deficient TCRβ$^{-/-}$ mice, subsequently immunized with OVA-IFA or βLG-IFA in the footpad. (**b**) Gating strategy to analyze the presence of OT-II.Rag$^+$ TCR-transgenic cells within Tfh and Tfr populations based on Thy1.1 staining. (**c**) Under these conditions, there was a higher representation of OVA-specific cells within Tfh and Tconv populations (* $p<0.05$; ** $p<0.01$). Although ~20% of Tfr cells derived from the OVA-specific TCR-transgenic population, that frequency remained similar in mice immunized with OVA or βLG. (**d**) Gating strategy to determine the percentage of OT-II Treg and Tconv cells that differentiate into Tfr and Tfh cells, respectively. (**e**) While the percentage of OT-II Tconv that differentiate into Tfh is higher in mice immunized with OVA than βLG (left), the same percentage of OT-II Treg originates Tfr cells in both immunizations (right). (**f**) Frequency of Vα2 Vβ5 double-positive cells within Tfr, Treg, Tfh, and Tconv populations from several conditions. Mean ± SD are presented in all graphs. Similar results were obtained in two additional independent experiments, with n=5.

that differentiated into Tfr was approximately 8% for both immunizing antigens, although, as expected, in OVA-immunized mice the percentage of OT-II.Rag$^+$ Tconv that differentiate into Tfh was much higher than in βLG-immunized mice (Figure 13d,e).

FOXP3$^+$ Treg cells from OT-II.Rag$^+$ mice co-express endogenous TCR-chains, in addition to the transgene, that allow their thymic selection as Treg cells[380]. In fact, WT T cells can also express more than one TCR, due to recombination of both TCRα-chains[381]. Accordingly, and as previously described, a proportion of Treg cells (~30%) do not co-express the transgenic TCR chains Vα2 and Vβ5, unlike Tconv cells which are virtually all double-positive. Nevertheless, upon adoptive transfer into TCRβ$^{-/-}$ mice and immunization,

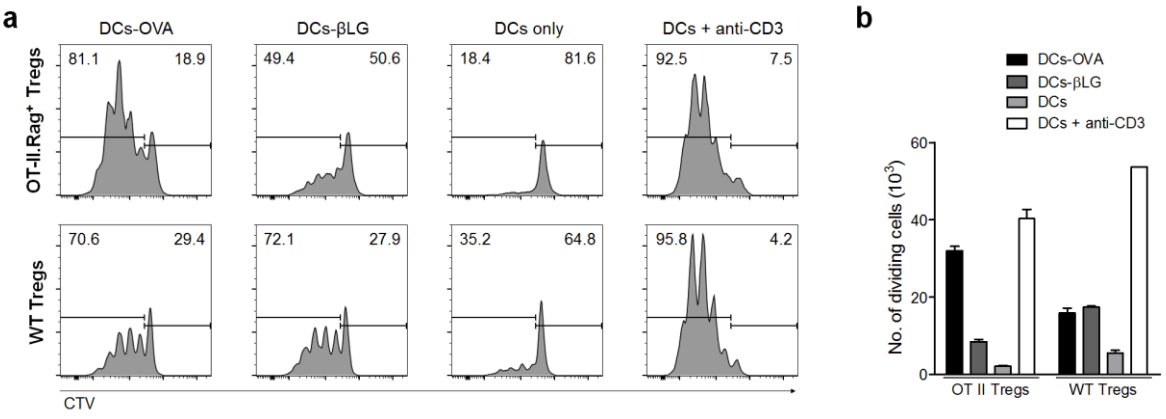

Figure 14– OT-II Treg cells specifically proliferate with OVA signals.
(**a**) FACS-sorted C57BL/6 and OT-II.Rag$^+$ Treg (CD4$^+$CD25$^+$GITR$^+$) cells were labelled with CellTrace Violet (CTV) and cultured for three days in presence of IL-2 and bone marrow DCs loaded with OVA or βLG. In control groups, T cells were cultured with unloaded DCs with or without soluble anti-CD3. Histograms are representative of Treg (CD4$^+$TCRβ$^+$CD25$^+$FOXP3$^+$) cell proliferation at the end of the culture. (**b**) Quantification of the number of proliferating cells. Data are representative of three independent experiments. Mean ± SD are presented in all graphs.

the percentage of OT-II.Rag$^+$ Treg and Tfr cells co-expressing Vα2 and Vβ5 remained unchanged (Figure 13f). Therefore, a preferential enrichment of Vα2 Vβ5 double-positive cells, more likely to be specific for the immunizing antigen, could not be found within the regulatory populations after OVA immunization.

The above observations may question the ability of FOXP3$^+$ cells from OT-II.Rag$^+$ mice to properly respond to OVA stimulation. Therefore, the capacity of OT-II Treg cells to be activated by OVA was confirmed *in vitro*. Sorted Treg (CD4$^+$CD25$^+$GITR$^+$) cells from OT-II.Rag$^+$ and C57BL/6 WT mice were cultured in presence of IL-2 and OVA-pulsed bone marrow-derived DCs. As control, Treg cells from both strains were also cultured with DCs loaded with a control antigen (βLG) or unloaded DCs with or without anti-CD3. As can be observed on Figure 14, OVA-loaded DCs, unlike DCs loaded with a control antigen, promoted proliferation of OT-II Treg cells to a similar extent as stimulation with anti-CD3. Moreover, WT Treg cells show lower proliferation and cell numbers when cultured in the presence of both antigens compared to cultures with anti-CD3. These data demonstrate that OT-II Treg cells, in spite of the co-expression of endogenous TCR chains, specifically recognize and proliferate in presence of OVA stimulation. As a consequence, the poor differentiation of OT-II Treg cells into Tfr cells following OVA-immunization does not appear to be due to loss of OVA-reactivity.

The low recruitment of OT-II.Rag$^+$ Treg cells as Tfr cells could be due to an intrinsic characteristic of OT-II transgenic cells. Thus, to test this possibility, the experiment was

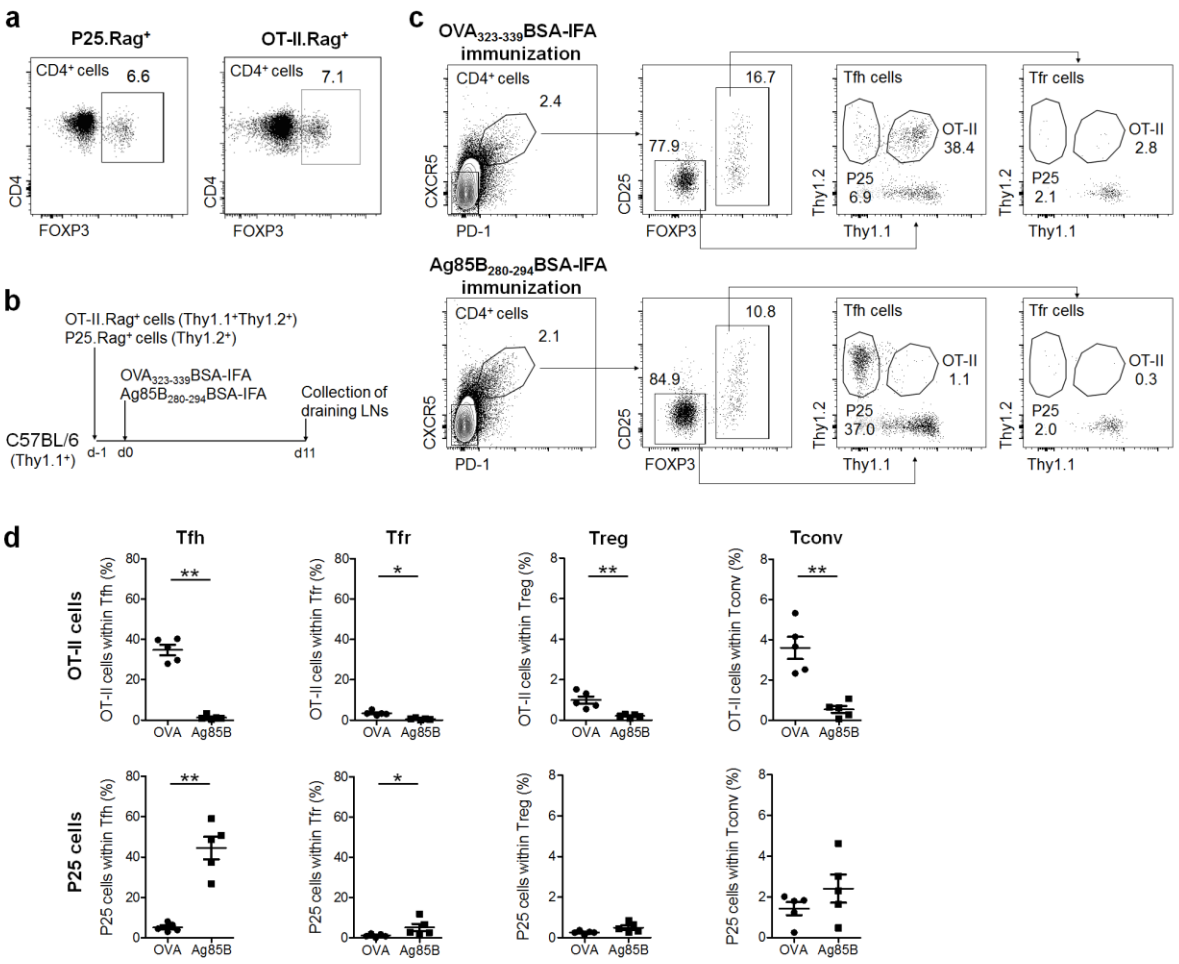

Figure 15– Recruitment of P25-specific and OVA-specific T cells into the Tfh pool of mice immunized with the corresponding antigens.

(**a**) P25.Rag$^+$ mice have similar frequency of tTreg cells as OT-II.Rag$^+$ mice. (**b**) C57BL/6 mice were transferred simultaneously with 10^7 CD4 T cells from OT-II.Rag$^+$ and P25.Rag$^+$ mice, and subsequently immunized with either $OVA_{323-339}$BSA-IFA or $Ag85B_{280-294}$BSA-IFA in the footpad. (**c**) Gating strategy to determine the percentage of OT-II.Rag$^+$ and P25.Rag$^+$ cells within Tfh and Tfr populations in mice immunized with $OVA_{323-339}$BSA-IFA (upper panel) or $Ag85B_{280-294}$BSA-IFA (bottom panel). (**d**) T cell subsets from draining LNs show that mice immunized with $OVA_{323-339}$ have a large accumulation of OVA-specific cells within the Tfh and Tconv populations while, conversely, $Ag85B_{280-294}$-immunized mice accumulate P25-specific T cells among Tfh cells (* p<0.05; ** p<0.01). A very small increase of T cells specific for the immunizing antigen is observed among the Tfr population. Mean ± SD are presented in all graphs. Data are representative of three independent experiments, each with n=5.

repeated with a second TCR-transgenic model. P25.Rag$^+$ is a TCR-transgenic mouse strain which CD4 cells are specific for the residues 280-294 of Mycobacterium tuberculosis Ag85B[382]. Like OT-II.Rag$^+$, this strain is on a RAG sufficient background and also has thymic-derived Treg cells (Figure 15a). An equal number of CD4 T cells from OT-II.Rag$^+$ and P25.Rag$^+$ mice was transferred, simultaneously, into WT C57BL/6 hosts (Figure 15b). Recipient mice were immunized in the footpad with $OVA_{323-339}$ or $Ag85B_{280-294}$ peptides coupled to BSA in IFA 1 day later. Eleven days after immunization, Tfh cells from the

draining LNs had a substantial increase in the frequency of TCR-transgenic cells specific for the immunizing antigen: a high frequency of OT-II cells in mice immunized with $OVA_{323-339}$BSA, and P25 cells in mice immunized with $Ag85B_{280-294}$BSA (Figure 15c,d). However, within the Tfr population there was only a small increase of TCR-transgenic cells specific for the immunizing antigen.
These results suggest that, while TCR-transgenic cells specific for the immunizing antigen are able to become Tfr cells, those cells are not preferentially selected into the Tfr pool.

To obtain an independent validation *in vivo* of the previous results, without the use of TCR-transgenic mice, two MHC-II I-A^b tetramers were used to detect antigen-specific CD4 T cells: a PE-labelled tetramer containing an OVA peptide sequence (AAHAEINEA) to identify OVA-specific T cells and an APC-labelled tetramer containing the Ag85B peptide sequence (FQDAYNAAGGHNAVF) to identify Ag85B-specific T cells. Antigen-specific tetramer$^+$ Tfr and Tfh cells were detected on draining LNs of WT C57BL/6 mice 11 days after immunization with several combinations of antigens and adjuvants: $OVA_{323-339}$-CFA, $OVA_{323-339}$-IFA, $OVA_{323-339}$BSA-IFA, $Ag85B_{280-294}$-CFA, $Ag85B_{280-294}$-IFA, and $Ag85B_{280-294}$BSA-IFA (Figure 16a). In all immunizations with $OVA_{323-339}$, the number of OVA-tetramer$^+$ cells was increased among the Tfh population compared to mice that were not immunized with this peptide (Figure 16b,c). The same pattern was observed in mice immunized with $Ag85B_{280-294}$, where larger populations of Ag85B-tetramer$^+$ Tfh cells were also found (Figure 16d,e). However, in neither case there was an enrichment of tetramer$^+$ cells within the Tfr population (Figure 16b-e). Once again, these results demonstrate that Tfr cells, unlike Tfh cells, do not preferentially contain cells specific for the non-self antigen driving the GC response.

Lastly, it was investigated whether Tfr and Tfh TCRs recognize different peptides from the same antigen. Proliferation of Tfh, Tfr, and Treg cells was assessed after stimulation with DCs loaded with the immunizing antigen in order to verify if they specifically proliferate with these signals. Sorted Tfh ($CD4^+CXCR5^{high}PD1^{high}FOXP3^-$) cells from OVA-immunized C57BL/6 FOXP3 reporter mice ($FOXP3^{hCD2}$)[383] were cultured with DCs loaded with OVA or βLG as a control antigen. Tfh cells showed higher proliferation and survival on OVA cultures compared to DCs loaded with βLG or unloaded DCs, demonstrating that this population specifically recognizes antigen signals (Figure 17a,b). On the contrary, Tfr ($CD4^+CXCR5^{high}PD1^{high}FOXP3^+$) cells cultured with OVA presented some proliferation, but that proliferation was not higher than in the presence of βLG or unloaded DCs (Figure

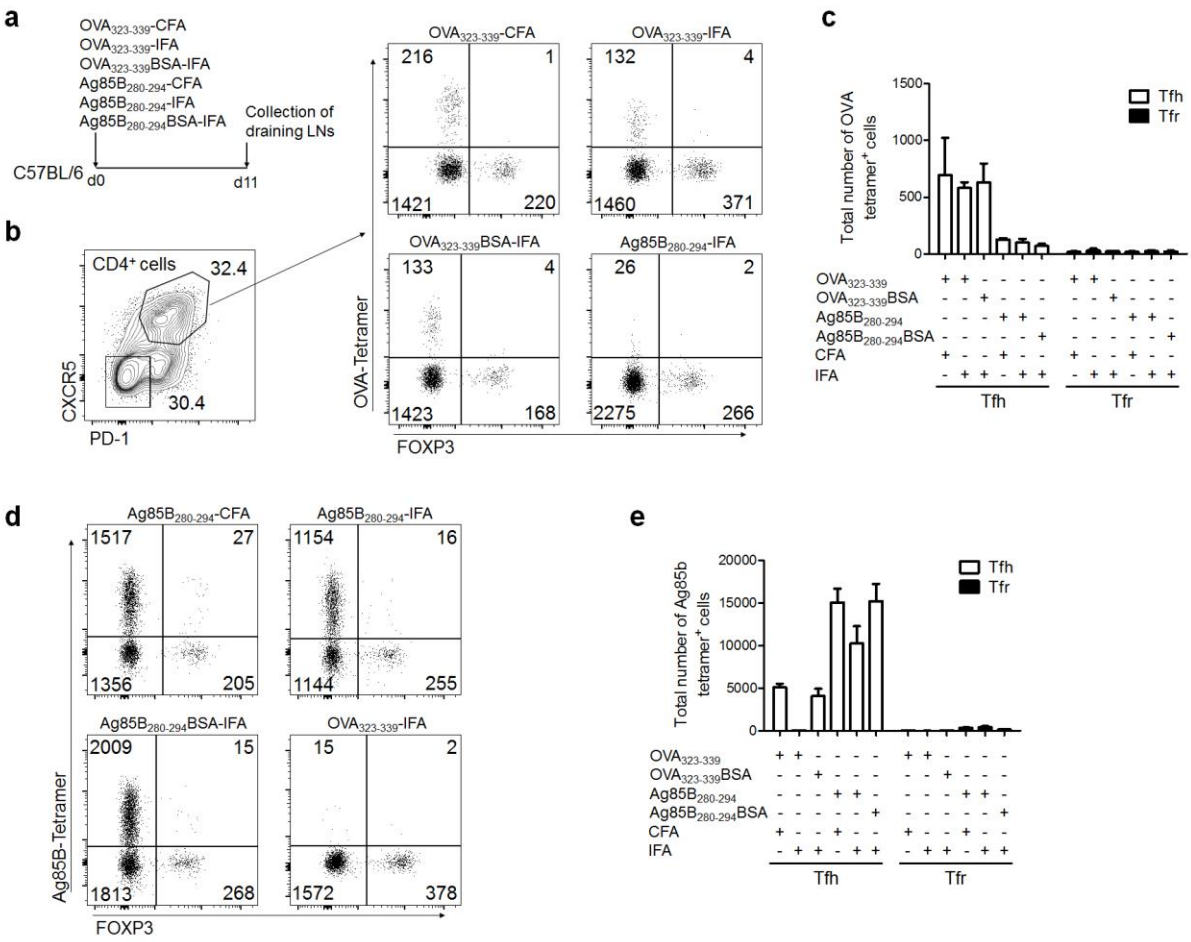

Figure 16– Tetramer$^+$ cells are predominantly found among the Tfh population.
(**a**) C57BL/6 mice were immunized with four different antigens combined with two different adjuvants on a total of 6 different immunizations. On day 11, draining LNs were collected for tetramer binding cells detection by flow cytometry. (**b**) Gating strategy to identify OVA-tetramer$^+$ Tfh and Tfr cells within CD4$^+$ cells. The Tfh population from mice immunized with the three conditions containing $OVA_{323-339}$ (independently of the adjuvant) was enriched on cells with TCRs capable of binding the OVA-tetramer. Such OVA-tetramer$^+$ cells were almost absent in the Tfr population in all immunizations. Scatter plots are representative of the results obtained for each of the immunizations with $OVA_{323-339}$ and of the controls immunized with different formulations of $Ag85B_{280-294}$. (**c**) Total number of OVA-tetramer$^+$ cells in the draining LNs. (**d**) Scatter plots of Ag85B-tetramer$^+$ Tfh and Tfr cells within follicular CD4 T cells. As observed for the OVA-tetramer, a large population on tetramer$^+$ Tfh cells was observed on immunizations containing $Ag85B_{280-294}$ peptide while low number of tetramer$^+$ Tfr cells were found in all immunizations. Scatter plots are representative of the results obtained for each of the immunizations with $Ag85B_{280-294}$ and of the controls immunized with $OVA_{323-339}$. (**e**) Total number of $Ag85B_{280-294}$-tetramer$^+$ cells in the draining LNs. Mean±SEM are presented. Data are representative of two independent experiments, each with n=5.

17c,d). Nevertheless, the presence of anti-CD3 induced a higher proliferation rate and survival of Tfr cells that translated into higher number of cells at the end of culture compared to the other conditions, indicating that if Tfr cells were specific for the immunizing antigen, TCR signaling would have led to higher proliferation and cell numbers in the end of culture (Figure 17d). As Tfr cells, Treg ($CD4^+CXCR5^-PD1^-FOXP3^+$) cells sorted from immunized

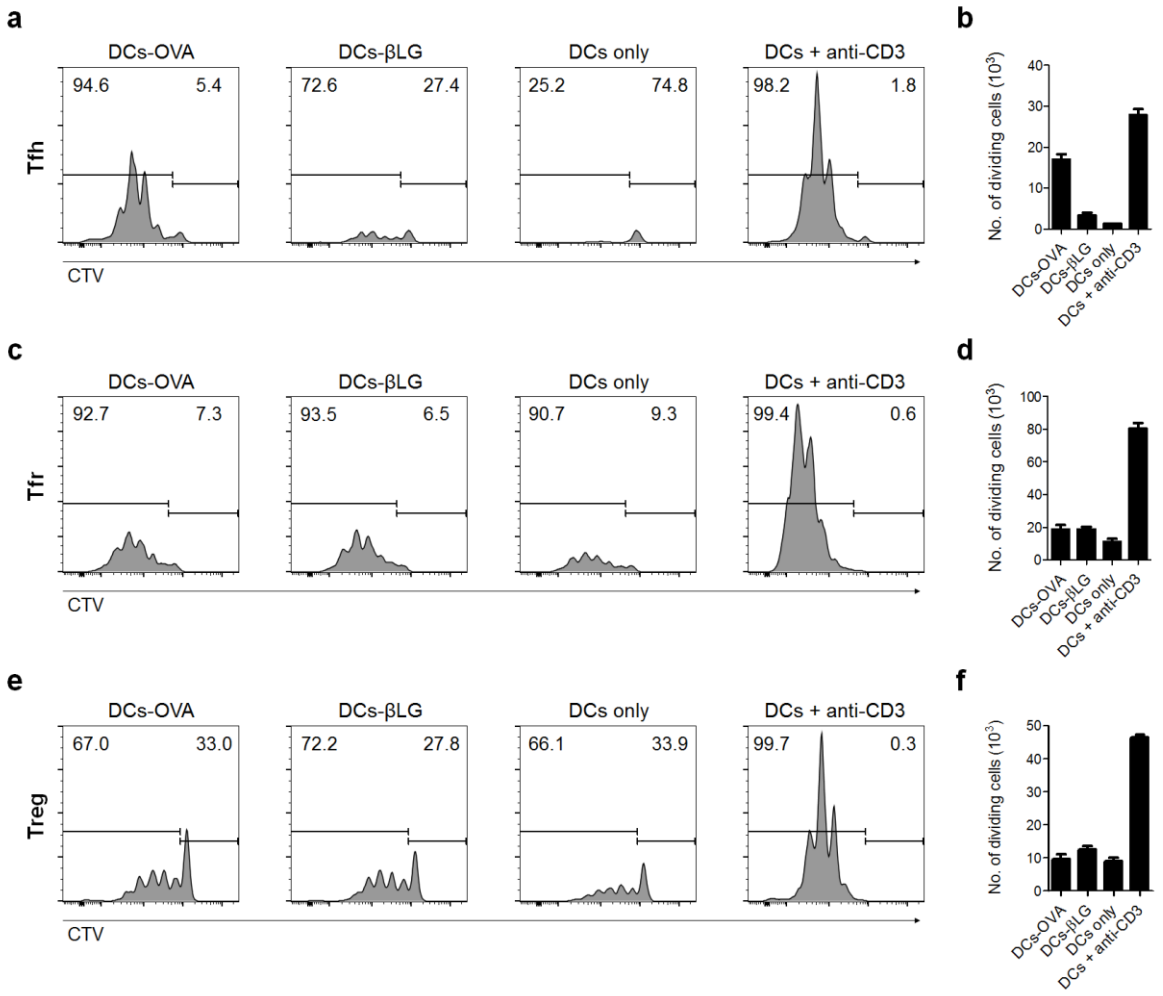

Figure 17– Tfr cells do not proliferate or survive with antigen signals.
(**a**)(**c**)(**e**) Histograms of proliferation of sorted (**a**) Tfh ($CD4^+CXCR5^{high}PD\text{-}1^{high}FOXP3^-$), (**c**) Tfr ($CD4^+CXCR5^{high}PD\text{-}1^{high}FOXP3^+$), and (**e**) Treg ($CD4^+CXCR5^-PD\text{-}1^-FOXP3^+$) cells from OVA-immunized $FOXP3^{hCD2}$ reporter mice cultured for three days with DCs loaded with OVA or βLG proteins. Cultures with unloaded DCs with or without anti-CD3 were performed as positive and negative controls, respectively. All cultures were performed in the presence of exogenous IL-2. (**b**)(**d**)(**f**) Bar graphs of total cell numbers obtained at end of culture as in (**a**), (**b**), and (**c**), respectively. Only Tfh cells show higher proliferation and total number on OVA-pulsed DCs cultures compared to βLG-pulsed ones, demonstrating that only this cell population specifically recognizes OVA-signals. Triplicate cultures were performed: one replicate is presented on the histograms; triplicates Mean±SD are presented on the bar graphs.

mice also show lower proliferation and cell numbers when cultured with DCs loaded with OVA or βLG compared to the culture in the presence of anti-CD3 (Figure 17e,f) and as previously observed on Figure 14. Taken together, these results demonstrate that, while Tfh cells specifically benefit from signals derived from the immunizing antigen, Tfr and Treg cells do not.

2.3. Discussion

Three different approaches were used to address the question of whether Tfr cells are specific for the non-self antigen driving the GC response.

The first set of experiments, with TCR-transgenic cells, suggests that tTreg cells specific to the non-self immunizing antigen do not preferentially differentiate into Tfr cells. TCR-transgenic models on RAG-sufficient background were used since, as shown on the previous results chapter, these mice harbor a population of TCR-transgenic FOXP3^{+} tTreg cells that can differentiate into Tfr cells. The differentiation as tTreg cells in the thymus is possible due to the expression of more than one TCR. Nevertheless, OT-II.Rag^{+} tTreg cells still expressed the transgene and were capable of recognizing and specifically proliferating with OVA signals, the antigen to which the transgene is specific.

Of note, on the experiments where the capacity of OT-II Treg cells proliferation with OVA signals was assessed, some unspecific proliferation of WT Treg cells was observed regardless of the antigen in culture. This observation is in line with previous reports describing that Treg cells do proliferate *in vitro* when cultured with activated DCs and in the presence of exogenous IL-2[384,385]. Furthermore, this unspecific proliferation is observed without providing the specific antigen to the culture, but it is dependent on TCR signaling (possibly driven by self antigens present in the APC).

Another important aspect of the results presented with this approach is the percentage of TCR-transgenic cells recruited into the GC when cells are transferred into the TCRβ$^{-/-}$ mice. In this case, approximately 1/5 of Tfr cells originated from TCR-transgenic cells independently of the immunizing antigen. These results are an indication that, in physiological conditions, some Tfr cells may present some reactivity against the antigen driving the GC response. However, such antigen-specificity is not the main driver of these cells towards the GC.

On the second approach MHC-II I-A^{b} tetramers were used to detect antigen-specific Tfr and Tfh cells on mice immunized with the corresponding peptides in different formats and adjuvants. As expected, high numbers of tetramer^{+} Tfh cells were detected in all immunizations where the corresponding antigen was present. However, Tfr cells were almost not detected even in conditions described as propitious for pTreg differentiation and induction of antigen-specific Tfr cells. These data suggest that, contrary to Tfh cells, the Tfr population does not preferentially contain cells specific for the immunizing antigen.

This approach has been extensively used by others to detect antigen-specific cells using MHC-II tetramers within activated CD4 T cells. Moreover, it has also been used to detect antigen-specific Tfh and Tfr cells. The presented results regarding tetramer$^+$ Tfh cells are in agreement with other groups' results, which have shown the presence of antigen-specific Tfh cells, detectable with MHC-II tetramers upon infection or immunization[118,229,346,349]. However, the two reports that also verified the existence of antigen-specific tetramer$^+$ Tfr cells upon immunization are contradictory: while one detected a tetramer$^+$ Tfr population, the other did not[346,349]. Thus, the data presented here are in line with the first published report as almost no tetramer$^+$ Tfr cells could be detected.

Interestingly, Ag85B-tetramer$^+$ Tfh cells were not only detected on mice immunized with Ag85B$_{280\text{-}294}$ or CFA (which contains *Mycobacterium tuberculosis* and consequently the antigen), but also on mice immunized with OVA$_{323\text{-}339}$BSA. These results suggest the existence of some cross-reactivity between Ag85B$_{280\text{-}294}$ and a BSA epitope (in OVA$_{323\text{-}339}$ immunization this population was not detected). The presence of this tetramer$^+$ population on every mouse (in all experiments performed) immunized with OVA$_{323\text{-}339}$BSA is quite striking. However, it may be explained by the size of the initial tetramer$^+$ population prior to immunization. It has been described that the size of a response to an antigen depends on the size of the initial population capable of recognizing that antigen[386,387]. Moreover, it has been also shown that Tconv cells that suffer low clonal deletion in the thymus, and consequently have a higher initial population, are associated with highly cross-reactive TCRs[388]. Accordingly, in the results presented, the number of Ag85B-tetramer$^+$ cells found after immunization with the corresponding antigen was higher than in the case of OVA-tetramer$^+$ cells. This indicates that a relatively larger population of Ag85B-tetramer$^+$ cells was present in steady-state. Thus, these Ag85B-tetramer$^+$ cells have an advantage in getting activated during an immune response, since they are initially present in higher numbers and potentially have cross-reactive TCRs.

As last approach, the capacity of Tfr cells to recognize and proliferate with signals from the immunizing antigen was assessed *in vitro*. As expected, Tfr cells showed the same level of proliferation, reaching similar cell numbers, at end of culture independently of the antigen provided in the DCs. Like before, background proliferation was observed when Treg cells were cultured with DCs and exogenous IL-2. This background proliferation was even higher in the case of Tfr cells, but this result may be due to Tfr cells activated and proliferative state

(compared to Treg cells). Once again, these results suggest that the Tfr population is not enriched in cells specific for the immunizing antigen.

Therefore, the overall results presented here are in line with the hypothesis that Tfr cells are not specific for the non-self immunizing antigen driving the GC response.

3. Tfr and Tfh populations have different TCR repertoires

3.1. Introduction

The TCR and its ability to specifically recognize antigens is one of the key characteristics of T cells and their ability to mount specific immune responses. Accordingly, the study of the TCR repertoire of a population may provide insights into the specificity, origin, and biology of those cells.

Numerous studies have described the TCR usage of distinct cell populations using different methods. However, the TCR usage of Tfr and Tfh cells as not been addressed yet.

The results presented on previous sections support a model where Tfr cells originate from tTreg cells and are not specific to the antigen driving the immune response. Thus, by obtaining information on the repertoire of these cells, further data supporting the model may be obtained.

To study the repertoire of Tfr, Treg, Tfh, and Tconv cells, the TRBV CDR3 length usage of these populations was initially analyzed by spectratyping.

Additionally, to obtain further information on the repertoire of Tfr, Treg, and Tfh, as well as activated and non-activated CD4 T cells, the TRA of these populations from immunized 1D2 β was also sequenced. These mice express a transgenic (fixed) TRB chain and only one *Tra* allele is available for recombination; thus, the TRA sequencing provides a complete insight to the repertoire of the analyzed populations.

3.2. Results

TCR usage analysis of Tfr cells from C57BL/6 mice immunized with OVA-IFA was performed to obtain a better insight into the TCR specificity of Tfr cells. Taking advantage of the $FOXP3^{gfp}$ reporter system, Tfh ($CD4^+CXCR5^+PD1^+FOXP3^-$), Tfr ($CD4^+CXCR5^+PD1^+FOXP3^+$), Treg ($CD4^+CXCR5^-PD1^-FOXP3^+$), and Tconv ($CD4^+CXCR5^-PD1^-FOXP3^-$) cells from the draining LNs were sorted, and the TRBV repertoire of the different populations analyzed by CDR3 spectratyping/Immunoscope®[366].

Once again, in order to minimize the contamination with pre-existent Tfh and Tfr cells from prior immune responses, mice were immunized in the footpad and popliteal LNs were collected at the peak of the GC response: in this anatomical location there are negligible numbers of Tfh and Tfr cells prior to immunization.

As described before and shown in Figure 18, TRBV CDR3 length profiles (or spectratypes) obtained from naïve polyclonal CD4 T cells resemble Gaussian distributions[366]. Therefore, by comparing the CDR3 length usage for each TRBV of the four sorted populations to naïve

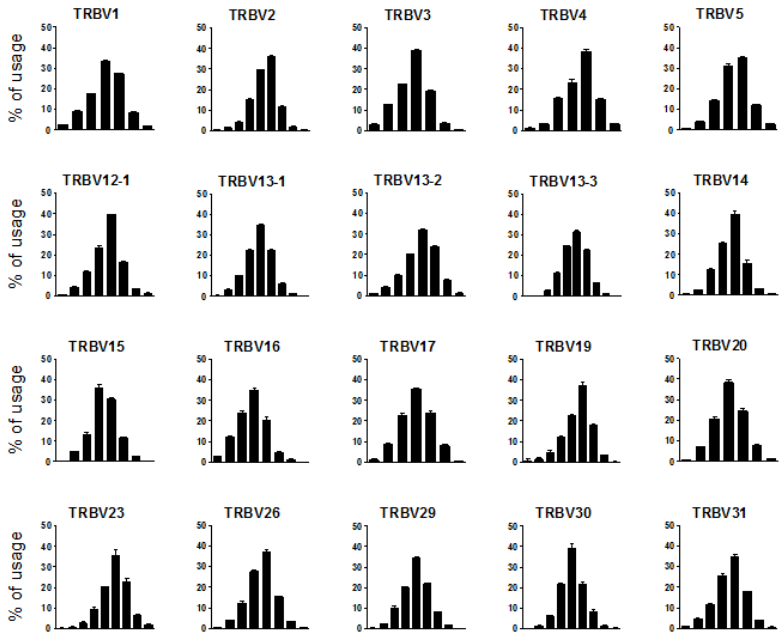

Figure 18 – CDR3 spectratypes obtained from naïve CD4 T cells.
CDR3-length usage distribution of 20 TRBV segments from $CD4^+$ T cells from naïve mice that presents a Gaussian-like distribution. Bar graphs present Mean±SEM of four samples.

CD4 T cells (used as control population), variations to this polyclonal distribution can be detected which, in the case of over-representations in specific CDR3 lengths, may be an indication of clonal expansions. Indeed, in the Tfh population there was an over-representation of specific CDR3 lengths, demonstrated by the red lines on Figure 19a. There were also some over-representations for the Tfr cells, but the majority of these, besides not being common to Tfh cells, were also present in Treg cells. In fact, detailed analysis of specific TRBV segments allowed the identification of clonal expansions among Tfh cells that were absent in the other T cell subsets (Figure 19b, arrows). Moreover, perturbation scores[369] were calculated for every TRBV segment between all samples and the naïve CD4 T cells average or the Tfr group average. The perturbation scores reveal the divergent TRBV usage of all cell populations compared to the chosen control group. Hierarchical clustering was then performed using the calculated perturbation scores to reveal the divergent TRBV usage of all cell populations compared to the control populations. As shown on Figure 20, independently of the population used as control group (the naïve CD4 T cells average on Figure 20a and the Tfr group average on Figure 20b), Tfh cells are clustered separately from Tfr cells, while there is a proximity between Tfr and Treg cells). Also, as it can be observed on Figure 20b, Tfh samples present the higher perturbation scores when Tfr cells are used as control group which indicates a more divergent TRBV CDR3 length usage compared to Tfr

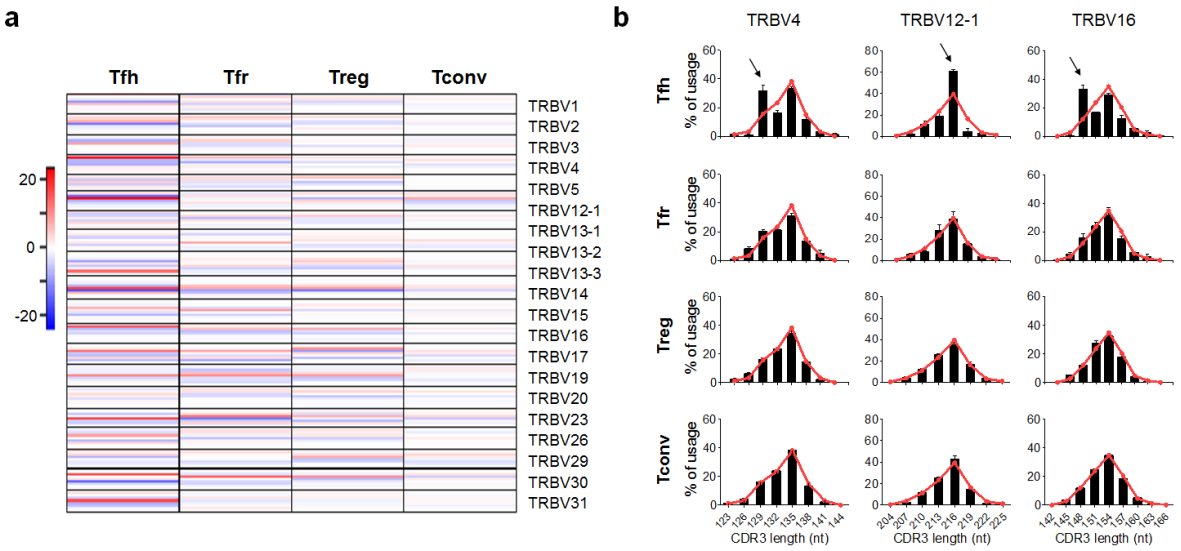

Figure 19 – Tfh cells present over-representations of specific CDR3 lengths.
(a) Heatmap showing the differences between the percentage of usage for each CDR3 length of each TRBV of Tfh, Tfr, Treg, and Tconv populations compared to the Gaussian-like distribution of CD4 naïve T cells (used as a control population). Similar TRBV CDR3 length frequencies (compared to control population) are displayed in white, while higher/lower frequencies of specific CDR3 lengths are represented, respectively, in red and blue. The heatmap is representative of, at least, three independent experiments per population. (b) Distribution of CDR3-length usage for three representative TRBV segments where greatest variation is observed. Bars represent CDR3-length usage distribution for indicated populations, with the reference values (naïve CD4 T cells) superimposed in red. Arrows indicate over-representation of a specific CDR3 length on Tfh cells, a putative consequence of clonal selection and expansion. Neither Treg nor Tfr cells show similar expansions (bar graphs below). Mean ± SEM of at least three independent replicates are represented on the bar graphs. Cells from 10 – 15 mice were sorted to obtain each replicated.

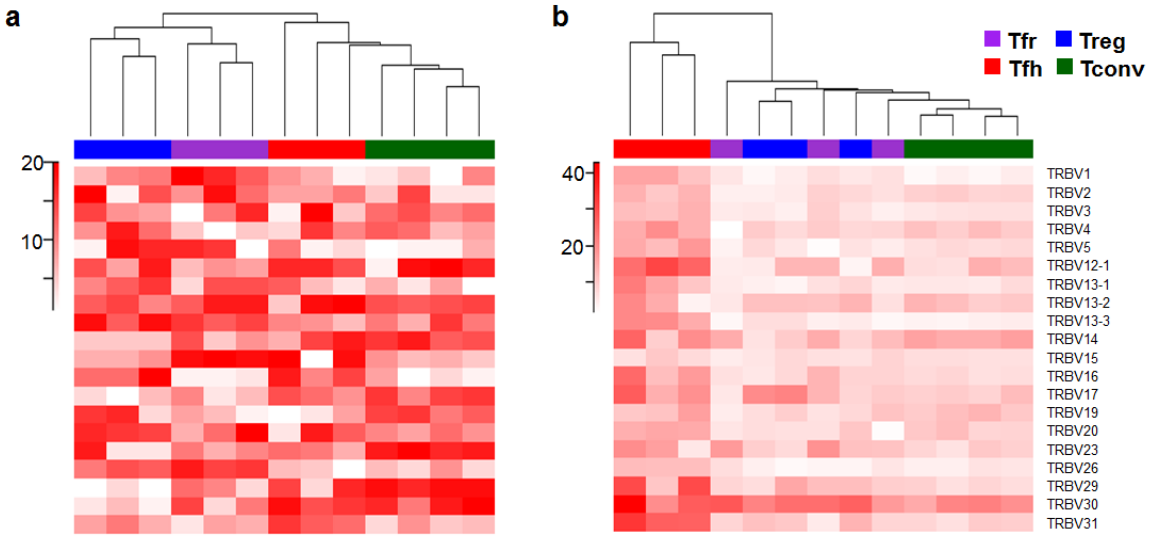

Figure 20 – Analysis of CDR3 length distribution unveils a closeness between Tfr and Treg cells.
(**a**) Hierarchical clustering of the samples from the four populations based on their TRBV perturbation scores, calculated using the naïve CD4 T cells CDR3 length distributions as control group. Heatmap color code indicates variations of TRBV scores between each sample and the average of naïve CD4 T cells, while the dendrogram shows distance between sample populations. (**b**) Same as in (**a**) but using the Tfr group average as reference. For each T cell population, at least three independent replicates were used, each one with cells sorted from 10 – 15 mice.

Table 6 – Pairwise Multiple Comparison Analysis with Holm-Bonferroni Correction between Samples TRBV Perturbation Scores[a]

	Tconv1	*Tconv2*	*Tconv3*	*Tconv4*	*Tfh1*	*Tfh2*	*Tfh3*	*Tfr1*	*Tfr2*	*Tfr3*	*Treg1*	*Treg2*
Tconv2	1	-	-	-	-	-	-	-	-	-	-	-
Tconv3	1	1	-	-	-	-	-	-	-	-	-	-
Tconv4	1	1	1	-	-	-	-	-	-	-	-	-
Tfh1	0,014 *	0,015 *	0,033 *	0,016 *	-	-	-	-	-	-	-	-
Tfh2	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,673	-	-	-	-	-	-	-
Tfh3	0,007 **	0,009 **	0,012 *	0,009 **	1	0,473	-	-	-	-	-	-
Tfr1	1	1	1	1	0,017 *	0,000 ***	0,007 **	-	-	-	-	-
Tfr2	1	1	1	1	0,162	0,000 ***	0,101	0,495	-	-	-	-
Tfr3	1	1	1	1	0,002 **	0,000 ***	0,002 **	1	1	-	-	-
Treg1	1	1	1	1	0,046 *	0,000 ***	0,004 **	1	1	1	-	-
Treg2	1	1	1	1	0,037 *	0,000 ***	0,018 *	1	1	1	1	-
Treg3	1	1	1	1	0,007 **	0,000 ***	0,007 **	1	1	1	1	1

[a] Perturbation scores calculated using Tfr group average as reference
** p<0.05, ** p<0.01, *** p<0.001*

cells. Moreover, as depicted on Table 6, ANOVA and multiple comparison analysis with Holm-Bonferroni correction for multiple sampling established the significance of the differences observed between populations, and between Tfh samples and each sample from the other populations.

Taken together, our data show that, within LNs draining the immunizing site, Tfh cells exhibit clear oligoclonal expansions of specific TRBV CDR3 lengths. The same pattern of TRBV CDR3 usage is not observed within the Tfr population that retain distributions of CDR3 lengths usage similar to Treg cells.

To further verify the different TCR usage between Tfr and Tfh cells, the TRA of 1D2β mice, which express a fixed TCRβ chain and variable TCRα chains, was sequenced.

This 1D2β mouse line (kindly provided by Dr Shohei Hori, RIKEN, Japan), of C57BL/6 background, was established using nuclear-transferred ES (NT-ES) cells that had been generated using peripheral $CD25^{high}CD4^{+}$ T cells as donor of nuclei. The productively rearranged TRB gene of one NT-ES cell line was successfully transmitted to germline and the resulting 1D2β mice were mated with $FOXP3^{hCD2}$ and $TRA^{-/-}$ mice to generate $FOXP3^{hCD2}.TRB^{1D2}.TRA^{-/WT}$ mice. Thus, 1D2β mice TRA sequencing provides a complete

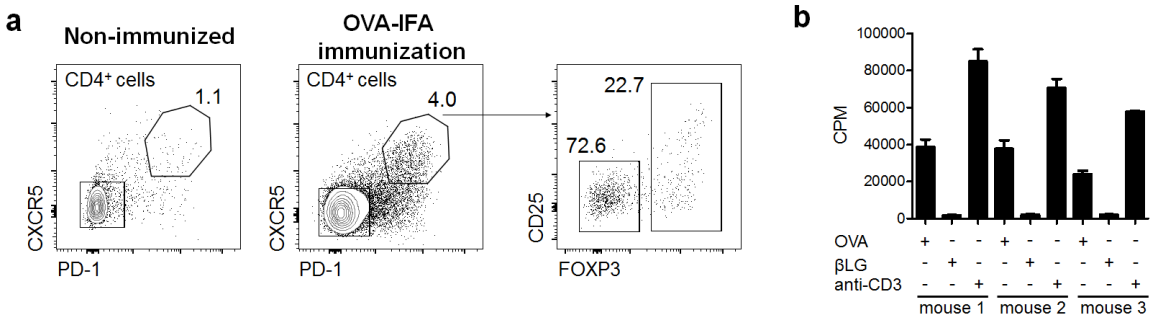

Figure 21 – 1D2β mice can mount specific responses against OVA.
(**a**) Frequency of follicular $CD4^+CXCR5^+PD1^+$ T cells on popliteal LNs of 1D2β mice before (left), and 11 days after OVA-IFA immunization (middle), when both Tfr and Tfh cells are present (right). (**b**) Proliferation of Tfh cells from OVA-IFA immunized 1D2β mice cultured with DCs pulsed with OVA or βLG (anti-CD3 was used as positive control). Cell proliferation was measured by 3H-thymidine incorporation. Mean±SEM of culture triplicates are presented and are representative of two independent experiments.

insight to the repertoire of the analyzed populations, while WT mice do not, as the corresponding TRB does not vary and only one *Tra* allele is available for recombination.

Although these mice have a restricted TRB repertoire, it was confirmed that their CD4 T cells were able to recognize OVA and differentiate into Tfh cells following OVA-immunization (Figure 21a). Furthermore, to show that 1D2β Tfh cells that arise after OVA-immunization can specifically recognize the antigen, *in vitro* proliferation assays were performed (Figure 21b). 1D2β mice were immunized with OVA-IFA and 11 days later Tfh ($CD4^+CXCR5^{high}PD1^{high}FOXP3^-$) cells from three individual mice were sorted and cultured with DCs pulsed with OVA. Tfh cells from all three mice were able to proliferate with OVA signals, but not with the control antigen βLG (Figure 21b). Thus, these mice, while not being able to recombine the TRB, still have a repertoire capable of specifically recognizing OVA.

Five T cell populations were sorted for TRA repertoire analysis on day 11 following OVA-IFA immunization from draining LNs of individual mice: Tfr ($CD4^+CXCR5^{high}PD1^{high}FOXP3^+$), Tfh ($CD4^+CXCR5^{high}PD1^{high}FOXP3^-$), Treg ($CD4^+CXCR5^-PD1^-FOXP3^+$), activated CD4 T (Tact: $CD4^+CXCR5^-PD1^-FOXP3^-CD44^+$) cells, and non-activated CD4 T (Tconv: $CD4^+CXCR5^-PD1^-FOXP3^-CD44^-$) cells. TRA gene was then specifically amplified and sequenced on an illumina MiSeq platform. In order to perform an unbiased analysis, 9,000 TCR sequences were randomly selected from each dataset per sample (9,000 being the lowest number of TCR sequences identified on a sample).

As first analysis, the number of common clonotypes between Tfr cells and other populations was verified. As showed on Figure 22a, the number of shared clonotypes is higher between

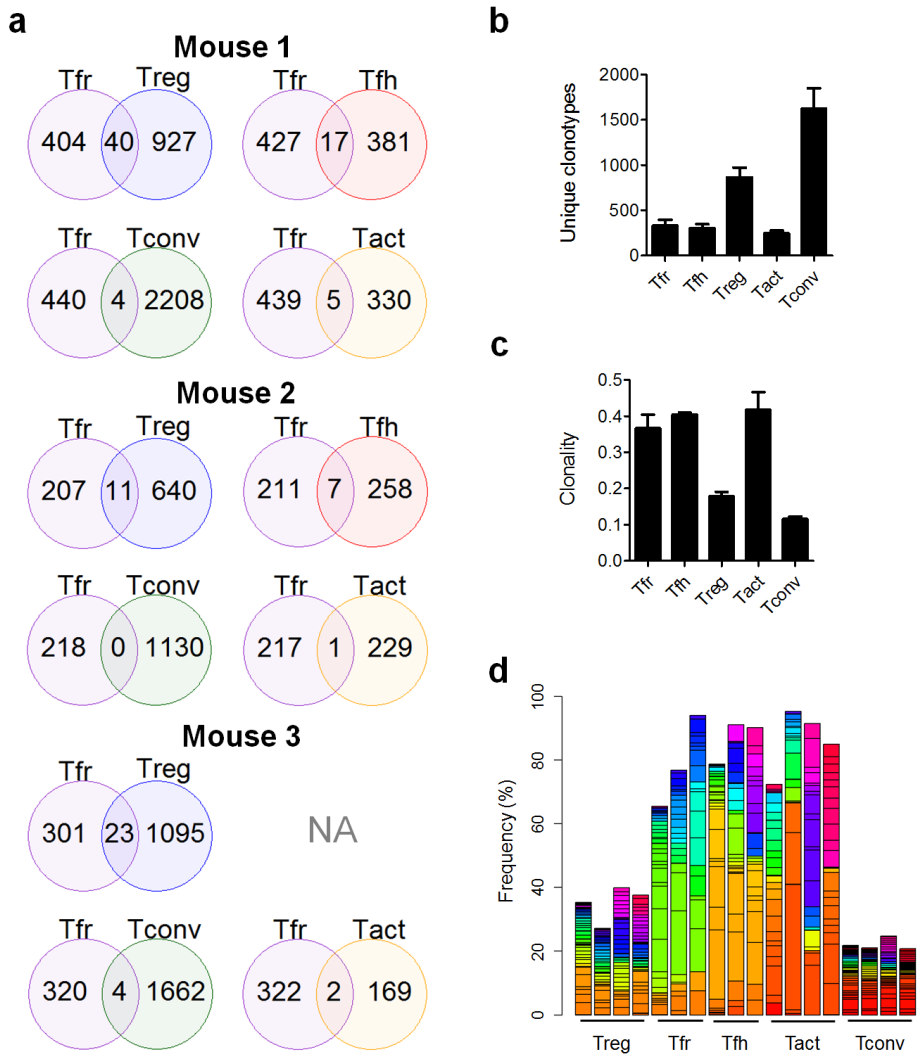

Figure 22 – Tfr cells are oligoclonal and have more common clonotypes with Treg than Tfh cells. (**a**) Venn diagrams showing the number of shared clonotypes between Tfr cells and the other four populations for mouse 1, 2, and 3. Sequencing results for Tfh sample of mouse 3 are not available (NA). Tfr cells have more common clonotypes with Treg cells than any other cell population. (**b**) Number of unique clonotypes identified for the five populations. (**c**) Clonality score for the five populations. (**d**) Histogram of cumulative frequency of the 20 most predominant clonotypes for each sample across all samples. Each color corresponds to a unique clonotype. In (**b**) and (**c**) Mean±SEM are presented with n=4, except for Tfh and Tfr where n=3.

Tfr and Treg cells than any other population. However, from the 9,000 TCR sequences, it was also observable that the number of identifiable clonotypes was lower for Tfr, Tfh, and Tact when compared to Treg and Tconv (Figure 22a,b). To determine if this observation was in line with a different clonality in the populations, a clonality score was calculated for each sample (Figure 22c). Indeed, the clonality score was higher for the populations with lower numbers of identifiable clonotypes. Furthermore, from the frequency distribution of the 20 most predominant clonotypes for each sample across all samples on Figure 22d, it was

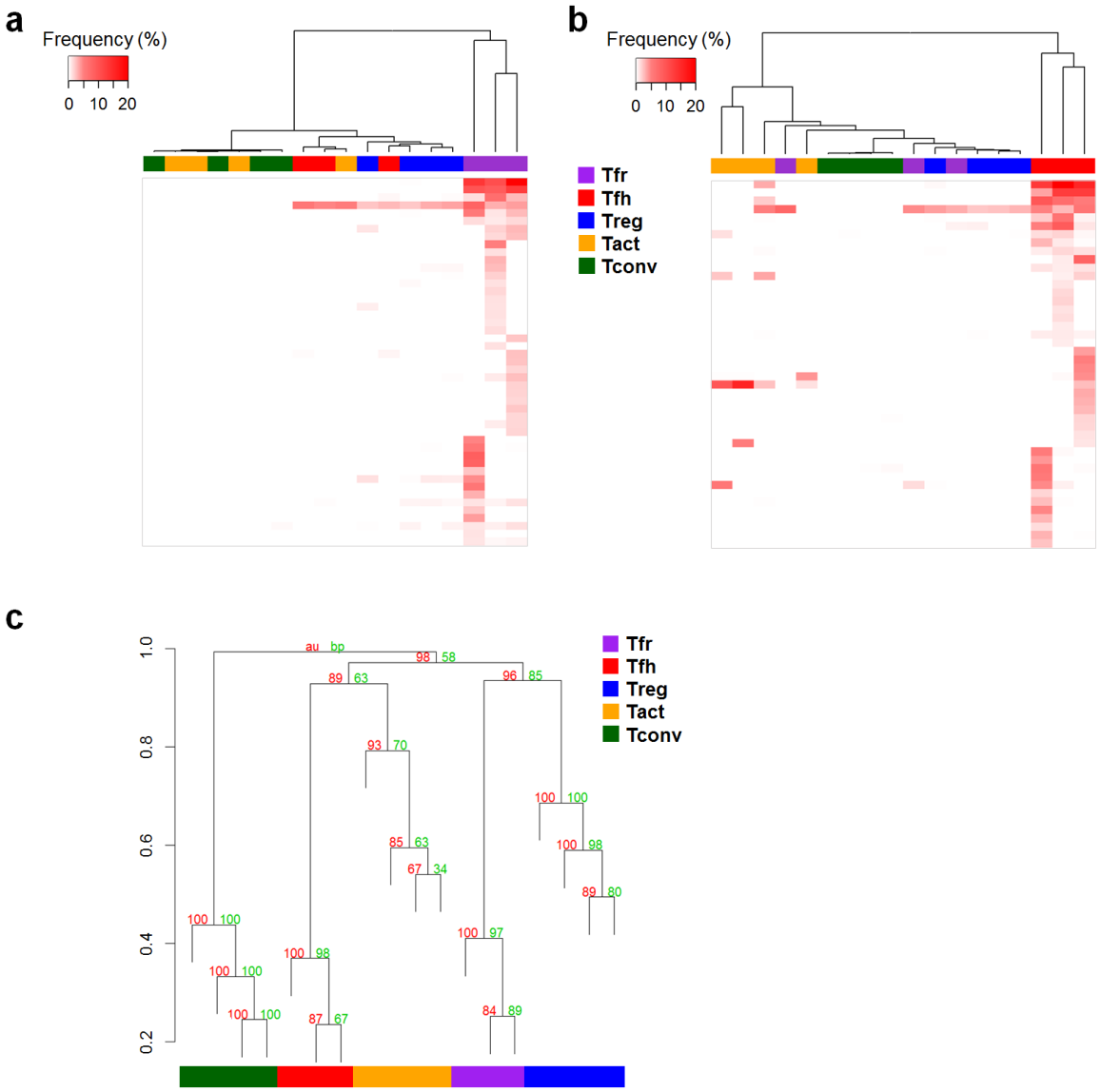

Figure 23 – Tfr cells are closer to Treg cells than to any other population.
(**a**) Heatmap and hierarchical clustering of the 20 most predominant clonotypes of Tfr replicates across all samples. Tfr most predominant clonotypes are mostly shared with Treg samples, with the exception of one sequence that is common with Treg and Tfh samples. (**b**) Same as in (**a**) but for the 20 most predominant clonotypes of Tfh replicates. The predominant clonotypes are mainly shared between Tfh and Tact samples. (**c**) Dendrogram showing the overall relation of all sequenced samples using Horn-Morisita index distance method. Bootstrap resampling was performed to calculate approximately unbiased (AU, left in red) p-values and bootstrap probability (BP, right in green) values for each cluster. In the case of the Tfr and Treg cluster, AU = 96% thus the existence of the cluster is strongly supported by the data.

observable that the 5 most abundant clonotypes represented in average up to ~50% of the total frequency for Tfh and Tact and ~40% for Tfr cells. Remarkably, the most common clonotypes from Tfh cells are shared in different mice (yellow), and the same happens for the most common Tfr clonotypes (green), although without a significant overlap between Tfh and Tfr clonotypes (Figure 22d). To further investigate the proximity between Tfr and

Tfh TCR repertoire, it was investigated if the 20 most predominant clonotypes from each Tfr sample are shared with other populations. The results, depicted on Figure 23a, show that the cell population that shares more clonotypes with the predominant Tfr clonotypes is the Treg population. Among the most abundant clonotypes, there is only one shared between all Tfr and Tfh samples, but that clonotype is also present on all Treg samples. The same approach was performed to obtain the 20 most predominant clonotypes for each Tfh sample (Figure 23b) where it is shown that Tfh cells present more shared clonotypes with Tact. Lastly, to establish the closeness between all samples regardless of the clonality of the populations, hierarchical clustering using Horn-Morisita index was performed[376]. This index has been described as appropriate to compare immune repertoires, since it is able to assess the similarity between samples, while taking into account the abundance of each clonotype in each sample. As shown on Figure 23c, Tfr and Treg samples clustered together (AU = 96%), indicating that Tfr samples have a repertoire closer to Treg than to any other population (Figure 23c).

These results indicate that, although Tfr cells undergo proliferation, their TCR is not specific for the antigen driving the GC reaction, since the TCR usage is almost not common with Tfh cells. Instead, Tfr cells present a TCR repertoire closer to Treg cells.

3.3. Discussion

The initial assessment of the TCR usage by CDR3 spectratyping showed that over-representation of specific CDR3 lengths was shared between Tfh samples. These results are in agreement with published data, demonstrating that these cells undergo selection and clonal expansion. Yet, such expansions were not observable on the Tfr population even though some CDR3 lengths were over-represented in some TRBVs. Moreover, on the analysis of the overall differences between the CDR3 length distributions of the four analyzed populations, using naïve CD4 T cells or Tfr cells as reference group, Tfh and Tfr cells were never clustered together. This observation suggests a different repertoire between these two cell populations. On the contrary, on both hierarchical clustering analyses, Tfr and Treg cells clustered together, which indicates a close relation between these two $Foxp3^+$ cell populations. Regarding Tconv cell distributions, the small deviations detected to the distributions of naïve CD4 T cells may be due to the presence of Th cells within this population, as overall $CD4^+CXCR5^-PD1^-FOXP3^-$ cells were sorted without the exclusion of non-follicular activated CD4 T cells.

There are some limitations to CDR3 spectratyping/immunoscope technique. For once, the analysis is only made on the TRBV, and the corresponding TRA usage is not known. Also, although there is an indication of expanding clones of specific CDR3 lengths within each TRBV analyzed, the actual number of clones that composed the population is not assessed. Furthermore, the number of cells required to have a representation of all possible CDR3 lengths in the parallel PCRs requires the pooling of LNs from several mice which may also introduce bias on the analysis.

Thus, to obtain more detailed data on the TCR repertoire of the populations of interest, the next approach was to sequence the TCR of five cell populations (Tfr, Treg, Tfh, Tact, and Tconv) from individual immunized mice. The 1D2β mouse allows a complete insight into the repertoire of the analyzed populations by only sequencing the TRA: these mice express a transgenic TCRβ chain and only one of the *Tra* alleles is available for recombination. The disruption of one of the *Tra* alleles is important to avoid the existence of cells expressing more than one TCRα which would influence the sequence results. Although the variability of the repertoire on these mice only depends on the recombination of one *Tra* gene, these mice were capable of mounting immune responses upon immunization with OVA-IFA. Of note, for this experiment, activated CD4 T (Tact) cells were analyzed separately from non-activated T (Tconv) cells in order not to have mixed information as occurred in the spectratyping analysis.

The sequencing data obtained gave a new understanding into Tfr cells biology. Tfr cells had a high clonality score meaning that they suffer oligoclonal expansion as Tfh and Tact cells; this characteristic was somehow expected since these three populations almost do not exist in non-immunized mice and must expand upon immunization. However, the most expanded clonotypes of Tfr and Tfh cells are not shared between them (with exception of one clonotype) indicating that these two populations, besides having different specificities, originate from different precursors. This idea is even more evident when the similarity of the five populations is assessed and the Tfr and Treg cells are clustered together. Thus, the results obtained further confirm the previous findings that Tfr cells originate from tTreg cells and are not specific for the antigen driving the GC response.

Another interesting observation from the sequencing data is that some clonotypes are shared between Tfh and Tact cells, but have different frequencies in the two populations. This observation is in line with previous published data[118]. Indeed, it has been described that the same CD4 T cells can originate Tfh and Th cells. However, different clones favor one of the

differentiation pathways, as higher affinities for the antigen, and consequently longer TCR interactions with peptide-MHC-II, favor the Tfh differentiation.

V. General Discussion

The ontogeny of Tfr cells has remained an important issue and it may be related to the function of this population. In the agreement with the first published reports, the data presented here supports the hypothesis that Tfr cells originate from tTreg cells: by using a model of cell transfer of T cells with defined TCR specificity, it was shown that, in GC induced upon immunization, Tfr cells differentiated exclusively from pre-existing tTreg cells. Moreover, analysis of the TCR usage, by CDR3 spectratyping and sequencing, revealed a proximity between Tfr and Treg cells repertoire.

Upon determining the origin of Tfr cells, the question if Tfr, as Tfh cells, were specific for the immunizing antigen remained to be answered. Four distinct approaches were used to address that issue: one based on cell transfer of T cells with defined TCR specificity; an approach using tetramers to identify antigen-specific Tfh and Tfr cells in WT mice; another strategy based on the capacity of Tfr cells to proliferate *in vitro* with immunizing antigen signals; and a final approach based on the analysis of the TCR repertoire. All experiments led to the same conclusion that T cell clones specific for the immunizing antigen are enriched within the Tfh pool, but not within Tfr cells.

Thus, the results presented in this thesis answered the questions regarding Tfr cells ontogeny and specificity. The answers to these two questions, especially the second, are of major importance since it has implications on possible mechanisms by which Tfr cells prevent the development of auto-reactive B cells. Moreover, further understanding of these mechanisms, as well as the antigen-specificity requirements for Tfr function, may unveil new strategies to prevent the onset of auto-antibody-mediated autoimmune diseases.

The findings that Tfr cells originate from tTreg cells and are not specific for the immunizing antigen are in line with the idea that Tfr cells, as Treg cells from which they derive, have TCR specificity towards self-antigens. However, these new observations rise new questions regarding Tfr cells differentiation and function.

During GC reaction, the BCR undergoes affinity maturation leading to formation of higher affinity receptors selected by Tfh cells. However, some mutations may lead to auto-reactive receptors or receptors that are cross-reactive with auto-antigens. Tfr cells, as regulatory cells with TCR specificity biased towards self-antigens, may be involved in preventing the generation of auto-antibody-mediated autoimmunity. In agreement with this hypothesis, recent reports have associated Tfr cells with the onset of auto-antibody-mediated

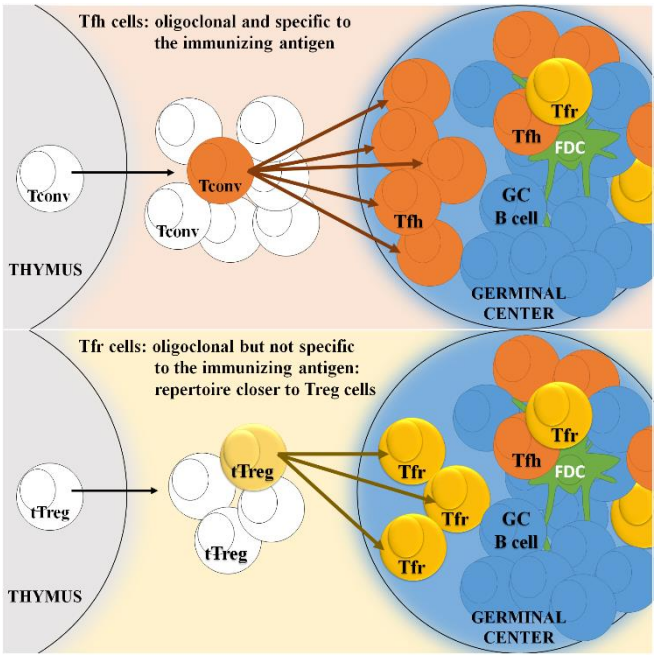

Figure 24 – Tfr cells origin and specificity.
The results obtained on this thesis demonstrate that Tfr cells originate from tTreg cells. Moreover, although Tfr cells suffer clonal expansion and have an oligoclonal repertoire as Tfh cells, they are not specific for the non-self antigen driving the GC response, and keep a repertoire closer to Treg cells.

autoimmunity in human diseases and mouse models[342-345,353,360-364]. But how do Tfr cells, by having a specificity towards self-antigens, prevent the selection of auto-reactive B cells that originate due to SHM in the GC? The most reasonable hypothesis would be the recognition, by Tfr cells, of self-antigens presented by auto-reactive B cells in their MHC-II molecules. As a result, Tfr would induce cell death on such B cells. This mechanism would be even more important in the case of B cells that acquired cross-reactivity to a self-antigen. In that case, even though those B cells are able to uptake and present the antigen driving the GC response to Tfh cells, their ability to also recognize self-antigen, and consequent presentation of those self-antigens to Tfr cells, would allow their deletion. One of the limitations of this hypothesis lies on the limited variety of self antigens present within the B cell follicle and, consequently, available for uptake and presentation by GC B cells. Reactivity to self-antigens that are not present could not be tested. However, this limitation may be in line with the results obtained. The sequencing data showed that a few Tfr clones, some of which shared between samples, represented more than half of the population. Consequently, Tfr cells have a diminished capability of multiple self-antigens recognition. However, this over-representation of some clones may be due to Tfr cells selection and/or expansion based on their affinity to self-antigens present in the GC. Since reactivity to self-antigens that are not present in the GC cannot be tested by Tfr cells, these cells may retain specificity to the ones

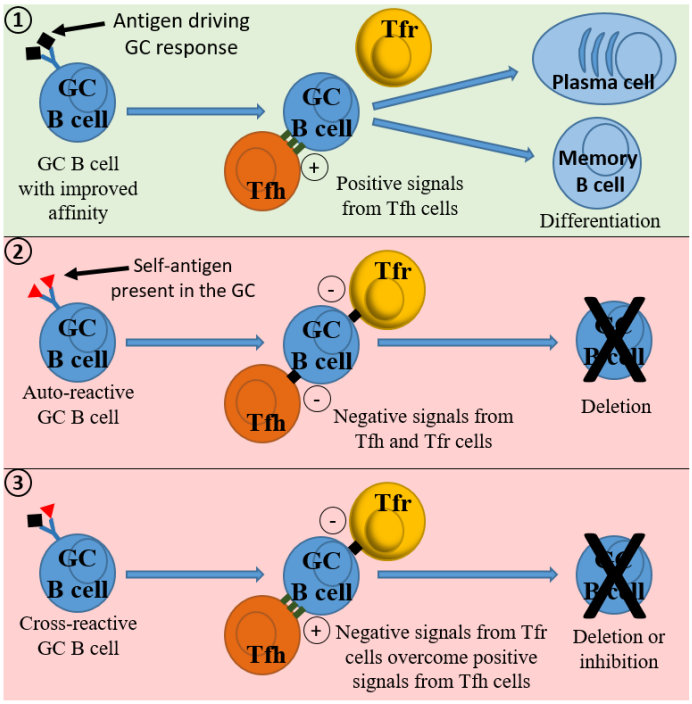

Figure 25 – Speculative hypothesis on how Tfr cells prevent the selection of auto-reactive B cells. Tfh and Tfr cells are involved in the positive and negative selection of GC B cells. GC B cells undergo affinity maturation in the GC: (1) if GC B cells successfully improve their affinity to the non-self antigen driving the GC response, they receive positive signals from Tfh cells and may differentiate into plasma or memory B cells; (2) if they lose their specificity to the antigen GC driving the response and gain specificity to self-antigen, GC B cells receive negative signals from Tfh – due to lack of non-self antigen presentation – and Tfr cells – due to presentation of self-antigen present within the GC; (3) lastly, if GC B cells maintain or improve their affinity to the non-self antigen driving the GC response but also gain affinity to self-antigens present in the GC, although Tfh cells may provide positive signals, Tfr cells detect the presentation of self-antigen and induce cell death or functional inactivation of these cells.

that are present. In that scenario, B cells that gain affinity to self-antigen present in the GC would uptake and present such antigens, in the attempt to obtain positive signals from Tfh cells; however, those B cells would instead be detected and eliminated or functionally inactivated by Tfr cells (Figure 25). Nevertheless, further studies are necessary to unveil the mechanism through which Tfr cells may prevent auto-antibody-mediated autoimmunity.

Tfh cells differentiate by antigen recognition of peptides presented first by DCs followed by B cells. Moreover, the differentiation process requires long-lived interactions between Tfh and GC B cells. Thus, antigen recognition plays a major role in Tfh differentiation and migration into the GC. Since Tfr cells are not specific for the antigen driving the immune response, what are the signals that drive Treg cells to differentiate into Tfr cells? There are several common requirements for Tfh and Tfr differentiation: both require priming from DCs and express common transcription factors like BCL-6 and STAT3. However, there are several evidences that the initial steps of activation are different between Tfr and Tfh cells.

Pre-Tfh cells upregulate ASCL2 that induces CXCR5 expression, while expression of CXCR5 in Tfr cells is dependent on NFAT2[227,353]. Tfr cells express BLIMP-1, a repressor of BCL-6 expression, that in Foxp3$^-$ CD4 T cells repress Tfh differentiation[329]. Moreover, Tfh and Tfr cells may require different cytokine signals since IL-21, an important cytokine for Tfh, has a detrimental impact on Tfr differentiation and function[363]. Thus, the results presented, as well as published data support, the hypothesis of different signaling requirements for Tfr and Tfh differentiation. However, further studies are required to elucidate which signals drive the Tfr differentiation.

Overall this thesis have established that Tfh and Tfr populations from the same GCs have different ontogeny and specificity: while Tfh cells originate from Tconv cells and are specific to the non-self antigen driving the GC response, Tfr cells originate from tTreg cells and are not specific for the antigen driving the GC response. Moreover, Tfr cells have a repertoire closer to tTreg thus with specificity towards self-antigens.

VI. References

1 Gay, N. J. & Gangloff, M. Structure and function of Toll receptors and their ligands. *Annu Rev Biochem* **76**, 141-165, doi:10.1146/annurev.biochem.76.060305.151318 (2007).

2 Geijtenbeek, T. B. H. & Gringhuis, S. I. Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol* **9**, 465-479 (2009).

3 Canton, J., Neculai, D. & Grinstein, S. Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol* **13**, 621-634, doi:10.1038/nri3515 (2013).

4 Ohashi, K., Burkart, V., Flohe, S. & Kolb, H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol* **164**, 558-561 (2000).

5 Asea, A. *et al.* Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* **277**, 15028-15034, doi:10.1074/jbc.M200497200 (2002).

6 Leadbetter, E. A. *et al.* Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* **416**, 603-607, doi:10.1038/416603a (2002).

7 Stone, K. D., Prussin, C. & Metcalfe, D. D. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* **125**, S73-80, doi:10.1016/j.jaci.2009.11.017 (2010).

8 Artis, D. & Spits, H. The biology of innate lymphoid cells. *Nature* **517**, 293-301, doi:10.1038/nature14189 (2015).

9 Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat Immunol* **9**, 503-510, doi:10.1038/ni1582 (2008).

10 Dunkelberger, J. R. & Song, W. C. Complement and its role in innate and adaptive immune responses. *Cell Res* **20**, 34-50, doi:10.1038/cr.2009.139 (2010).

11 Hozumi, N. & Tonegawa, S. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc Natl Acad Sci U S A* **73**, 3628-3632 (1976).

12 Burnet, F. M. & Fenner, F. *The Production of Antibodies*. 2nd edn, (Melbourne: Macmillan, 1949).

13 Goodnow, C. C. *et al.* Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676-682, doi:10.1038/334676a0 (1988).

14 Goodnow, C. C. *et al.* Clonal silencing of self-reactive B lymphocytes in a transgenic mouse model. *Cold Spring Harb Symp Quant Biol* **54 Pt 2**, 907-920 (1989).

15 Nemazee, D. A. & Burki, K. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* **337**, 562-566, doi:10.1038/337562a0 (1989).

16 Goodnow, C. C., Brink, R. & Adams, E. Breakdown of self-tolerance in anergic B lymphocytes. *Nature* **352**, 532-536, doi:10.1038/352532a0 (1991).

17 Brombacher, F., Kohler, G. & Eibel, H. B cell tolerance in mice transgenic for anti-CD8 immunoglobulin mu chain. *J Exp Med* **174**, 1335-1346 (1991).

18 Erikson, J. *et al.* Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature* **349**, 331-334, doi:10.1038/349331a0 (1991).

19 Tiegs, S. L., Russell, D. M. & Nemazee, D. Receptor editing in self-reactive bone marrow B cells. *J Exp Med* **177**, 1009-1020 (1993).

20 Melamed, D. & Nemazee, D. Self-antigen does not accelerate immature B cell apoptosis, but stimulates receptor editing as a consequence of developmental arrest. *Proc Natl Acad Sci U S A* **94**, 9267-9272 (1997).

21 Pelanda, R. *et al.* Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification. *Immunity* **7**, 765-775 (1997).
22 Grandien, A., Fucs, R., Nobrega, A., Andersson, J. & Coutinho, A. Negative selection of multireactive B cell clones in normal adult mice. *Eur J Immunol* **24**, 1345-1352, doi:10.1002/eji.1830240616 (1994).
23 Muramatsu, M. *et al.* Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553-563 (2000).
24 Baumgarth, N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* **11**, 34-46, doi:10.1038/nri2901 (2011).
25 Loder, F. *et al.* B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J Exp Med* **190**, 75-89 (1999).
26 Martin, F., Oliver, A. M. & Kearney, J. F. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* **14**, 617-629 (2001).
27 Balazs, M., Martin, F., Zhou, T. & Kearney, J. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity* **17**, 341-352 (2002).
28 Taylor, J. J., Pape, K. A. & Jenkins, M. K. A germinal center-independent pathway generates unswitched memory B cells early in the primary response. *J Exp Med* **209**, 597-606, doi:10.1084/jem.20111696 (2012).
29 Jacob, J. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J Exp Med* **176**, 679-687 (1992).
30 Paus, D. *et al.* Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J Exp Med* **203**, 1081-1091, doi:10.1084/jem.20060087 (2006).
31 O'Connor, B. P. *et al.* Imprinting the fate of antigen-reactive B cells through the affinity of the B cell receptor. *J Immunol* **177**, 7723-7732 (2006).
32 Gatto, D., Paus, D., Basten, A., Mackay, C. R. & Brink, R. Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses. *Immunity* **31**, 259-269, doi:10.1016/j.immuni.2009.06.016 (2009).
33 Pereira, J. P., Kelly, L. M., Xu, Y. & Cyster, J. G. EBI2 mediates B cell segregation between the outer and centre follicle. *Nature* **460**, 1122-1126, doi:10.1038/nature08226 (2009).
34 Blink, E. J. *et al.* Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J Exp Med* **201**, 545-554, doi:10.1084/jem.20042060 (2005).
35 Kabashima, K. *et al.* Plasma cell S1P1 expression determines secondary lymphoid organ retention versus bone marrow tropism. *J Exp Med* **203**, 2683-2690, doi:10.1084/jem.20061289 (2006).
36 Benner, R., Hijmans, W. & Haaijman, J. J. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. *Clin Exp Immunol* **46**, 1-8 (1981).
37 Slifka, M. K., Matloubian, M. & Ahmed, R. Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol* **69**, 1895-1902 (1995).
38 Manz, R. A., Thiel, A. & Radbruch, A. Lifetime of plasma cells in the bone marrow. *Nature* **388**, 133-134, doi:10.1038/40540 (1997).

39 Dogan, I. *et al.* Multiple layers of B cell memory with different effector functions. *Nat Immunol* **10**, 1292-1299, doi:10.1038/ni.1814 (2009).

40 Pape, K. A., Taylor, J. J., Maul, R. W., Gearhart, P. J. & Jenkins, M. K. Different B cell populations mediate early and late memory during an endogenous immune response. *Science* **331**, 1203-1207, doi:10.1126/science.1201730 (2011).

41 Jacob, J., Kassir, R. & Kelsoe, G. 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**, 1165-1175 (1991).

42 Jacob, J., Kelsoe, G., Rajewsky, K. & Weiss, U. Intraclonal generation of antibody mutants in germinal centres. *Nature* **354**, 389-392, doi:10.1038/354389a0 (1991).

43 Kraal, G., Weissman, I. L. & Butcher, E. C. Germinal centre B cells: antigen specificity and changes in heavy chain class expression. *Nature* **298**, 377-379 (1982).

44 Liu, Y. J. *et al.* Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation. *Immunity* **4**, 241-250 (1996).

45 Rajewsky, K. Clonal selection and learning in the antibody system. *Nature* **381**, 751-758, doi:10.1038/381751a0 (1996).

46 Okada, T. *et al.* Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol* **3**, e150, doi:10.1371/journal.pbio.0030150 (2005).

47 Qi, H., Cannons, J. L., Klauschen, F., Schwartzberg, P. L. & Germain, R. N. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* **455**, 764-769, doi:10.1038/nature07345 (2008).

48 Dal Porto, J. M., Haberman, A. M., Kelsoe, G. & Shlomchik, M. J. Very Low Affinity B Cells Form Germinal Centers, Become Memory B Cells, and Participate in Secondary Immune Responses When Higher Affinity Competition Is Reduced. *The Journal of Experimental Medicine* **195**, 1215 (2002).

49 Shih, T. A., Meffre, E., Roederer, M. & Nussenzweig, M. C. Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat Immunol* **3**, 570-575, doi:10.1038/ni803 (2002).

50 Kerfoot, S. M. *et al.* Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* **34**, 947-960, doi:10.1016/j.immuni.2011.03.024 (2011).

51 Kitano, M. *et al.* Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* **34**, 961-972, doi:10.1016/j.immuni.2011.03.025 (2011).

52 Ochiai, K. *et al.* Transcriptional regulation of germinal center B and plasma cell fates by dynamical control of IRF4. *Immunity* **38**, 918-929, doi:10.1016/j.immuni.2013.04.009 (2013).

53 Willis, S. N. *et al.* Transcription factor IRF4 regulates germinal center cell formation through a B cell-intrinsic mechanism. *J Immunol* **192**, 3200-3206, doi:10.4049/jimmunol.1303216 (2014).

54 Cattoretti, G. *et al.* BCL-6 protein is expressed in germinal-center B cells. *Blood* **86**, 45-53 (1995).

55 Onizuka, T. *et al.* BCL-6 gene product, a 92- to 98-kD nuclear phosphoprotein, is highly expressed in germinal center B cells and their neoplastic counterparts. *Blood* **86**, 28-37 (1995).

56 Dent, A. L., Shaffer, A. L., Yu, X., Allman, D. & Staudt, L. M. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* **276**, 589-592 (1997).

57 Huang, C. *et al.* The BCL6 RD2 domain governs commitment of activated B cells to form germinal centers. *Cell Rep* **8**, 1497-1508, doi:10.1016/j.celrep.2014.07.059 (2014).
58 Cinamon, G. *et al.* Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat Immunol* **5**, 713-720, doi:10.1038/ni1083 (2004).
59 Ci, W. *et al.* The BCL6 transcriptional program features repression of multiple oncogenes in primary B cells and is deregulated in DLBCL. *Blood* **113**, 5536-5548, doi:10.1182/blood-2008-12-193037 (2009).
60 Saito, M. *et al.* BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma. *Proc Natl Acad Sci U S A* **106**, 11294-11299, doi:10.1073/pnas.0903854106 (2009).
61 Phan, R. T. & Dalla-Favera, R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* **432**, 635-639, doi:10.1038/nature03147 (2004).
62 Ranuncolo, S. M. *et al.* Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nat Immunol* **8**, 705-714, doi:10.1038/ni1478 (2007).
63 Shaffer, A. L. *et al.* BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity* **13**, 199-212 (2000).
64 Basso, K. *et al.* Integrated biochemical and computational approach identifies BCL6 direct target genes controlling multiple pathways in normal germinal center B cells. *Blood* **115**, 975-984, doi:10.1182/blood-2009-06-227017 (2010).
65 Kosco-Vilbois, M. H. & Scheidegger, D. Follicular dendritic cells: antigen retention, B cell activation, and cytokine production. *Curr Top Microbiol Immunol* **201**, 69-82 (1995).
66 Allen, C. D. & Cyster, J. G. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Semin Immunol* **20**, 14-25, doi:10.1016/j.smim.2007.12.001 (2008).
67 MacLennan, I. C. Germinal centers. *Annu Rev Immunol* **12**, 117-139, doi:10.1146/annurev.iy.12.040194.001001 (1994).
68 Victora, G. D. & Nussenzweig, M. C. Germinal centers. *Annu Rev Immunol* **30**, 429-457, doi:10.1146/annurev-immunol-020711-075032 (2012).
69 De Silva, N. S. & Klein, U. Dynamics of B cells in germinal centres. *Nat Rev Immunol* **15**, 137-148, doi:10.1038/nri3804 (2015).
70 Nieuwenhuis, P. & Opstelten, D. Functional anatomy of germinal centers. *Am J Anat* **170**, 421-435, doi:10.1002/aja.1001700315 (1984).
71 Szakal, A. K., Gieringer, R. L., Kosco, M. H. & Tew, J. G. Isolated follicular dendritic cells: cytochemical antigen localization, Nomarski, SEM, and TEM morphology. *J Immunol* **134**, 1349-1359 (1985).
72 Cyster, J. G. *et al.* Follicular stromal cells and lymphocyte homing to follicles. *Immunol Rev* **176**, 181-193 (2000).
73 Wu, Y. *et al.* IL-6 produced by immune complex-activated follicular dendritic cells promotes germinal center reactions, IgG responses and somatic hypermutation. *Int Immunol* **21**, 745-756, doi:10.1093/intimm/dxp041 (2009).
74 Fliedner, T. M. in *Germinal Centers in Immune Responses: Proceedings of a Symposium held, at the University of Bern, Switzerland, June 22–24, 1966* (eds H. Cottier, N. Odartchenko, R. Schindler, & C. C. Congdon) 218-224 (Springer Berlin Heidelberg, 1967).
75 Baumann, I. *et al.* Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum*

46, 191-201, doi:10.1002/1529-0131(200201)46:1<191::AID-ART10027>3.0.CO;2-K (2002).

76 Hanayama, R. *et al.* Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* **304**, 1147-1150, doi:10.1126/science.1094359 (2004).

77 Rahman, Z. S., Shao, W. H., Khan, T. N., Zhen, Y. & Cohen, P. L. Impaired apoptotic cell clearance in the germinal center by Mer-deficient tingible body macrophages leads to enhanced antibody-forming cell and germinal center responses. *J Immunol* **185**, 5859-5868, doi:10.4049/jimmunol.1001187 (2010).

78 Allen, C. D. *et al.* Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* **5**, 943-952, doi:10.1038/ni1100 (2004).

79 Victora, G. D. *et al.* Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* **143**, 592-605, doi:10.1016/j.cell.2010.10.032 (2010).

80 Snapper, C. M. & Paul, W. E. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* **236**, 944-947 (1987).

81 Hasbold, J., Hong, J. S., Kehry, M. R. & Hodgkin, P. D. Integrating signals from IFN-gamma and IL-4 by B cells: positive and negative effects on CD40 ligand-induced proliferation, survival, and division-linked isotype switching to IgG1, IgE, and IgG2a. *J Immunol* **163**, 4175-4181 (1999).

82 Mitsdoerffer, M. *et al.* Proinflammatory T helper type 17 cells are effective B-cell helpers. *Proc Natl Acad Sci U S A* **107**, 14292-14297, doi:10.1073/pnas.1009234107 (2010).

83 Reinhardt, R. L., Liang, H. E. & Locksley, R. M. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* **10**, 385-393, doi:10.1038/ni.1715 (2009).

84 Meyer-Hermann, M. E., Maini, P. K. & Iber, D. An analysis of B cell selection mechanisms in germinal centers. *Math Med Biol* **23**, 255-277, doi:10.1093/imammb/dql012 (2006).

85 Shulman, Z. *et al.* Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* **345**, 1058-1062, doi:10.1126/science.1257861 (2014).

86 Gitlin, A. D., Shulman, Z. & Nussenzweig, M. C. Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature* **509**, 637-640, doi:10.1038/nature13300 (2014).

87 Zhang, Y. *et al.* Germinal center B cells govern their own fate via antibody feedback. *J Exp Med* **210**, 457-464, doi:10.1084/jem.20120150 (2013).

88 Shokat, K. M. & Goodnow, C. C. Antigen-induced B-cell death and elimination during germinal-centre immune responses. *Nature* **375**, 334-338, doi:10.1038/375334a0 (1995).

89 Han, S., Zheng, B., Dal Porto, J. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J Exp Med* **182**, 1635-1644 (1995).

90 Weisel, F. J., Zuccarino-Catania, G. V., Chikina, M. & Shlomchik, M. J. A Temporal Switch in the Germinal Center Determines Differential Output of Memory B and Plasma Cells. *Immunity* **44**, 116-130, doi:10.1016/j.immuni.2015.12.004 (2016).

91 Davis, M. M. & Bjorkman, P. J. T-cell antigen receptor genes and T-cell recognition. *Nature* **334**, 395-402, doi:10.1038/334395a0 (1988).

92 Vantourout, P. & Hayday, A. Six-of-the-best: unique contributions of γδ T cells to immunology(). *Nature reviews. Immunology* **13**, 88-100, doi:10.1038/nri3384 (2013).
93 Godfrey, D. I., Stankovic, S. & Baxter, A. G. Raising the NKT cell family. *Nat Immunol* **11**, 197-206, doi:10.1038/ni.1841 (2010).
94 von Boehmer, H., Teh, H. S. & Kisielow, P. The thymus selects the useful, neglects the useless and destroys the harmful. *Immunology Today* **10**, 57-61, doi:10.1016/0167-5699(89)90307-1 (1989).
95 Moon, J. J. *et al.* Quantitative impact of thymic selection on Foxp3+ and Foxp3- subsets of self-peptide/MHC class II-specific CD4+ T cells. *Proc Natl Acad Sci U S A* **108**, 14602-14607, doi:10.1073/pnas.1109806108 (2011).
96 Ellmeier, W., Sawada, S. & Littman, D. R. The regulation of CD4 and CD8 coreceptor gene expression during T cell development. *Annu Rev Immunol* **17**, 523-554, doi:10.1146/annurev.immunol.17.1.523 (1999).
97 Wang, L. & Bosselut, R. CD4-CD8 lineage differentiation: Thpok-ing into the nucleus. *J Immunol* **183**, 2903-2910, doi:10.4049/jimmunol.0901041 (2009).
98 Geppert, T. D. & Lipsky, P. E. Antigen presentation by interferon-gamma-treated endothelial cells and fibroblasts: differential ability to function as antigen-presenting cells despite comparable Ia expression. *J Immunol* **135**, 3750-3762 (1985).
99 Steimle, V., Siegrist, C. A., Mottet, A., Lisowska-Grospierre, B. & Mach, B. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science* **265**, 106-109 (1994).
100 Romieu-Mourez, R., Francois, M., Boivin, M. N., Stagg, J. & Galipeau, J. Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-gamma, TGF-beta, and cell density. *J Immunol* **179**, 1549-1558 (2007).
101 Kurts, C., Robinson, B. W. & Knolle, P. A. Cross-priming in health and disease. *Nat Rev Immunol* **10**, 403-414, doi:10.1038/nri2780 (2010).
102 Crotzer, V. L. & Blum, J. S. Autophagy and adaptive immunity. *Immunology* **131**, 9-17, doi:10.1111/j.1365-2567.2010.03321.x (2010).
103 John, B. *et al.* Dynamic Imaging of CD8(+) T cells and dendritic cells during infection with Toxoplasma gondii. *PLoS Pathog* **5**, e1000505, doi:10.1371/journal.ppat.1000505 (2009).
104 Butz, E. A. & Bevan, M. J. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* **8**, 167-175 (1998).
105 Murali-Krishna, K. *et al.* Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8**, 177-187 (1998).
106 Badovinac, V. P., Haring, J. S. & Harty, J. T. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* **26**, 827-841, doi:10.1016/j.immuni.2007.04.013 (2007).
107 Watts, T. H. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* **23**, 23-68, doi:10.1146/annurev.immunol.23.021704.115839 (2005).
108 Mescher, M. F. *et al.* Signals required for programming effector and memory development by CD8+ T cells. *Immunol Rev* **211**, 81-92, doi:10.1111/j.0105-2896.2006.00382.x (2006).
109 Groom, J. R. & Luster, A. D. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* **89**, 207-215, doi:10.1038/icb.2010.158 (2011).

110 Nakanishi, Y., Lu, B., Gerard, C. & Iwasaki, A. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* **462**, 510-513, doi:10.1038/nature08511 (2009).

111 Boyman, O. & Sprent, J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol* **12**, 180-190, doi:10.1038/nri3156 (2012).

112 Lieberman, J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* **3**, 361-370, doi:10.1038/nri1083 (2003).

113 Green, D. R., Droin, N. & Pinkoski, M. Activation-induced cell death in T cells. *Immunol Rev* **193**, 70-81 (2003).

114 Palmer, E. M., Holbrook, B. C., Arimilli, S., Parks, G. D. & Alexander-Miller, M. A. IFNgamma-producing, virus-specific CD8+ effector cells acquire the ability to produce IL-10 as a result of entry into the infected lung environment. *Virology* **404**, 225-230, doi:10.1016/j.virol.2010.05.004 (2010).

115 Trandem, K., Zhao, J., Fleming, E. & Perlman, S. Highly activated cytotoxic CD8 T cells express protective IL-10 at the peak of coronavirus-induced encephalitis. *J Immunol* **186**, 3642-3652, doi:10.4049/jimmunol.1003292 (2011).

116 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-2357 (1986).

117 Fazilleau, N., McHeyzer-Williams, L. J., Rosen, H. & McHeyzer-Williams, M. G. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol* **10**, 375-384, doi:10.1038/ni.1704 (2009).

118 Tubo, N. J. *et al.* Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* **153**, 785-796, doi:10.1016/j.cell.2013.04.007 (2013).

119 Macatonia, S. E. *et al.* Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* **154**, 5071-5079 (1995).

120 Scharton, T. M. & Scott, P. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. *J Exp Med* **178**, 567-577 (1993).

121 Lighvani, A. A. *et al.* T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A* **98**, 15137-15142, doi:10.1073/pnas.261570598 (2001).

122 Afkarian, M. *et al.* T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* **3**, 549-557, doi:10.1038/ni794 (2002).

123 Lugo-Villarino, G., Maldonado-López, R., Possemato, R., Peñaranda, C. & Glimcher, L. H. T-bet is required for optimal production of IFN-γ and antigen-specific T cell activation by dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 7749-7754, doi:10.1073/pnas.1332767100 (2003).

124 Thierfelder, W. E. *et al.* Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* **382**, 171-174, doi:10.1038/382171a0 (1996).

125 Hwang, E. S., Szabo, S. J., Schwartzberg, P. L. & Glimcher, L. H. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* **307**, 430-433, doi:10.1126/science.1103336 (2005).

126 Lazarevic, V. *et al.* T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. *Nat Immunol* **12**, 96-104, doi:10.1038/ni.1969 (2011).

127 Djuretic, I. M. *et al.* Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nat Immunol* **8**, 145-153, doi:10.1038/ni1424 (2007).
128 Williams, M. A., Tyznik, A. J. & Bevan, M. J. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* **441**, 890-893, doi:10.1038/nature04790 (2006).
129 Davidson, N. J. *et al.* T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. *J Exp Med* **184**, 241-251 (1996).
130 Parronchi, P. *et al.* Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol* **150**, 823-832 (1997).
131 Hu, H. Z., Li, G. L., Lim, Y. K., Chan, S. H. & Yap, E. H. Kinetics of interferon-gamma secretion and its regulatory factors in the early phase of acute graft-versus-host disease. *Immunology* **98**, 379-385 (1999).
132 Wang, B. *et al.* Interferon-gamma impacts at multiple points during the progression of autoimmune diabetes. *Proc Natl Acad Sci U S A* **94**, 13844-13849 (1997).
133 Leung, B. P., McInnes, I. B., Esfandiari, E., Wei, X. Q. & Liew, F. Y. Combined effects of IL-12 and IL-18 on the induction of collagen-induced arthritis. *J Immunol* **164**, 6495-6502 (2000).
134 Kaplan, M. H., Schindler, U., Smiley, S. T. & Grusby, M. J. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* **4**, 313-319 (1996).
135 Shimoda, K. *et al.* Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* **380**, 630-633, doi:10.1038/380630a0 (1996).
136 Zhu, J., Guo, L., Watson, C. J., Hu-Li, J. & Paul, W. E. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. *J Immunol* **166**, 7276-7281 (2001).
137 Zhu, J. *et al.* Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol* **5**, 1157-1165, doi:10.1038/ni1128 (2004).
138 Zhu, J., Yamane, H., Cote-Sierra, J., Guo, L. & Paul, W. E. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res* **16**, 3-10 (0000).
139 Horiuchi, S. *et al.* Genome-wide analysis reveals unique regulation of transcription of Th2-specific genes by GATA3. *J Immunol* **186**, 6378-6389, doi:10.4049/jimmunol.1100179 (2011).
140 Stritesky, G. L. *et al.* The transcription factor STAT3 is required for T helper 2 cell development. *Immunity* **34**, 39-49, doi:10.1016/j.immuni.2010.12.013 (2011).
141 Zhu, J., Cote-Sierra, J., Guo, L. & Paul, W. E. Stat5 activation plays a critical role in Th2 differentiation. *Immunity* **19**, 739-748 (2003).
142 Cote-Sierra, J. *et al.* Interleukin 2 plays a central role in Th2 differentiation. *Proc Natl Acad Sci U S A* **101**, 3880-3885, doi:10.1073/pnas.0400339101 (2004).
143 Zhu, J. *et al.* Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. *Immunity* **16**, 733-744 (2002).
144 Rengarajan, J. *et al.* Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *J Exp Med* **195**, 1003-1012 (2002).
145 Lohoff, M. *et al.* Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci U S A* **99**, 11808-11812, doi:10.1073/pnas.182425099 (2002).
146 Pawankar, R., Okuda, M., Yssel, H., Okumura, K. & Ra, C. Nasal mast cells in perennial allergic rhinitics exhibit increased expression of the Fc epsilonRI, CD40L,

IL-4, and IL-13, and can induce IgE synthesis in B cells. *J Clin Invest* **99**, 1492-1499, doi:10.1172/JCI119311 (1997).

147 Dabbagh, K. *et al.* IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *J Immunol* **162**, 6233-6237 (1999).

148 Doucet, C. *et al.* IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *Int Immunol* **10**, 1421-1433 (1998).

149 Dent, L. A., Strath, M., Mellor, A. L. & Sanderson, C. J. Eosinophilia in transgenic mice expressing interleukin 5. *J Exp Med* **172**, 1425-1431 (1990).

150 Kouro, T. & Takatsu, K. IL-5- and eosinophil-mediated inflammation: from discovery to therapy. *Int Immunol* **21**, 1303-1309, doi:10.1093/intimm/dxp102 (2009).

151 Little, F. F., Cruikshank, W. W. & Center, D. M. Il-9 stimulates release of chemotactic factors from human bronchial epithelial cells. *Am J Respir Cell Mol Biol* **25**, 347-352, doi:10.1165/ajrcmb.25.3.4349 (2001).

152 Fort, M. M. *et al.* IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* **15**, 985-995 (2001).

153 Zaiss, D. M. *et al.* Amphiregulin, a TH2 cytokine enhancing resistance to nematodes. *Science* **314**, 1746, doi:10.1126/science.1133715 (2006).

154 Fiorentino, D. F., Bond, M. W. & Mosmann, T. R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* **170**, 2081-2095 (1989).

155 Couper, K. N., Blount, D. G. & Riley, E. M. IL-10: the master regulator of immunity to infection. *J Immunol* **180**, 5771-5777, doi:10.4049/jimmunol.180.9.5771 (2008).

156 Harrington, L. E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* **6**, 1123-1132, doi:10.1038/ni1254 (2005).

157 Matusevicius, D. *et al.* Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler* **5**, 101-104 (1999).

158 Kotake, S. *et al.* IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest* **103**, 1345-1352, doi:10.1172/JCI5703 (1999).

159 Fujino, S. *et al.* Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* **52**, 65-70 (2003).

160 Ivanov, II *et al.* The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* **126**, 1121-1133, doi:10.1016/j.cell.2006.07.035 (2006).

161 Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. & Stockinger, B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* **24**, 179-189, doi:10.1016/j.immuni.2006.01.001 (2006).

162 Mangan, P. R. *et al.* Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* **441**, 231-234, doi:10.1038/nature04754 (2006).

163 Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235-238, doi:10.1038/nature04753 (2006).

164 Yang, X. O. *et al.* T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* **28**, 29-39, doi:10.1016/j.immuni.2007.11.016 (2008).

165 Chen, Z. *et al.* Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A* **103**, 8137-8142, doi:10.1073/pnas.0600666103 (2006).
166 Yang, X. O. *et al.* STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* **282**, 9358-9363, doi:10.1074/jbc.C600321200 (2007).
167 Schraml, B. U. *et al.* The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* **460**, 405-409, doi:10.1038/nature08114 (2009).
168 Brustle, A. *et al.* The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* **8**, 958-966, doi:10.1038/ni1500 (2007).
169 Korn, T. *et al.* IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* **448**, 484-487, doi:10.1038/nature05970 (2007).
170 Nurieva, R. *et al.* Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* **448**, 480-483, doi:10.1038/nature05969 (2007).
171 Langrish, C. L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* **201**, 233-240, doi:10.1084/jem.20041257 (2005).
172 Yao, Z. *et al.* Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* **3**, 811-821 (1995).
173 Hymowitz, S. G. *et al.* IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J* **20**, 5332-5341, doi:10.1093/emboj/20.19.5332 (2001).
174 Moseley, T. A., Haudenschild, D. R., Rose, L. & Reddi, A. H. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev* **14**, 155-174 (2003).
175 Leonard, W. J. & Spolski, R. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat Rev Immunol* **5**, 688-698, doi:10.1038/nri1688 (2005).
176 Aujla, S. J. *et al.* IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* **14**, 275-281, doi:10.1038/nm1710 (2008).
177 Zheng, Y. *et al.* Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* **14**, 282-289, doi:10.1038/nm1720 (2008).
178 Hirota, K. *et al.* Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat Immunol* **14**, 372-379, doi:10.1038/ni.2552 (2013).
179 Veldhoen, M. *et al.* Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* **9**, 1341-1346, doi:10.1038/ni.1659 (2008).
180 Dardalhon, V. *et al.* IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol* **9**, 1347-1355, doi:10.1038/ni.1677 (2008).
181 Schmitt, E. *et al.* IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *J Immunol* **153**, 3989-3996 (1994).
182 Elyaman, W. *et al.* Notch receptors and Smad3 signaling cooperate in the induction of interleukin-9-producing T cells. *Immunity* **36**, 623-634, doi:10.1016/j.immuni.2012.01.020 (2012).
183 Goswami, R. *et al.* STAT6-dependent regulation of Th9 development. *J Immunol* **188**, 968-975, doi:10.4049/jimmunol.1102840 (2012).
184 Lin, J. X. & Leonard, W. J. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. *Oncogene* **19**, 2566-2576, doi:10.1038/sj.onc.1203523 (2000).

185 Yang, X. O. *et al.* The signaling suppressor CIS controls proallergic T cell development and allergic airway inflammation. *Nat Immunol* **14**, 732-740, doi:10.1038/ni.2633 (2013).
186 Gorelik, L., Constant, S. & Flavell, R. A. Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. *J Exp Med* **195**, 1499-1505 (2002).
187 Chang, H. C. *et al.* The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol* **11**, 527-534, doi:10.1038/ni.1867 (2010).
188 Goswami, R. & Kaplan, M. H. Gcn5 is required for PU.1-dependent IL-9 induction in Th9 cells. *J Immunol* **189**, 3026-3033, doi:10.4049/jimmunol.1201496 (2012).
189 Staudt, V. *et al.* Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* **33**, 192-202, doi:10.1016/j.immuni.2010.07.014 (2010).
190 Jager, A., Dardalhon, V., Sobel, R. A., Bettelli, E. & Kuchroo, V. K. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol* **183**, 7169-7177, doi:10.4049/jimmunol.0901906 (2009).
191 Tan, C. *et al.* Antigen-specific Th9 cells exhibit uniqueness in their kinetics of cytokine production and short retention at the inflammatory site. *J Immunol* **185**, 6795-6801, doi:10.4049/jimmunol.1001676 (2010).
192 Nowak, E. C. *et al.* IL-9 as a mediator of Th17-driven inflammatory disease. *J Exp Med* **206**, 1653-1660, doi:10.1084/jem.20090246 (2009).
193 Elyaman, W. *et al.* IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci U S A* **106**, 12885-12890, doi:10.1073/pnas.0812530106 (2009).
194 Lu, L. F. *et al.* Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* **442**, 997-1002, doi:10.1038/nature05010 (2006).
195 Wilhelm, C. *et al.* An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat Immunol* **12**, 1071-1077, doi:10.1038/ni.2133 (2011).
196 Trifari, S., Kaplan, C. D., Tran, E. H., Crellin, N. K. & Spits, H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol* **10**, 864-871, doi:10.1038/ni.1770 (2009).
197 Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. & Sallusto, F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol* **10**, 857-863, doi:10.1038/ni.1767 (2009).
198 Eyerich, S. *et al.* Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest* **119**, 3573-3585, doi:10.1172/JCI40202 (2009).
199 Jia, L. & Wu, C. The biology and functions of Th22 cells. *Adv Exp Med Biol* **841**, 209-230, doi:10.1007/978-94-017-9487-9_8 (2014).
200 Azizi, G., Yazdani, R. & Mirshafiey, A. Th22 cells in autoimmunity: a review of current knowledge. *Eur Ann Allergy Clin Immunol* **47**, 108-117 (2015).
201 Wolk, K. *et al.* IL-22 increases the innate immunity of tissues. *Immunity* **21**, 241-254, doi:10.1016/j.immuni.2004.07.007 (2004).
202 Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* **192**, 1545-1552 (2000).

203 Schaerli, P. *et al.* CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* **192**, 1553-1562 (2000).
204 Kim, C. H. *et al.* Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* **193**, 1373-1381 (2001).
205 Miller, J. F. Effect of thymectomy in adult mice on immunological responsiveness. *Nature* **208**, 1337-1338 (1965).
206 Claman, H. N., Chaperon, E. A. & Triplett, R. F. Thymus-marrow cell combinations. Synergism in antibody production. *Proc Soc Exp Biol Med* **122**, 1167-1171 (1966).
207 Mitchell, G. F. & Miller, J. F. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J Exp Med* **128**, 821-837 (1968).
208 Nurieva, R. I. *et al.* Bcl6 mediates the development of T follicular helper cells. *Science* **325**, 1001-1005, doi:10.1126/science.1176676 (2009).
209 Johnston, R. J. *et al.* Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* **325**, 1006-1010, doi:10.1126/science.1175870 (2009).
210 Yu, D. *et al.* The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* **31**, 457-468, doi:10.1016/j.immuni.2009.07.002 (2009).
211 Crotty, S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* **29**, 621-663, doi:10.1146/annurev-immunol-031210-101400 (2011).
212 Deenick, E. K. *et al.* Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* **33**, 241-253, doi:10.1016/j.immuni.2010.07.015 (2010).
213 Goenka, R. *et al.* Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. *J Immunol* **187**, 1091-1095, doi:10.4049/jimmunol.1100853 (2011).
214 Choi, Y. S. *et al.* ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* **34**, 932-946, doi:10.1016/j.immuni.2011.03.023 (2011).
215 Barnett, L. G. *et al.* B cell antigen presentation in the initiation of follicular helper T cell and germinal center differentiation. *J Immunol* **192**, 3607-3617, doi:10.4049/jimmunol.1301284 (2014).
216 Shahinian, A. *et al.* Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261**, 609-612 (1993).
217 Ferguson, S. E., Han, S., Kelsoe, G. & Thompson, C. B. CD28 is required for germinal center formation. *J Immunol* **156**, 4576-4581 (1996).
218 Choi, Y. S., Eto, D., Yang, J. A., Lao, C. & Crotty, S. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol* **190**, 3049-3053, doi:10.4049/jimmunol.1203032 (2013).
219 Jacquemin, C. *et al.* OX40 Ligand Contributes to Human Lupus Pathogenesis by Promoting T Follicular Helper Response. *Immunity* **42**, 1159-1170, doi:10.1016/j.immuni.2015.05.012 (2015).
220 Kopf, M. *et al.* OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL Responses after virus infection. *Immunity* **11**, 699-708 (1999).
221 Kusam, S., Toney, L. M., Sato, H. & Dent, A. L. Inhibition of Th2 differentiation and GATA-3 expression by BCL-6. *J Immunol* **170**, 2435-2441 (2003).

222 Hatzi, K. *et al.* BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. *J Exp Med* **212**, 539-553, doi:10.1084/jem.20141380 (2015).
223 Weber, J. P. *et al.* ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med* **212**, 217-233, doi:10.1084/jem.20141432 (2015).
224 Nurieva, R. I. *et al.* Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* **29**, 138-149, doi:10.1016/j.immuni.2008.05.009 (2008).
225 Poholek, A. C. *et al.* In vivo regulation of Bcl6 and T follicular helper cell development. *J Immunol* **185**, 313-326, doi:10.4049/jimmunol.0904023 (2010).
226 Eto, D. *et al.* IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS One* **6**, e17739, doi:10.1371/journal.pone.0017739 (2011).
227 Liu, X. *et al.* Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* **507**, 513-518, doi:10.1038/nature12910 (2014).
228 Choi, Y. S. *et al.* LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol* **16**, 980-990, doi:10.1038/ni.3226 (2015).
229 Xu, L. *et al.* The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection. *Nat Immunol* **16**, 991-999, doi:10.1038/ni.3229 (2015).
230 Wu, T. *et al.* TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection. *Cell Rep* **12**, 2099-2110, doi:10.1016/j.celrep.2015.08.049 (2015).
231 Akiba, H. *et al.* The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. *J Immunol* **175**, 2340-2348 (2005).
232 Bauquet, A. T. *et al.* The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* **10**, 167-175, doi:10.1038/ni.1690 (2009).
233 Rolf, J. *et al.* Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. *J Immunol* **185**, 4042-4052, doi:10.4049/jimmunol.1001730 (2010).
234 Vogelzang, A. *et al.* A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* **29**, 127-137, doi:10.1016/j.immuni.2008.06.001 (2008).
235 Xiao, N. *et al.* The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells. *Nat Immunol* **15**, 657-666, doi:10.1038/ni.2912 (2014).
236 Lee, J. Y. *et al.* The transcription factor KLF2 restrains CD4(+) T follicular helper cell differentiation. *Immunity* **42**, 252-264, doi:10.1016/j.immuni.2015.01.013 (2015).
237 Xu, H. *et al.* Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* **496**, 523-527, doi:10.1038/nature12058 (2013).
238 Cannons, J. L. *et al.* Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* **32**, 253-265, doi:10.1016/j.immuni.2010.01.010 (2010).
239 Kageyama, R. *et al.* The receptor Ly108 functions as a SAP adaptor-dependent on-off switch for T cell help to B cells and NKT cell development. *Immunity* **36**, 986-1002, doi:10.1016/j.immuni.2012.05.016 (2012).
240 Liu, D. *et al.* T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* **517**, 214-218, doi:10.1038/nature13803 (2015).
241 Takahashi, Y., Dutta, P. R., Cerasoli, D. M. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity

maturation develops in two stages of clonal selection. *J Exp Med* **187**, 885-895 (1998).
242 Liu, Y. J. *et al.* Mechanism of antigen-driven selection in germinal centres. *Nature* **342**, 929-931, doi:10.1038/342929a0 (1989).
243 Choe, J., Kim, H. S., Zhang, X., Armitage, R. J. & Choi, Y. S. Cellular and molecular factors that regulate the differentiation and apoptosis of germinal center B cells. Anti-Ig down-regulates Fas expression of CD40 ligand-stimulated germinal center B cells and inhibits Fas-mediated apoptosis. *J Immunol* **157**, 1006-1016 (1996).
244 Arpin, C. *et al.* Generation of memory B cells and plasma cells in vitro. *Science* **268**, 720-722 (1995).
245 Zotos, D. *et al.* IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* **207**, 365-378, doi:10.1084/jem.20091777 (2010).
246 Linterman, M. A. *et al.* IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* **207**, 353-363, doi:10.1084/jem.20091738 (2010).
247 Ozaki, K. *et al.* Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol* **173**, 5361-5371 (2004).
248 Takahashi, Y., Ohta, H. & Takemori, T. Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity* **14**, 181-192 (2001).
249 Butt, D. *et al.* FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production. *Immunity* **42**, 890-902, doi:10.1016/j.immuni.2015.04.010 (2015).
250 King, I. L. & Mohrs, M. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med* **206**, 1001-1007, doi:10.1084/jem.20090313 (2009).
251 Yusuf, I. *et al.* Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* **185**, 190-202, doi:10.4049/jimmunol.0903505 (2010).
252 Dorfman, D. M., Brown, J. A., Shahsafaei, A. & Freeman, G. J. Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. *Am J Surg Pathol* **30**, 802-810, doi:10.1097/01.pas.0000209855.28282.ce (2006).
253 Haynes, N. M. *et al.* Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol* **179**, 5099-5108 (2007).
254 Agata, Y. *et al.* Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* **8**, 765-772 (1996).
255 Good-Jacobson, K. L. *et al.* PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* **11**, 535-542, doi:10.1038/ni.1877 (2010).
256 Fife, B. T. *et al.* Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nat Immunol* **10**, 1185-1192, doi:dx.doi.org/10.1038/ni.1790 (2009).
257 Kashiwakuma, D. *et al.* B and T lymphocyte attenuator suppresses IL-21 production from follicular Th cells and subsequent humoral immune responses. *J Immunol* **185**, 2730-2736, doi:10.4049/jimmunol.0903839 (2010).

258 Murphy, T. L. & Murphy, K. M. Slow down and survive: Enigmatic immunoregulation by BTLA and HVEM. *Annu Rev Immunol* **28**, 389-411, doi:10.1146/annurev-immunol-030409-101202 (2010).

259 Betz, B. C. *et al.* Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. *J Exp Med* **207**, 933-942, doi:10.1084/jem.20091548 (2010).

260 Bollig, N. *et al.* Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci U S A* **109**, 8664-8669, doi:10.1073/pnas.1205834109 (2012).

261 Weber, J. P., Fuhrmann, F. & Hutloff, A. T-follicular helper cells survive as long-term memory cells. *European Journal of Immunology* **42**, 1981-1988, doi:10.1002/eji.201242540 (2012).

262 Luthje, K. *et al.* The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol* **13**, 491-498, doi:10.1038/ni.2261 (2012).

263 He, J. *et al.* Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* **39**, 770-781, doi:10.1016/j.immuni.2013.09.007 (2013).

264 Sage, P. T., Alvarez, D., Godec, J., von Andrian, U. H. & Sharpe, A. H. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest* **124**, 5191-5204, doi:10.1172/JCI76861 (2014).

265 Lindqvist, M. *et al.* Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest* **122**, 3271-3280, doi:10.1172/JCI64314 (2012).

266 Perreau, M. *et al.* Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med* **210**, 143-156, doi:10.1084/jem.20121932 (2013).

267 Xu, H. *et al.* Increased frequency of circulating follicular helper T cells in lupus patients is associated with autoantibody production in a CD40L-dependent manner. *Cell Immunol* **295**, 46-51, doi:10.1016/j.cellimm.2015.01.014 (2015).

268 Ma, J. *et al.* Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol* **2012**, 827480, doi:10.1155/2012/827480 (2012).

269 Szabo, K. *et al.* Follicular helper T cells may play an important role in the severity of primary Sjogren's syndrome. *Clin Immunol* **147**, 95-104, doi:10.1016/j.clim.2013.02.024 (2013).

270 Luo, C. *et al.* Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. *J Neuroimmunol* **256**, 55-61, doi:10.1016/j.jneuroim.2012.12.001 (2013).

271 Mazerolles, F., Picard, C., Kracker, S., Fischer, A. & Durandy, A. Blood CD4+CD45RO+CXCR5+ T cells are decreased but partially functional in signal transducer and activator of transcription 3 deficiency. *J Allergy Clin Immunol* **131**, 1146-1156, 1156 e1141-1145, doi:10.1016/j.jaci.2012.12.1519 (2013).

272 Liu, Y. *et al.* Novel NFKB2 mutation in early-onset CVID. *J Clin Immunol* **34**, 686-690, doi:10.1007/s10875-014-0064-x (2014).

273 Ma, C. S. *et al.* Monogenic mutations differentially affect the quantity and quality of T follicular helper cells in patients with human primary immunodeficiencies. *J Allergy Clin Immunol* **136**, 993-1006 e1001, doi:10.1016/j.jaci.2015.05.036 (2015).

274 Ma, C. S. *et al.* Unique and shared signaling pathways cooperate to regulate the differentiation of human CD4+ T cells into distinct effector subsets. *J Exp Med* **213**, 1589-1608, doi:10.1084/jem.20151467 (2016).

275 Schmitt, N. *et al.* The cytokine TGF-beta co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFH cells. *Nat Immunol* **15**, 856-865, doi:10.1038/ni.2947 (2014).
276 McCarron, M. J. & Marie, J. C. TGF-beta prevents T follicular helper cell accumulation and B cell autoreactivity. *J Clin Invest* **124**, 4375-4386, doi:10.1172/JCI76179 (2014).
277 Morita, R. *et al.* Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* **34**, 108-121, doi:10.1016/j.immuni.2010.12.012 (2011).
278 Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. 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**, 1151-1164 (1995).
279 Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4**, 330-336, doi:10.1038/ni904 (2003).
280 Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* **8**, 191-197, doi:10.1038/ni1428 (2007).
281 Thiault, N. *et al.* Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors. *Nat Immunol* **16**, 628-634, doi:10.1038/ni.3150 (2015).
282 Hsieh, C. S., Lee, H. M. & Lio, C. W. Selection of regulatory T cells in the thymus. *Nat Rev Immunol* **12**, 157-167, doi:10.1038/nri3155 (2012).
283 Brunkow, M. E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* **27**, 68-73, doi:10.1038/83784 (2001).
284 Chatila, T. A. *et al.* JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest* **106**, R75-81, doi:10.1172/JCI11679 (2000).
285 Bennett, C. L. *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* **27**, 20-21, doi:10.1038/83713 (2001).
286 Wildin, R. S. *et al.* X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* **27**, 18-20, doi:10.1038/83707 (2001).
287 Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057-1061, doi:10.1126/science.1079490 (2003).
288 Khattri, R., Cox, T., Yasayko, S. A. & Ramsdell, F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* **4**, 337-342, doi:10.1038/ni909 (2003).
289 Wan, Y. Y. & Flavell, R. A. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* **445**, 766-770, doi:10.1038/nature05479 (2007).
290 Williams, L. M. & Rudensky, A. Y. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* **8**, 277-284, doi:10.1038/ni1437 (2007).
291 Maloy, K. J. & Powrie, F. Regulatory T cells in the control of immune pathology. *Nat Immunol* **2**, 816-822 (2001).

292 Josefowicz, S. Z., Lu, L. F. & Rudensky, A. Y. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* **30**, 531-564, doi:10.1146/annurev.immunol.25.022106.141623 (2012).

293 Bautista, J. L. *et al.* Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nat Immunol* **10**, 610-617, doi:10.1038/ni.1739 (2009).

294 Leung, M. W., Shen, S. & Lafaille, J. J. TCR-dependent differentiation of thymic Foxp3+ cells is limited to small clonal sizes. *J Exp Med* **206**, 2121-2130, doi:10.1084/jem.20091033 (2009).

295 Hsieh, C. S. *et al.* Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity* **21**, 267-277, doi:10.1016/j.immuni.2004.07.009 (2004).

296 Pacholczyk, R., Ignatowicz, H., Kraj, P. & Ignatowicz, L. Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity* **25**, 249-259, doi:10.1016/j.immuni.2006.05.016 (2006).

297 Tai, X., Cowan, M., Feigenbaum, L. & Singer, A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* **6**, 152-162, doi:10.1038/ni1160 (2005).

298 Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. & Rudensky, A. Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* **6**, 1142-1151, doi:10.1038/ni1263 (2005).

299 Vang, K. B. *et al.* IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development. *J Immunol* **181**, 3285-3290 (2008).

300 Lio, C. W. & Hsieh, C. S. A two-step process for thymic regulatory T cell development. *Immunity* **28**, 100-111, doi:10.1016/j.immuni.2007.11.021 (2008).

301 Gottschalk, R. A., Corse, E. & Allison, J. P. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. *J Exp Med* **207**, 1701-1711, doi:10.1084/jem.20091999 (2010).

302 Zheng, S. G. *et al.* TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. *J Immunol* **176**, 3321-3329 (2006).

303 Benson, M. J., Pino-Lagos, K., Rosemblatt, M. & Noelle, R. J. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* **204**, 1765-1774, doi:10.1084/jem.20070719 (2007).

304 Chen, W. *et al.* 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**, 1875-1886, doi:10.1084/jem.20030152 (2003).

305 Oliveira, V. G., Caridade, M., Paiva, R. S., Demengeot, J. & Graca, L. Sub-optimal CD4+ T-cell activation triggers autonomous TGF-beta-dependent conversion to Foxp3+ regulatory T cells. *Eur J Immunol* **41**, 1249-1255, doi:10.1002/eji.201040896 (2011).

306 Coombes, J. L. *et al.* A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* **204**, 1757-1764, doi:10.1084/jem.20070590 (2007).

307 Sun, C. M. *et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* **204**, 1775-1785, doi:10.1084/jem.20070602 (2007).

308 Malek, T. R. *et al.* IL-2 family of cytokines in T regulatory cell development and homeostasis. *J Clin Immunol* **28**, 635-639, doi:10.1007/s10875-008-9235-y (2008).
309 Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M. J. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* **8**, 1353-1362, doi:10.1038/ni1536 (2007).
310 Rubtsov, Y. P. *et al.* Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546-558, doi:10.1016/j.immuni.2008.02.017 (2008).
311 Collison, L. W. *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **450**, 566-569, doi:10.1038/nature06306 (2007).
312 Li, M. O., Wan, Y. Y. & Flavell, R. A. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* **26**, 579-591, doi:10.1016/j.immuni.2007.03.014 (2007).
313 Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S. & Noelle, R. J. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* **174**, 1783-1786 (2005).
314 Zhao, D. M., Thornton, A. M., DiPaolo, R. J. & Shevach, E. M. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood* **107**, 3925-3932, doi:10.1182/blood-2005-11-4502 (2006).
315 Walker, L. S. & Sansom, D. M. Confusing signals: recent progress in CTLA-4 biology. *Trends Immunol* **36**, 63-70, doi:10.1016/j.it.2014.12.001 (2015).
316 Huang, C. T. *et al.* Role of LAG-3 in regulatory T cells. *Immunity* **21**, 503-513, doi:10.1016/j.immuni.2004.08.010 (2004).
317 Liang, B. *et al.* Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol* **180**, 5916-5926 (2008).
318 Kobie, J. J. *et al.* T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J Immunol* **177**, 6780-6786 (2006).
319 Bopp, T. *et al.* Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* **204**, 1303-1310, doi:10.1084/jem.20062129 (2007).
320 Borsellino, G. *et al.* Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* **110**, 1225-1232, doi:10.1182/blood-2006-12-064527 (2007).
321 Deaglio, S. *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* **204**, 1257-1265, doi:10.1084/jem.20062512 (2007).
322 Yu, F., Sharma, S., Edwards, J., Feigenbaum, L. & Zhu, J. Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. *Nat Immunol* **16**, 197-206, doi:10.1038/ni.3053 (2015).
323 Koch, M. A. *et al.* The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* **10**, 595-602, doi:10.1038/ni.1731 (2009).
324 Zheng, Y. *et al.* Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* **458**, 351-356, doi:10.1038/nature07674 (2009).
325 Wang, Y., Su, M. A. & Wan, Y. Y. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity* **35**, 337-348, doi:10.1016/j.immuni.2011.08.012 (2011).

326 Wohlfert, E. A. *et al.* GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest* **121**, 4503-4515, doi:10.1172/JCI57456 (2011).
327 Yang, B. H. *et al.* Foxp3(+) T cells expressing RORgammat represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol* **9**, 444-457, doi:10.1038/mi.2015.74 (2016).
328 Chung, Y. *et al.* Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* **17**, 983-988, doi:10.1038/nm.2426 (2011).
329 Linterman, M. A. *et al.* Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* **17**, 975-982, doi:10.1038/nm.2425 (2011).
330 Wollenberg, I. *et al.* Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* **187**, 4553-4560, doi:10.4049/jimmunol.1101328 (2011).
331 Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A. & Weiner, H. L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* **265**, 1237-1240 (1994).
332 Groux, H. *et al.* A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389**, 737-742, doi:10.1038/39614 (1997).
333 Awasthi, A. *et al.* A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* **8**, 1380-1389, doi:10.1038/ni1541 (2007).
334 Gagliani, N. *et al.* Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* **19**, 739-746, doi:10.1038/nm.3179 (2013).
335 Pot, C. *et al.* Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol* **183**, 797-801, doi:10.4049/jimmunol.0901233 (2009).
336 Apetoh, L. *et al.* The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol* **11**, 854-861, doi:10.1038/ni.1912 (2010).
337 Miller, A., Lider, O., Roberts, A. B., Sporn, M. B. & Weiner, H. L. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci U S A* **89**, 421-425 (1992).
338 Weiner, H. L. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* **182**, 207-214 (2001).
339 Godfrey, V. L., Wilkinson, J. E. & Russell, L. B. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol* **138**, 1379-1387 (1991).
340 Lin, W. *et al.* Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice. *J Allergy Clin Immunol* **116**, 1106-1115, doi:10.1016/j.jaci.2005.08.046 (2005).
341 Lim, H. W., Hillsamer, P., Banham, A. H. & Kim, C. H. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol* **175**, 4180-4183 (2005).
342 Shan, Y. *et al.* Higher frequency of peripheral blood follicular regulatory T cells in patients with new onset ankylosing spondylitis. *Clin Exp Pharmacol Physiol* **42**, 154-161, doi:10.1111/1440-1681.12330 (2015).
343 Wen, Y. *et al.* Imbalance of circulating CD4(+)CXCR5(+)FOXP3(+) Tfr-like cells and CD4(+)CXCR5(+)FOXP3(-) Tfh-like cells in myasthenia gravis. *Neurosci Lett* **630**, 176-182, doi:10.1016/j.neulet.2016.07.049 (2016).

344 Charbonnier, L. M. *et al.* Regulatory T-cell deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like disorder caused by loss-of-function mutations in LRBA. *J Allergy Clin Immunol* **135**, 217-227, doi:10.1016/j.jaci.2014.10.019 (2015).
345 Dhaeze, T. *et al.* Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis. *J Immunol* **195**, 832-840, doi:10.4049/jimmunol.1500759 (2015).
346 Aloulou, M. *et al.* Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun* **7**, 10579, doi:10.1038/ncomms10579 (2016).
347 Gerner, M. Y., Torabi-Parizi, P. & Germain, R. N. Strategically localized dendritic cells promote rapid T cell responses to lymph-borne particulate antigens. *Immunity* **42**, 172-185, doi:10.1016/j.immuni.2014.12.024 (2015).
348 Sage, P. T., Francisco, L. M., Carman, C. V. & Sharpe, A. H. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* **14**, 152-161, doi:10.1038/ni.2496 (2013).
349 Wing, J. B., Ise, W., Kurosaki, T. & Sakaguchi, S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* **41**, 1013-1025, doi:10.1016/j.immuni.2014.12.006 (2014).
350 Sage, P. T., Paterson, A. M., Lovitch, S. B. & Sharpe, A. H. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* **41**, 1026-1039, doi:10.1016/j.immuni.2014.12.005 (2014).
351 Ray, J. P. *et al.* Transcription factor STAT3 and type I interferons are corepressive insulators for differentiation of follicular helper and T helper 1 cells. *Immunity* **40**, 367-377, doi:10.1016/j.immuni.2014.02.005 (2014).
352 Wu, H., Xie, M. M., Liu, H. & Dent, A. L. Stat3 Is Important for Follicular Regulatory T Cell Differentiation. *PLoS One* **11**, e0155040, doi:10.1371/journal.pone.0155040 (2016).
353 Vaeth, M. *et al.* Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med* **211**, 545-561, doi:10.1084/jem.20130604 (2014).
354 Chang, J. H. *et al.* TRAF3 regulates the effector function of regulatory T cells and humoral immune responses. *J Exp Med* **211**, 137-151, doi:10.1084/jem.20131019 (2014).
355 Kawamoto, S. *et al.* Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* **41**, 152-165, doi:10.1016/j.immuni.2014.05.016 (2014).
356 Sage, P. T. & Sharpe, A. H. T follicular regulatory cells. *Immunol Rev* **271**, 246-259, doi:10.1111/imr.12411 (2016).
357 Sage, P. T. *et al.* Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol*, doi:10.1038/ni.3578 (2016).
358 Sage, P. T. & Sharpe, A. H. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol* **36**, 410-418, doi:10.1016/j.it.2015.05.005 (2015).
359 Levy, Y. & Brouet, J. C. Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. *J Clin Invest* **93**, 424-428, doi:10.1172/JCI116977 (1994).
360 Huynh, A. *et al.* Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. *Nat Immunol* **16**, 188-196, doi:10.1038/ni.3077 (2015).

361 Shrestha, S. *et al.* Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat Immunol* **16**, 178-187, doi:10.1038/ni.3076 (2015).

362 Leavenworth, J. W., Verbinnen, B., Yin, J., Huang, H. & Cantor, H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol* **16**, 96-106, doi:10.1038/ni.3050 (2015).

363 Ding, Y. *et al.* Interleukin-21 promotes germinal center reaction by skewing the follicular regulatory T cell to follicular helper T cell balance in autoimmune BXD2 mice. *Arthritis Rheumatol* **66**, 2601-2612, doi:10.1002/art.38735 (2014).

364 Kim, Y. U., Lim, H., Jung, H. E., Wetsel, R. A. & Chung, Y. Regulation of autoimmune germinal center reactions in lupus-prone BXD2 mice by follicular helper T cells. *PLoS One* **10**, e0120294, doi:10.1371/journal.pone.0120294 (2015).

365 Moon, J. J. *et al.* Tracking epitope-specific T cells. *Nat Protoc* **4**, 565-581, doi:10.1038/nprot.2009.9 (2009).

366 Pannetier, C. *et al.* The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments. *Proc Natl Acad Sci U S A* **90**, 4319-4323 (1993).

367 Collette, A. & Six, A. ISEApeaks: an Excel platform for GeneScan and Immunoscope data retrieval, management and analysis. *Bioinformatics* **18**, 329-330 (2002).

368 Collette, A., Cazenave, P. A., Pied, S. & Six, A. New methods and software tools for high throughput CDR3 spectratyping. Application to T lymphocyte repertoire modifications during experimental malaria. *J Immunol Methods* **278**, 105-116 (2003).

369 Gorochov, G. *et al.* Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. *Nat Med* **4**, 215-221 (1998).

370 Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30**, 614-620, doi:10.1093/bioinformatics/btt593 (2014).

371 Plessy, C., Mariotti-Ferrandiz, E., Manabe, R.-I. & Hori, S. clonotypeR--high throughput analysis of T cell antigen receptor sequences. *bioRxiv*, doi:10.1101/028696 (2015).

372 Pielou, E. C. The measurement of diversity in different types of biological collections. *Journal of Theoretical Biology* **13**, 131-144, doi:10.1016/0022-5193(66)90013-0 (1966).

373 Shannon, C. E. A Mathematical Theory of Communication. *At&T Tech J* **27**, 379-423 (1948).

374 Stewart, J. J. *et al.* A Shannon entropy analysis of immunoglobulin and T cell receptor. *Mol Immunol* **34**, 1067-1082 (1997).

375 Horn, H. S. Measurement of Overlap in Comparative Ecological Studies. *Am Nat* **100**, 419-&, doi:Doi 10.1086/282436 (1966).

376 Venturi, V. *et al.* Method for assessing the similarity between subsets of the T cell receptor repertoire. *J Immunol Methods* **329**, 67-80, doi:10.1016/j.jim.2007.09.016 (2008).

377 Suzuki, R. & Shimodaira, H. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* **22**, 1540-1542, doi:10.1093/bioinformatics/btl117 (2006).

378 Vanderleyden, I., Linterman, M. A. & Smith, K. G. Regulatory T cells and control of the germinal centre response. *Arthritis Res Ther* **16**, 471 (2014).

379 Korn, T. *et al.* IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* **105**, 18460-18465, doi:10.1073/pnas.0809850105 (2008).
380 Zal, T., Weiss, S., Mellor, A. & Stockinger, B. Expression of a second receptor rescues self-specific T cells from thymic deletion and allows activation of autoreactive effector function. *Proc Natl Acad Sci U S A* **93**, 9102-9107 (1996).
381 Padovan, E. *et al.* Expression of two T cell receptor alpha chains: dual receptor T cells. *Science* **262**, 422-424 (1993).
382 Tamura, T. *et al.* The role of antigenic peptide in CD4+ T helper phenotype development in a T cell receptor transgenic model. *Int Immunol* **16**, 1691-1699, doi:10.1093/intimm/dxh170 (2004).
383 Komatsu, N. *et al.* 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**, 1903-1908, doi:10.1073/pnas.0811556106 (2009).
384 Zou, T., Caton, A. J., Koretzky, G. A. & Kambayashi, T. Dendritic cells induce regulatory T cell proliferation through antigen-dependent and -independent interactions. *J Immunol* **185**, 2790-2799, doi:10.4049/jimmunol.0903740 (2010).
385 Levine, A. G., Arvey, A., Jin, W. & Rudensky, A. Y. Continuous requirement for the TCR in regulatory T cell function. *Nat Immunol* **15**, 1070-1078, doi:10.1038/ni.3004 (2014).
386 Moon, J. J. *et al.* Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* **27**, 203-213, doi:10.1016/j.immuni.2007.07.007 (2007).
387 Nelson, R. W. *et al.* T cell receptor cross-reactivity between similar foreign and self peptides influences naive cell population size and autoimmunity. *Immunity* **42**, 95-107, doi:10.1016/j.immuni.2014.12.022 (2015).
388 Huseby, E. S. *et al.* How the T cell repertoire becomes peptide and MHC specific. *Cell* **122**, 247-260, doi:10.1016/j.cell.2005.05.013 (2005).

VII. Appendix

List of publications resulting from this thesis

1. **Maceiras, A. R.**, Almeida, S.C.P., Mariotti-Ferrandiz, E., Chaara, W., Jebbawi, F., Six, A., Hori, S., Klatzmann, D., Faro, J. & Graca L. T follicular helper and T follicular regulatory cells have different TCR specificity. *Nat Commun*. **8**, 15067, doi:10.1038/ncomms15067 (2017) (*in press*).

2. **Maceiras, A. R.** & Graca L. Identification of Foxp3+ T Follicular Regulatory (Tfr) Cells by Flow Cytometry. *Methods Mol Biol* **1291**, 143-150, doi:10.1007/978-1-4939-2498-1_12 (2015).

T follicular helper and T follicular regulatory cells have different TCR specificity

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ABSTRACT

Immunization leads to the formation of germinal centers (GCs) that contain both T follicular helper (Tfh) and T follicular regulatory (Tfr) cells. Whether T cell receptor (TCR) specificity defines the differential functions of Tfh and Tfr cells is unclear. Here we show that antigen-specific T cells after immunization are preferentially recruited to the GC to become Tfh cells, but not Tfr cells. Tfh cells, but not Tfr cells, also proliferate efficiently upon restimulation with the same immunizing antigen *in vitro*. *Ex vivo* TCR repertoire analysis shows that immunization induces oligoclonal expansion of Tfh cells; by contrast, the Tfr pool has a TCR repertoire that more closely resembles that of regulatory T (Treg) cells. Our data thus indicate that the GC Tfh and Tfr pools are generated from distinct TCR repertoires, with Tfh cells expressing antigen-responsive TCRs to promote antibody responses, and Tfr cells expressing potentially autoreactive TCRs to suppress autoimmunity.

INTRODUCTION

Thymus-dependent humoral immune responses are not only critical for protection against pathogens, but are also a central protective mechanism of most vaccines. These antibody-mediated responses depend on germinal centers (GCs) - anatomical structures inside the B cell zone – where T follicular helper (Tfh) cells interact with and provide help to B cells, enabling affinity maturation and isotype switching[1]. Affinity maturation is a critical event in the GC reaction in which B cells edit their B cell receptor (BCR) and undergo a selection process leading to higher receptor affinity. However, during affinity maturation, autoreactive BCRs may be generated, resulting in production of autoantibodies and the potential for autoimmune disease. Several autoimmune diseases are characterized by formation of ectopic GCs and production of autoantibodies[2].

Tfh cells are required for GC formation and maintenance[3-7], and Foxp3$^+$ T follicular regulatory (Tfr) cells participate in the regulation of GC reactions[8-12]. Lack of Tfr cells or an altered Tfr:Tfh ratio can increase the risk of autoimmunity and autoantibody production[13-16]. This contribution of Tfr cells to the prevention of autoimmunity has been detected in several experimental models of autoimmunity and inferred from human pathology[13-17].

Here we test the hypothesis that populations of Tfh and Tfr cells have different T cell receptor (TCR) repertoires, leading to different antigenic targets for effector versus regulatory action. Protective immune responses are promoted by Tfh cells which, with a TCR repertoire specific for an immunizing antigen, provide help to B cells and enable BCR affinity maturation, whereas the Tfr cell TCR repertoire, which is predominantly autoreactive, enables these cells to suppress autoreactive affinity-matured B cell clones, thus preventing autoantibody-mediated autoimmunity.

Using antigen-specific CD4$^+$ T cells from TCR-transgenic mice, we demonstrate that recruitment of Tfh cells into GCs is predominantly controlled by specificity for the immunizing antigen. By contrast, recruitment of Tfr cells for the same GCs was not biased towards specificity for the immunizing antigen. These findings are confirmed in wild-type (WT) mice

using MHC class II tetramers: while we detect a large population of tetramer-positive Tfh cells, almost no tetramer-positive Tfr cells are found. In addition, we use an independent approach, analysing the TCR diversity from sorted T cell subsets (including Tfh and Tfr) to demonstrate that Tfh cells from GCs induced by immunization with a defined antigen present oligoclonal expansions that are not observed on the Tfr subset. Moreover, the Tfr cell TCR repertoire closely resembles the thymic regulatory T (Treg) cell repertoire. Thus, our data not only confirm that Tfh cells differentiate predominantly from naïve Foxp3$^-$ T cells, and that Tfr cells originate from thymic Foxp3$^+$ Treg cells, but also show that the ontogeny of Tfh and Tfr cells corresponds to a distinct TCR usage.

RESULTS

Tfr cells differentiate from thymic Foxp3$^+$ Treg cells. We had previously shown that under lymphopenic conditions, immunization with a foreign antigen leads to GC formation containing Tfr cells that differentiate from adoptively transferred thymic Foxp3$^+$ Treg cells[8]. In order to exclude a potential artifact elicited from lymphopenic conditions we now investigated, using congenic markers, the precursors of Tfr cells following immunization in two distinct genetic backgrounds (Fig. 1). Magnetic-activated cell sorting (MACS)-purified OVA-specific TCR-transgenic CD4$^+$ T cells from OT-II.*Rag*$^{-/-}$ or DO11.10.*Rag*$^{-/-}$ mice, devoid of thymic Foxp3$^+$ Treg cells, were adoptively transferred into naïve C57BL/6 or Balb/c hosts, respectively (Fig. 1a,b). Recipient mice were subsequently immunized with OVA in incomplete Freund's adjuvant (IFA) in the footpad and draining popliteal lymph nodes (LNs) were analysed by flow cytometry, at the peak of GC response, when higher numbers of Tfh and Tfr cells can be obtained (day 11)[8, 18]. Popliteal LNs were found to contain Tfh populations derived to a great extent from transferred TCR-transgenic cells, while Tfr cells derived exclusively from endogenous T cells (Fig. 1c,d). On the contrary, TCR-transgenic CD4$^+$ T cells from OT-II.*Rag*$^+$ mice that, unlike *Rag*-deficient counterparts, harbour a population of TCR-transgenic thymic Foxp3$^+$ Treg cells (Fig. 1e), can support the differentiation of Tfr cells, following adoptive transfer into T cell

deficient mice (Fig. 1f). Indeed, when OT-II.*Rag*$^{+}$ cells were transferred into *TCRβ*$^{-/-}$ mice we found that they readily gave rise to a population of Tfr cells. Note that the popliteal LN allows the study of GCs driven by the immunizing antigen since Tfh and Tfr cells, abundant in immunized mice, are virtually absent on equivalent LNs of non-immunized C57BL/6 mice (Fig. 1g,h). These results show that, while adoptive transfer of thymic derived Foxp3^{+} Treg cells can differentiate into Tfr cells, Foxp3^{-} T cells only differentiate into Tfh.

No preferential recruitment of antigen-specific Tfr cells. In order to assess the specificity requirements of Tfr cells, we took advantage of OT-II.*Rag*$^{+}$ mice. OVA-specific TCR-transgenic CD4^{+} T cells from these mice were transferred into congenic C57BL/6 mice subsequently immunized with OVA-IFA or a control antigen (β-lactoglobulin, βLG) (Fig. 2a). Again, using congenic markers, it was possible to quantify the frequency of TCR-transgenic cells within the Tfh and Tfr populations (Fig. 2a,b). We found that a large fraction of Tfh cells in mice immunized with OVA were derived from OVA-specific precursors, while those TCR-transgenic precursors were almost excluded from Tfr cells even in OVA-immunized mice (Fig. 2b,c). To exclude that the low number of TCR-transgenic Foxp3^{+} Tfr cells observed was due to competition by a much larger number of endogenous cells, we performed adoptive cell transfers into T cell deficient mice, allowing competition of the same number of TCR-transgenic and WT T cells. Thus, the same number of CD4^{+} T cells from OT-II.*Rag*$^{+}$ and WT mice was co-transferred into *TCRβ*$^{-/-}$ mice followed by immunization with OVA or a control antigen (Fig. 2d). Under those conditions, and 11 days after immunization, TCR-transgenic cells became vastly over-represented among Tfh cells in mice immunized with OVA (Fig. 2e). In addition, we found that TCR-transgenic cells were able to differentiate into Tfr, but their frequency (approximately 1/5 of Tfr cells) did not change significantly regardless of the immunizing antigen. Additionally, the percentage of OT-II.*Rag*$^{+}$ Treg cells that differentiated into Tfr is approximately 8% in both immunizations although, as expected, in OVA-immunized mice the

percentage of OT-II.*Rag*$^{+}$ conventional T (Tconv) cells that differentiate into Tfh is higher than in βLG-immunized mice (Supplementary Fig. 1).

Foxp3^{+} Treg cells from OT-II.*Rag*$^{+}$ mice co-express endogenous TCR chains, in addition to the transgene, that allow their thymic selection as Treg cells[19]. In fact, WT T cells can also express more than one TCR, due to recombination of both TCRα chains[20]. We found that, as previously described, a proportion of Treg cells (~30%) do not co-express the transgenic TCR chains Vα2 and Vβ5 unlike Tconv cells which are virtually all double-positive. Nevertheless, upon adoptive transfer into *TCRβ*$^{-/-}$ mice and immunization, the percentage of OT-II.*Rag*$^{+}$ Treg and Tfr cells co-expressing Vα2 and Vβ5 remained unchanged (Fig. 2f). Therefore, we could not find a preferential enrichment of Vα2 Vβ5 double-positive cells, more likely to be specific for the immunizing antigen, within the regulatory populations after OVA immunization.

As the above observations may question the ability of Foxp3^{+} cells from OT-II.*Rag*$^{+}$ mice to properly respond to OVA stimulation, we confirmed that OT-II Treg cells can be activated by OVA. We cultured sorted Treg (CD4^{+}CD25^{+}GITR^{+}) cells from OT-II.*Rag*$^{+}$ and C57BL/6 WT mice in presence of IL-2 and OVA-pulsed bone marrow-derived DCs (Fig. 2g,h and Supplementary Fig. 2a). We found that OVA-loaded DCs, unlike DCs loaded with a control antigen, promoted proliferation of OT-II Treg cells to similar extent as stimulation with anti-CD3. Moreover, WT Treg cells show lower proliferation and cell numbers when cultured in the presence of both antigens compared to cultures with anti-CD3. Of note, it was previously described that some unspecific proliferation is induced on Treg cells when cultured with activated DCs in the presence of exogenous IL-2[21, 22], which is in line with what we observed in the cultures of WT Treg cells with OVA/βLG-loaded DCs and OT-II Treg cells with βLG-loaded DCs. These data demonstrate that OT-II Treg cells, in spite of the co-expression of endogenous TCR chains, specifically recognize and proliferate in presence of OVA stimulation. As a consequence, the poor differentiation of OT-II Treg cells into Tfr cells following OVA-immunization does not appear to be due to loss of OVA-reactivity.

The low recruitment of OT-II.*Rag*$^{+}$ Treg cells as Tfr cells could be due to an intrinsic characteristic of OT-II transgenic cells. To test this possibility, we repeated the experiment with a second TCR-transgenic model. We transferred, simultaneously, an equal number of CD4^{+} T cells from OT-II.*Rag*$^{+}$ and P25.*Rag*$^{+}$ mice (TCR-transgenic specific for the residues 280-294 of *Mycobacterium tuberculosis* Ag85B) that also have thymic-derived Treg cells (Fig. 3a,b)[23]. Recipient mice were immunized in the footpad with $OVA_{323-339}$ or $Ag85B_{280-294}$ peptides coupled to BSA in IFA. We used peptides coupled to a carrier protein (BSA) because it has been reported that immunization with linear epitopes may fail to generate optimal antibody responses[24]. We found that Tfh cells from the draining LNs have a substantial frequency increase of TCR-transgenic cells specific for the immunizing antigen (a high frequency of OT-II cells in mice immunized with $OVA_{323-339}$BSA, and P25 cells in mice immunized with $Ag85B_{280-294}$BSA; Fig. 3c,d). Within the Tfr population there was only a small increase of TCR-transgenic cells specific for the immunizing antigen.

These results suggest that, while TCR-transgenic cells specific for the immunizing antigen are able to become Tfr cells, those cells are not preferentially selected into the Tfr pool.

Tfr cells do not recognize the immunizing antigen. To obtain an independent validation of our findings without the use of TCR-transgenic mice, we used two MHC-II I-A^{b} tetramers: a phycoerythrin (PE)-labelled tetramer containing an OVA peptide sequence (AAHAEINEA) to identify OVA-specific T cells and an allophycocyanin (APC)-labelled tetramer containing the Ag85B peptide sequence (FQDAYNAAGGHNAVF) to identify Ag85B-specific T cells. Antigen-specific tetramer^{+} cells were detected on draining lymph nodes of C57BL/6 mice 11 days after immunization with several combinations of antigens and adjuvants (Fig. 4a). In all immunizations with $OVA_{323-339}$, the number of OVA-tetramer^{+} cells was increased among the Tfh population compared to mice that were not immunized with this peptide (Fig. 4b,c). The same pattern was observed in mice immunized with $Ag85B_{280-294}$, where we found larger populations of Ag85B-tetramer^{+} Tfh cells (Fig. 4d,e). However, in neither case did we observe

an enrichment of tetramer$^+$ cells within the Tfr population (Fig. 4b-e). Once again, these results demonstrate that Tfr cells, unlike Tfh cells, do not preferentially contain cells specific for the non-self antigen driving the GC response.

To investigate whether Tfr and Tfh TCRs recognize different peptides from the same antigen, we assessed proliferation of sorted Tfh (CD4$^+$CXCR5$^+$PD-1$^+$Foxp3$^-$), Tfr (CD4$^+$CXCR5$^+$PD-1$^+$Foxp3$^+$), and Treg (CD4$^+$CXCR5$^-$PD-1$^-$Foxp3$^+$) cells stimulated with DCs loaded with the immunizing antigen to verify if they specifically proliferate with these signals (Supplementary Fig. 2b). Tfh cells from OVA-immunized C57BL/6 Foxp3 reporter mice (*Foxp3*^{hCD2})[25] were cultured with DCs loaded with OVA or βLG as a control antigen. Tfh cells showed higher proliferation and survival on OVA cultures compared to DCs loaded with βLG or unloaded DCs, demonstrating that this population specifically recognizes antigen signals (Fig. 4f). On the contrary, Tfr cells cultured with OVA presented some proliferation (probably due to the same non-specific effect observed on Treg cell cultures on Fig. 2g,h) but that proliferation was not higher than in the presence of βLG or unloaded DCs (Fig. 4g). The presence of anti-CD3 induced a higher proliferation rate and survival of Tfr cells that translated into higher number of cells at the end of culture compared to the other conditions. Therefore, if Tfr cells were specific for the immunizing antigen, TCR signalling would have led to higher proliferation and cell numbers in the end of culture (Fig. 4g). Of note, follicular T cells seem to be fragile, with low survival capacity when they are not receiving TCR signalling. In fact, non-proliferating cells die quickly in culture and only cells that undergo some degree of background proliferation survive. Tfh, but not Tfr, can be rescued when the immunizing antigen is added to the culture. As Tfr cells, Treg cells sorted from immunized mice also show lower proliferation and cell numbers when cultured with DCs loaded with OVA or βLG compared to the culture in the presence of anti-CD3 (Fig. 4h) and as previously observed on Fig. 2g,h. Taken together, these results demonstrate that, while Tfh cells specifically benefit from signals derived from the immunizing antigen, Tfr and Treg cells do not.

Tfr and Tfh have different *TRBV* CDR3 length distributions. We next performed TCR usage analysis in C57BL/6 mice, bearing a *Foxp3*^{gfp} reporter system, immunized with a model antigen (OVA-IFA). In order to minimize the contamination with pre-existent Tfh and Tfr cells from prior immune responses, we used footpad immunization with collection of popliteal LNs at the peak of the GC response. As shown in Fig. 1g,h, there are negligible numbers of Tfh and Tfr cells in this anatomic location prior to immunization.

Taking advantage of the *Foxp3*^{gfp} reporter system, we sorted Tfh ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^-$), Tfr ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^+$), Treg ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^+$), and Tconv ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^-$) cells from the draining LNs (Supplementary Fig. 2c). The *TRBV* repertoire of the different populations was then analysed by CDR3 spectratyping/Immunoscope[26]. The *TRBV* CDR3 length profiles (or spectratypes) of naïve polyclonal $CD4^+$ T cells resemble Gaussian distributions[26] (Supplementary Fig. 3). Therefore, by comparing the CDR3 length usage for each *TRBV* of the four sorted populations to naïve $CD4^+$ T cells (used as control population), we can detect variations to this polyclonal distribution. Indeed, in the Tfh population there is an over representation of specific CDR3 lengths (in red, Fig. 5a).There are also some over representations for the Tfr cells but the majority of these, besides not being common to Tfh cells, are also present in Treg cells (Fig. 5a). In fact, detailed analysis of specific *TRBV* segments can identify clonal expansions among Tfh cells that are absent in the other T cell subsets (Fig. 5b, arrows). Also, we calculated a perturbation score[27] for every *TRBV* segment between all samples and the Tfr group average. Hierarchical clustering and principal component analysis (PCA) were performed using the calculated perturbation scores to reveal the divergent *TRBV* usage of all cell populations compared to Tfr group average. On the heatmap, Tfh cells present the higher perturbation scores (more divergent *TRBV* CDR3 length distributions from Tfr cells) and are clustered separately, while we observe a proximity between Tfr and Treg cells (Fig. 5c). Moreover, the same relation between sample populations can be observed when we perform PCA: the first two principal components, which describe 77.3% of the samples variability, separate the Tfh from the remaining samples, while clustering Tfr, Treg, and Tconv together (Fig. 5d). ANOVA

and multiple comparison analysis with Holm-Bonferroni correction for multiple sampling established the significance of the differences observed between populations, and between Tfh samples and each sample from the other populations (Supplementary Table 1).

Taken together, our data show that, within LNs draining the immunizing site, Tfh cells exhibit clear oligoclonal expansions of specific *TRBV* CDR3 lengths. The same pattern of *TRBV* CDR3 usage is not observed within the Tfr population that retain distributions of CDR3 lengths usage similar to Treg cells.

The TCR repertoire of Tfr cells resembles that of Treg cells. The spectratyping data demonstrate that, although Tfr and Tfh cell numbers increase after immunization, they present different *TRBV* CDR3 length usage. To further verify the different TCR usage between both populations, we sequenced the *TRA* of 1D2β mice, which express a fixed TCRβ chain and variable TCRα chains. This 1D2β mouse line, of C57BL/6 background, was established using nuclear-transferred ES (NT-ES) cells that had been generated using peripheral $CD25^{high}CD4^{+}$ T cells as donor of nuclei. The productively rearranged *TRB* gene of one NT-ES cell line was successfully transmitted to germline and the resulting 1D2β mice were mated with $Foxp3^{hCD2}$ and $TRA^{-/-}$ mice to generate $Foxp3^{hCD2}.TRB^{1D2}.TRA^{-/WT}$ mice. Thus, 1D2β mice *TRA* sequencing provides a complete insight to the repertoire of the analysed populations, compared to WT mice, as the corresponding *TRB* does not vary.

Although these mice have a restricted *TRB* repertoire, we confirmed that their $CD4^{+}$ T cells were able to recognize OVA and differentiate into Tfh cells following OVA-immunization (Fig. 6a). To show that 1D2β Tfh cells that arise after OVA-immunization can specifically recognize the antigen, we performed *in vitro* proliferation assays (Fig. 6b). 1D2β mice were immunized with OVA-IFA and 11 days later Tfh ($CD4^{+}CXCR5^{+}PD\text{-}1^{+}Foxp3^{-}$) cells from three individual mice were sorted and cultured with DCs pulsed with OVA (Supplementary Fig. 2d). Tfh cells from all three mice were able to proliferate with OVA signals but not with the control antigen

βLG (Fig. 6b). Thus, these mice, while not being able to recombine the *TRB*, still have a repertoire capable of specifically recognizing OVA.

Five T cell populations were sorted for *TRA* repertoire analysis on day 11 following OVA-IFA immunization from draining LNs of individual mice: Tfr ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^+$), Tfh ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^-$), Treg ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^+$), activated $CD4^+$ T (Tact: $CD4^+CXCR5^-PD\text{-}1^-Foxp3^-CD44^+$), and non-activated $CD4^+$ T (Tconv: $CD4^+CXCR5^-PD\text{-}1^-Foxp3^-CD44^-$) cells (Supplementary Fig. 2d). *TRA* gene was then specifically amplified and sequenced on an illumina MiSeq platform. In order to perform an unbiased analysis, 9,000 TCR sequences were randomly selected from each dataset per sample (9,000 being the lowest number of TCR sequences identified on a sample).

We started by verifying the number of common clonotypes between Tfr cells and other populations. The number of shared clonotypes is higher between Tfr and Treg cells than any other population (Fig. 6c and Supplementary Fig. 4a). However, we found that from the 9,000 TCR sequences the number of identifiable clonotypes was lower for Tfr, Tfh, and Tact when compared to Treg and Tconv. To determine if this observation was in line with different clonality in the populations, we calculated a clonality score for each sample (Fig. 6d). Indeed, the clonality score was higher for the populations with lower numbers of identifiable clonotypes, as expected, given the fact that Tfh, Tact, and Tfr have undergone cell proliferation, unlike Treg and Tconv. When we checked the frequency distribution of the 20 most frequent clonotypes for each sample across all samples, we observed that the 5 most abundant clonotypes represented in average up to ~50% of the total frequency for Tfh and Tact and ~40% for Tfr cells (Fig. 6e). This was somehow expected since Tfr, Tfh, and Tact almost do not exist prior to immunization and must expand upon immunization, in contrast to Treg and Tconv. (Fig 6a). Remarkably, the most common clonotypes from Tfh cells are shared in different mice (yellow), and the same happens for the most common Tfr clonotypes (green), although without a significant overlap between Tfh and Tfr clonotypes (Fig. 6e). To further investigate the proximity between Tfr and Tfh TCR repertoire, we investigated if the 20 most predominant clonotypes from each Tfr sample are shared with other populations. We found that the cell

population that shares more clonotypes with the predominant Tfr clonotypes is the Treg population (Fig. 6f). Among the most abundant clonotypes there is only one shared between all Tfr and Tfh samples, but that clonotype is also present on all Treg samples. The same approach was performed to obtain the 20 most predominant clonotypes for each Tfh sample (Supplementary Fig. 4b) where it is shown that Tfh cells present more shared clonotypes with Tact. Lastly, we wanted to establish the closeness between all samples regardless of the observed expansions. For that, we performed hierarchical clustering using Horn-Morisita index[28]. This index has been described as appropriate to compare immune repertoires since it is able to access the similarity between samples while taking into account the abundance of each clonotype in each sample. We found that Tfr and Treg samples are clustered together (AU = 96%), indicating that Tfr samples have a repertoire closer to that of Treg cells, rather than to that of any of the other populations (Fig. 6g).

These results indicate that, although Tfr cells undergo proliferation, their TCR is not specific for the antigen driving the GC reaction since the TCR usage has little in common with Tfh cells repertoire. Instead, Tfr cells present a TCR repertoire that closely resembles that of Treg cells.

DISCUSSION

During GC reaction the BCR undergoes affinity maturation leading to formation of higher affinity receptors selected by Tfh cells. However, some mutations may lead to autoreactive receptors or receptors that are cross-reactive with autoantigens. Given the documented capacity of Tfr cells to prevent auto-antibody-mediated autoimmunity[13-17], we investigated the possibility that the TCR usage by Tfh and Tfr populations from the same GCs is different: only the Tfh repertoire is biased towards the immunizing antigens.

We used three distinct approaches to address that issue: one approach based on cell transfer of T cells with defined TCR specificity; another approach using tetramers to identify antigen-specific Tfh and Tfr cells in WT mice; and a final approach based on the analysis of the TCR repertoire. All experiments led to similar conclusions, showing that T cell clones specific for the immunizing antigen are enriched within the Tfh pool, but not within Tfr cells. Our experiments

do not directly show that Tfr cells recognize self antigens and, as a consequence, the antigen-specificity of Tfr cells remains to be established. Nevertheless, as the TCR repertoire of Tfr cells is closer to that of Treg cells, rather than to that of any of the other populations, we speculate that Tfr cells share the same specificity as the Treg population which is biased towards self antigens.

We cannot exclude that, under certain conditions, Tfr cells may be specific for the immunizing antigen and differentiate from peripherally induced Treg cells. Indeed, a recent report claims that Tfr cells can differentiate from Foxp3$^-$ T cells and can be specific for the immunizing antigen[29]. However, even under those conditions tested, the percentage of tetramer binding Tfr cells was only ~3% of total Tfr cells (which we did not observe on our experiments even after enrichment) and only a percentage of those Tfr cells presented markers indicating their differentiation from Foxp3$^-$ T cells. Moreover, these cells alone do not seem to substitute Tfr cells that originate from thymic Treg cells: Tfr cells seem to influence the affinity maturation process[30, 31] and it has been also shown that mice lacking Treg cells able to migrate into the GC have impaired affinity maturation[31].

We studied popliteal LNs in mice immunized in the footpad. In that location nearly all Tfh and Tfr cells are derived from GCs induced by the immunizing antigen (with negligible contaminants). As a consequence, the different TCR usage between Tfr and Tfh cells, as shown in Fig. 5 and 6, suggests different antigen-specificity requirements. Furthermore, the fact that Tconv and Treg from draining LNs have Gaussian-like *TRBV* distributions and a low clonality score is in line with our observations with adoptively transferred TCR-transgenic cells: adoptive transfer of TCR-transgenic T cells into non-lymphopenic hosts (Fig. 2c and 3d) leads only to a minor expansion of antigen-specific cells among Tconv (4-6%), unlike what is observed within Tfh cells (30-40%). In addition, the results obtained with tetramers corroborated our hypothesis: we could detect antigen-specific tetramer binding Tfh, but not Tfr, cells indicating a different TCR usage.

The ontogenic origin of Tfh and Tfr cells has remained an important issue as it may be related to the distinct functional specialization of the two T follicular populations. Indeed, following

immunization with an antigen in adjuvant, a process similar to what is observed following vaccination, Tfr cells associated with those induced GCs originate from pre-existing thymic Treg cells (Fig. 1). Such ontogenic proximity between Tfr and Treg has a repercussion on the TCR usage by the two populations: the TCR usage by Tfr cells remains largely similar to the TCR usage of Treg cells.

The different ontogeny of Tfr and Tfh cells suggests an attractive model for distinct functions of the two populations: while Tfh cells predominantly promote humoral responses targeting non-self antigens, Tfr cells prevent the generation of auto-antibody-mediated autoimmunity. An expected consequence of this model is a different TCR repertoire of Tfh *vs* Tfr cells, which is confirmed by our present results. The ability of Tfr cells to regulate non-antigen-specific B cell clones has been suggested[9]. The distinct range of antigen targets of Tfh and Tfr cells from the same GCs provides a molecular basis for such differential behaviour. It should be noted, however, that such distinction is not complete: it was shown that Tfr cells can partially regulate the amount of antibodies produced targeting a foreign antigen[8].

In conclusion, our data establish a different antigen-specificity of Tfr and Tfh populations from the same GCs. Tfh cells repertoire comprises oligoclonal expansions in response to immunizing antigens. Such expansions are not observed in Tfr cells that bear a TCR repertoire resembling that of Treg cells, and thus, possibly, biased towards autoreactivity.

METHODS

Mice and animal procedures

C57BL/6, C57BL/6.*Thy1.1*, *Foxp3*^{gfp}, *Foxp3*^{hCD2}, Balb/c, *TCRβ*$^{-/-}$, DO11.10.*Rag*$^{-/-}$, OT-II.*Rag*$^{-/-}$, OT-II.*Rag*$^{+}$.*Thy1.1*.*Thy1.2*, P25, and 1D2β mice were bred and maintained in our institute specific pathogen-free facilities. Animals of both sex (same sex per experiment) and with age ranged from 2 to 6 months were used. For animal studies, no randomization or blinding was done. Permission for animal experimentation was granted by the ORBEA-iMM (the institutional Animal Welfare Body) and DGAV (Portuguese competent authority for animal protection). Animals were immunized subcutaneously in the footpad with different antigens: Ovalbumin (Sigma-Aldrich, Cat#A5503), β-lactoglobulin (Sigma-Aldrich, Cat#L3908), $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) peptide, $Ag85B_{280-294}$ (FQDAYNAAGGHNAVF) peptide (Schafer-N) and with $OVA_{323-339}$ or $Ag85B_{280-294}$ conjugated with BSA (synthesized by Thermo Fisher Scientific or Schafer-N). Antigens were prepared by mixing the antigen solution 1:1 (v:v) with incomplete or complete Freund's adjuvant (IFA or CFA, respectively) (Sigma-Aldrich, Cat#F5506 and Cat#F5881) to a final concentration of 1.6 mg ml^{-1} (proteins and peptides coupled to proteins) or 2 mg ml^{-1} (peptides alone). 50 µl of emulsion was injected in each footpad. For adoptive cell transfers, purified CD4^{+} T cells were injected i.v. in saline solution. In all experiments the immunization occurred 1 day after the adoptive cell transfer, and collection of popliteal LNs at day 11 following immunization.

Flow cytometry and cell sorting

For flow cytometry analysis and sorting, single cell suspensions were obtained and stained with the following mAbs: CD4 (RM4-5, dilution 1/200), CD19 (ebio1D3, dilution 1/100), CD25 (PC61.5, dilution 1/400), Foxp3 (FJK-16s, dilution 1/100), human CD2 (RPA-2.10, dilution 1/200), PD-1 (J43, dilution 1/100), Thy1.1 (HIS51, dilution 1/200), Thy1.2 (53-2,1, dilution 1/400), TCRβ (H57-597, dilution 1/100), and Vα2 (B20.1, dilution 1/200) from eBioscience; GITR (DTA.1, dilution 1/200), CXCR5 (2G8, dilution 1/50) and Vβ5.1, 5.2 (MR9-4, dilution 1/200) from BD Pharmingen; and CD4 (RM4-5, dilution 1/100) from Biolegend. Intracellular

Foxp3 staining was performed using the Foxp3 Staining Set (eBioscience, Cat#00-5523-00) according to manufacturer's instructions. $OVA_{323-339}$ or $Ag85B_{280-294}$ specific T cells were detected with a PE-(OVA) or APC-(Ag85B) conjugated MHC-II I-A^b tetramer containing an $OVA_{329-337}$ (AAHAEINEA) or $Ag85B_{280-294}$ (FQDAYNAAGGHNAVF) peptide, respectively. Staining was performed for 1h at RT[32]. Enrichment of tetramer$^+$ cells was performed using MACS cell separation system and anti-PE and anti-APC magnetic beads (Miltenyi Biotec, Cat#130-048-801 and Cat#130-090-855). Samples were acquired on a BD LSR Fortessa flow cytometer. Acquisition data was analysed on FlowJo software (Tree Star). For cell sorting: $CD4^+$ T cells for adoptive cell transfers were purified from spleen and mesenteric LNs using MACS cell separation system and anti-CD4 (L3T4) magnetic beads (Miltenyi Biotec, Cat#130-049-201). FACS-sorting, with mAbs mentioned above, was performed on a BD FACSAria cell sorter. For flow cytometry analysis of cultured cells and tetramer enriched cell samples, 10 µm latex counting beads were added to cell suspensions to obtain total cell counts (Counter Beckman).

***In vitro* cultures**

Bone marrow derived DCs were generated by culturing progenitors for 7 days in presence of GM-CSF (PeproTech). Specific antigen loading was performed for 3h at 37ºC in presence of 1 mg ml^{-1} of protein. In OT-II Treg/WT Treg and Tfr/Tfh/Treg cell cultures, T cells were pre-incubated with Cell Trace Violet (Life Technologies) for tracking cell proliferation according to manufacturer's instructions. A 3:2 ratio of $CD4^+$ T cells to DCs was used to a final number of $5x10^4$ cells per well in the case of OT-II Treg/WT Treg cell cultures and $2.5x10^4$ cells per well in the case of Tfr/Tfh/Treg cell cultures. Cells were co-cultured in the presence of 2 ng ml^{-1} IL-2 (eBioscience, Cat#14-8021-64) and, in some conditions, 3 µg ml^{-1}anti-CD3 (145-2C11, eBioscience) was added to the culture. After 3 days, cells were stained and analysed by flow cytometry. For Tfh proliferation assay, $2.0x10^4$ Tfh cells were cultured with the same number of DCs. In the wells where unloaded DCs were cultured, 3 µg ml^{-1} anti-CD3 was added to the culture. After 3 days of culture, cells were incubated with 1.0 µCi per well of 3H-thymidine

(Perkin Elmer, Cat#NET027W001MC) at 37ºC for 6 hours, harvested on a Tomtec Harvester (Tomtec) and scintillation counted on a Microbeta Trilux (PerkinElmer).

CDR3 length analysis

RNA extraction from cell-sorted populations ($2x10^5$ - $5x10^6$ cells) was performed using TRIzol (Life Technologies, Cat#15596). cDNA was amplified using Random Primers and SuperScript III Reverse Transcriptase (Invitrogen, Cat#48190011 and Cat#18080-044). Both RNA extraction and cDNA synthesis were performed following the manufacturer's instructions. To perform CDR3 spectratyping[26], each obtained cDNA was divided into 23 parallel PCR reactions with a common Cβ reverse primer and 23 Vβ-specific forward primers (GoTaq DNA Polymerase from Promega, Cat#M7801, and primers from Life Technologies). Run-off reactions were done using dye-labelled Cβ primer. All primer sequences can be found on Supplementary Table 2. Run-off products were run on ABI 3130XL Automatic Sequencer (Applied Biosystems) together with GeneScan 500 ROX dye Size Standard (Applied Biosystems, Cat#401734) and consequently separated based on their nucleotide size. Gene Mapper software (Applied Biosystems) was used to obtain nucleotide length and area of each peak.

Deep sequencing

RNA from 1D2β mice sorted cell populations ($1.1x10^4$ – $1.0x10^6$ cells) was extracted using RNeasy Mini kit (Qiagen, Cat#74104) following manufacturer's instructions. Full-cDNA library was prepared using Mint-2 kit (evrogen, Cat#SK005), which introduces 5-prime adapters to cDNA fragments, according to manufacturer's instructions. *TRA* was then specifically amplified using Pfx DNA polymerase (Invitrogen, Cat#11708013) and a primer pair (Life Technologies) specific for the 5'-adapter and the C region of *TRA* gene. Primers used for *TRA* amplification can be found on Supplementary Table 2. Sequencing library was prepared using the Nextera kit (Cat#FC-121-1030 and Cat#FC-131-1002), in which each sample was barcoded, and sequenced using 250bp paired-end illumina MiSeq technology (all illumina).

CDR3 data analysis

In order to have adequate representation of a complete *TRBV* repertoire, it is necessary to analyse at least $2x10^5$ cells. To achieve this number of cells, it was necessary to pool draining LNs from 15 mice for each biological replicate. In our experiments, we used at least three biological replicates for each T cell subset. To extract and analyse the data obtained for CDR3 fragment size we used ISEApeaks®[33, 34]. Briefly, this software quantifies the percentage of use of each CDR3 length, obtained by dividing the area of CDR3 peaks by the total area of all peaks within the profile. On C57BL/6 mice, *TRBV21* and *TRBV24* are pseudogenes and thus ignored on the analysis. *TRBV12-2* was also discarded since it could not be detected on three of the samples. To facilitate the comparison between samples and populations, a perturbation score[27] was computed to obtain the overall differences between *TRBV* CDR3 spectratypes of each sample and the average profiles of Tfr samples as control group. Calculated scores were used to perform the hierarchical clustering (using Euclidean distance and average linkage) and PCA.

Deep sequencing data analysis

Paired-end 250bp illumina sequencing data was initially trimmed and subsequently merged using PEAR[35]. clonotypeR[36] toolkit was then used to perform TCR sequence annotation. Two samples had to be discarded due to low sequencing quality (mouse 3 Tfh sample and mouse 4 Tfr sample). Out of the 7,547,998 raw reads obtained for the 18 remaining samples, we identified 25,099 TCR clonotypes from 949,729 productive TCR sequences. For the samples to be comparable, the analyses were performed on 9,000 randomly selected TCR sequences for each dataset as it was the lowest number of TCR sequences found in a dataset. We repeated this sampling process 100 times to obtain the Mean ± SD values presented on the Venn diagrams. The presented clonality metric is 1- Pielou's evenness index[37], and can vary from 0 to 1 (more diverse to less diverse). The Pielou's evenness corresponds to the Shannon's entropy[38, 39] (using log 2) for each sample divided by the number of unique clonotypes (in log 2) of the same sample. For the histogram of cumulative frequency, the 20

most predominant clonotypes were determined for each sample, and gathered across all samples to plot the cumulative frequency of those clonotypes for each sample. The 20 most predominant Tfr clonotypes were selected from each of the three Tfr samples and gathered into a list used to perform hierarchical clustering in all samples (using Euclidean distance and average linkage). The same was performed for the 20 most predominant clonotypes of each Tfh sample. Dendrogram of overall relation between all samples was obtained using Horn-Morisita index[28, 40] as distance and average linkage. This index assesses the similarity between samples taking into account the abundance of each clonotype in the sample. Approximately unbiased (AU) p-values were calculated for each cluster through 1000 bootstrap resampling iterations[41].

Statistical analysis

Scatter plots and column bar graphs were obtained using GraphPad PRISM. Unless stated otherwise, n represents the number of individual mice analysed per experiment. To determine statistical significance, two-tailed nonparametric Mann–Whitney U tests were performed, and $p<0.05$ was deemed significant (in figures: * $p<0.05$; ** $p<0.01$). A minimum of 5 mice per group were used on *in vivo* experiments to allow usage of non-parametric statistical tests. Clustering analysis, PCA, Venn diagrams, ANOVA, pairwise multiple comparison analysis with Holm-Bonferroni correction for multiple sampling, statistical analysis, and multivariate analysis were performed using R software (http://www.r-project.org/).

Data availability

Sequence data that support the findings of this study have been deposited in the Sequence Read Archive (SRA) with the accession code SRP096953. All other relevant data are available from the corresponding author upon reasonable request.

REFERENCES

1. Victora, G.D. & Nussenzweig, M.C. Germinal centers. *Annu Rev Immunol* **30**, 429-457 (2012).
2. Pitzalis, C., Jones, G.W., Bombardieri, M. & Jones, S.A. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. *Nat Rev Immunol* **14**, 447-462 (2014).
3. Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* **192**, 1545-1552 (2000).
4. Walker, L.S. *et al.* Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. *J Exp Med* **190**, 1115-1122 (1999).
5. Schaerli, P. *et al.* CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* **192**, 1553-1562 (2000).
6. Ramiscal, R.R. & Vinuesa, C.G. T-cell subsets in the germinal center. *Immunol Rev* **252**, 146-155 (2013).
7. Tangye, S.G., Ma, C.S., Brink, R. & Deenick, E.K. The good, the bad and the ugly - TFH cells in human health and disease. *Nat Rev Immunol* **13**, 412-426 (2013).
8. Wollenberg, I. *et al.* Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* **187**, 4553-4560 (2011).
9. Linterman, M.A. *et al.* Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* **17**, 975-982 (2011).
10. Chung, Y. *et al.* Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* **17**, 983-988 (2011).
11. Sage, P.T., Francisco, L.M., Carman, C.V. & Sharpe, A.H. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* **14**, 152-161 (2013).

12. Sage, P.T. & Sharpe, A.H. T Follicular Regulatory Cells in the Regulation of B cell Responses. *Trends Immunol* (2015).
13. Vaeth, M. *et al.* Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med* **211**, 545-561 (2014).
14. Leavenworth, J.W., Verbinnen, B., Yin, J., Huang, H. & Cantor, H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol* **16**, 96-106 (2015).
15. Ding, Y. *et al.* Interleukin-21 promotes germinal center reaction by skewing the follicular regulatory T cell to follicular helper T cell balance in autoimmune BXD2 mice. *Arthritis Rheumatol* **66**, 2601-2612 (2014).
16. Dhaeze, T. *et al.* Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis. *J Immunol* **195**, 832-840 (2015).
17. Charbonnier, L.M. *et al.* Regulatory T-cell deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like disorder caused by loss-of-function mutations in LRBA. *J Allergy Clin Immunol* **135**, 217-227 (2015).
18. Vanderleyden, I., Linterman, M.A. & Smith, K.G. Regulatory T cells and control of the germinal centre response. *Arthritis Res Ther* **16**, 471 (2014).
19. Zal, T., Weiss, S., Mellor, A. & Stockinger, B. Expression of a second receptor rescues self-specific T cells from thymic deletion and allows activation of autoreactive effector function. *Proc Natl Acad Sci U S A* **93**, 9102-9107 (1996).
20. Padovan, E. *et al.* Expression of two T cell receptor alpha chains: dual receptor T cells. *Science* **262**, 422-424 (1993).
21. Zou, T., Caton, A.J., Koretzky, G.A. & Kambayashi, T. Dendritic cells induce regulatory T cell proliferation through antigen-dependent and -independent interactions. *J Immunol* **185**, 2790-2799 (2010).
22. Levine, A.G., Arvey, A., Jin, W. & Rudensky, A.Y. Continuous requirement for the TCR in regulatory T cell function. *Nat Immunol* **15**, 1070-1078 (2014).

23. Tamura, T. *et al.* The role of antigenic peptide in CD4+ T helper phenotype development in a T cell receptor transgenic model. *Int Immunol* **16**, 1691-1699 (2004).

24. Agarwal, A., Nayak, B.P. & Rao, K.V. B cell responses to a peptide epitope. VII. Antigen-dependent modulation of the germinal center reaction. *J Immunol* **161**, 5832-5841 (1998).

25. Komatsu, N. *et al.* 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**, 1903-1908 (2009).

26. Pannetier, C. *et al.* The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments. *Proc Natl Acad Sci U S A* **90**, 4319-4323 (1993).

27. Gorochov, G. *et al.* Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. *Nat Med* **4**, 215-221 (1998).

28. Venturi, V. *et al.* Method for assessing the similarity between subsets of the T cell receptor repertoire. *J Immunol Methods* **329**, 67-80 (2008).

29. Aloulou, M. *et al.* Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun* **7**, 10579 (2016).

30. Sage, P.T., Paterson, A.M., Lovitch, S.B. & Sharpe, A.H. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* **41**, 1026-1039 (2014).

31. Kawamoto, S. *et al.* Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* **41**, 152-165 (2014).

32. Moon, J.J. *et al.* Tracking epitope-specific T cells. *Nat Protoc* **4**, 565-581 (2009).

33. Collette, A. & Six, A. ISEApeaks: an Excel platform for GeneScan and Immunoscope data retrieval, management and analysis. *Bioinformatics* **18**, 329-330 (2002).

34. Collette, A., Cazenave, P.A., Pied, S. & Six, A. New methods and software tools for high throughput CDR3 spectratyping. Application to T lymphocyte repertoire modifications during experimental malaria. *J Immunol Methods* **278**, 105-116 (2003).

35. Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30**, 614-620 (2014).

36. Plessy, C., Mariotti-Ferrandiz, E., Manabe, R.-I. & Hori, S. clonotypeR--high throughput analysis of T cell antigen receptor sequences. Preprint at http://biorxiv.org/content/early/2015/10/08/028696 (2015).

37. Pielou, E.C. The measurement of diversity in different types of biological collections. *Journal of Theoretical Biology* **13**, 131-144 (1966).

38. Shannon, C.E. A Mathematical Theory of Communication. *At&T Tech J* **27**, 379-423 (1948).

39. Stewart, J.J. *et al.* A Shannon entropy analysis of immunoglobulin and T cell receptor. *Mol Immunol* **34**, 1067-1082 (1997).

40. Horn, H.S. Measurement of "Overlap" in Comparative Ecological Studies. *The American Naturalist* **100**, 419-424 (1966).

41. Suzuki, R. & Shimodaira, H. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* **22**, 1540-1542 (2006).

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AUTHOR CONTRIBUTIONS

LG and JF conceived the idea and supervised the study. ARM, SCPA, EMF, and FJ performed experiments. ARM, SCPA, EMF, WC, DK, JF, and LG analysed the data. AS and SH contributed with key reagents and software. ARM and LG wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

FIGURES AND FIGURE LEGENDS

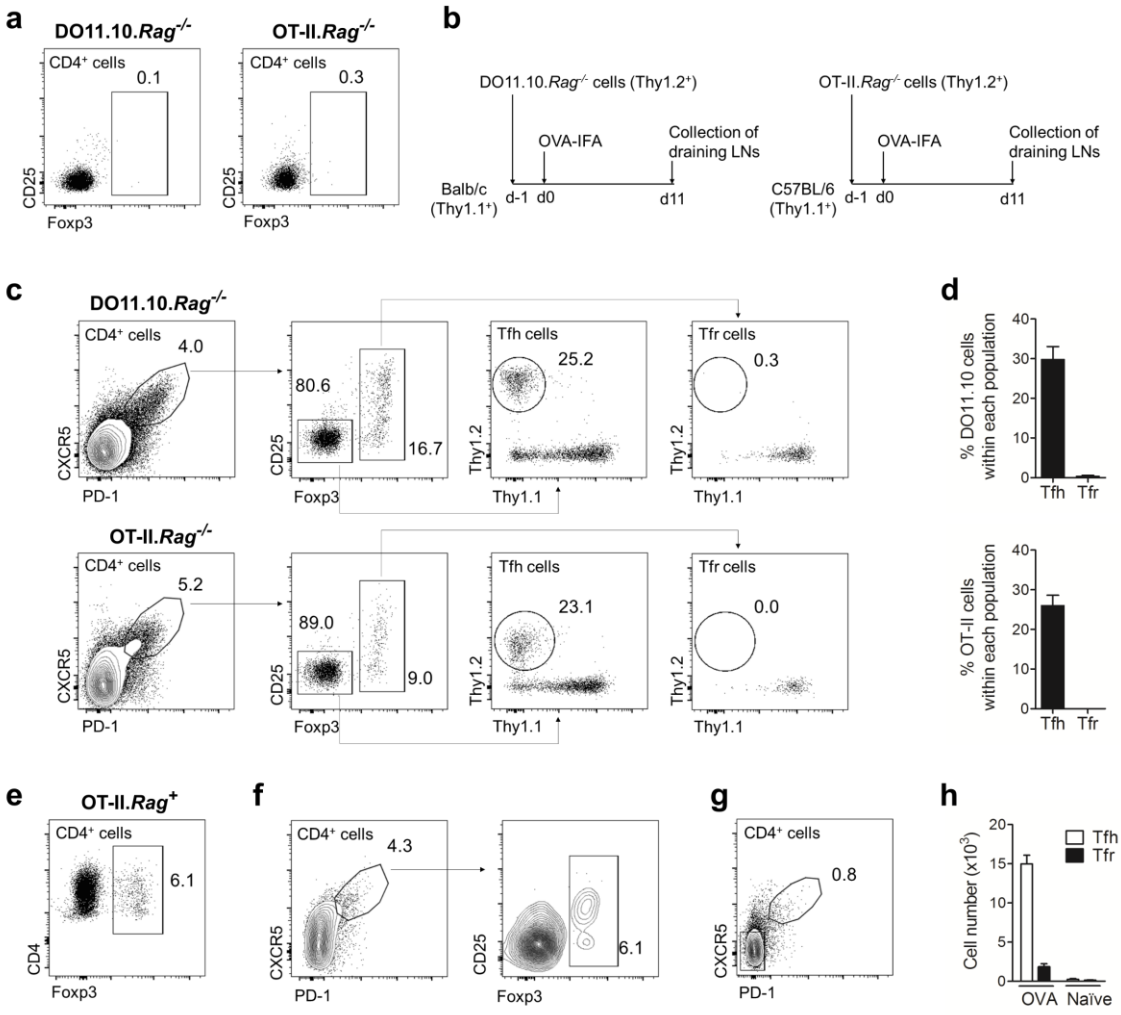


Figure 1. *Tfr cells do not differentiate from Foxp3⁻ T cell precursors*. (**a**) CD4⁺ T cells from OVA-specific OT-II.*Rag*$^{-/-}$ or DO11.10.*Rag*$^{-/-}$ mice are devoid of Foxp3⁺ Treg cells. (**b**) 10^6 CD4⁺ T cells from OT-II.*Rag*$^{-/-}$ or DO11.10.*Rag*$^{-/-}$ mice were adoptively transferred into, respectively, C57BL/6 or Balb/c hosts subsequently immunized with OVA-IFA in the footpad. At day 11 popliteal LNs were analysed by flow cytometry. (**c**) Gating strategy for detection of DO11.10.*Rag*$^{-/-}$ (upper panel) or OT-II.*Rag*$^{-/-}$ (bottom panel) within Tfh and Tfr cell populations. (**d**) While Tfh (CD4⁺CXCR5⁺PD-1⁺Foxp3⁻) cells contained ~25-30% TCR-transgenic cells, those adoptively transferred cells could not be detected among the Tfr (CD4⁺CXCR5⁺PD-1⁺Foxp3⁺) population in any of the two genetic backgrounds. Mean ± SEM are presented for n=5. (**e**) OT-II.*Rag*⁺ mice have Foxp3⁺ Treg cells. (**f**) Adoptive transfer of CD4⁺ T cells from

OT-II.*Rag*$^{+}$ mice into *TCRβ*$^{-/-}$ hosts followed by immunization as described in (**b**). Under these conditions the transferred TCR-transgenic cells originated both Tfh and Tfr cells. (**g**) Relative frequency of T follicular cells in popliteal LNs of non-immunized C57BL/6 mice. (**h**) Absolute number of Tfh and Tfr cells within popliteal LNs from non-immunized C57BL/6 mice compared to OVA-immunized mice. Mean ± SEM is presented for n=3.

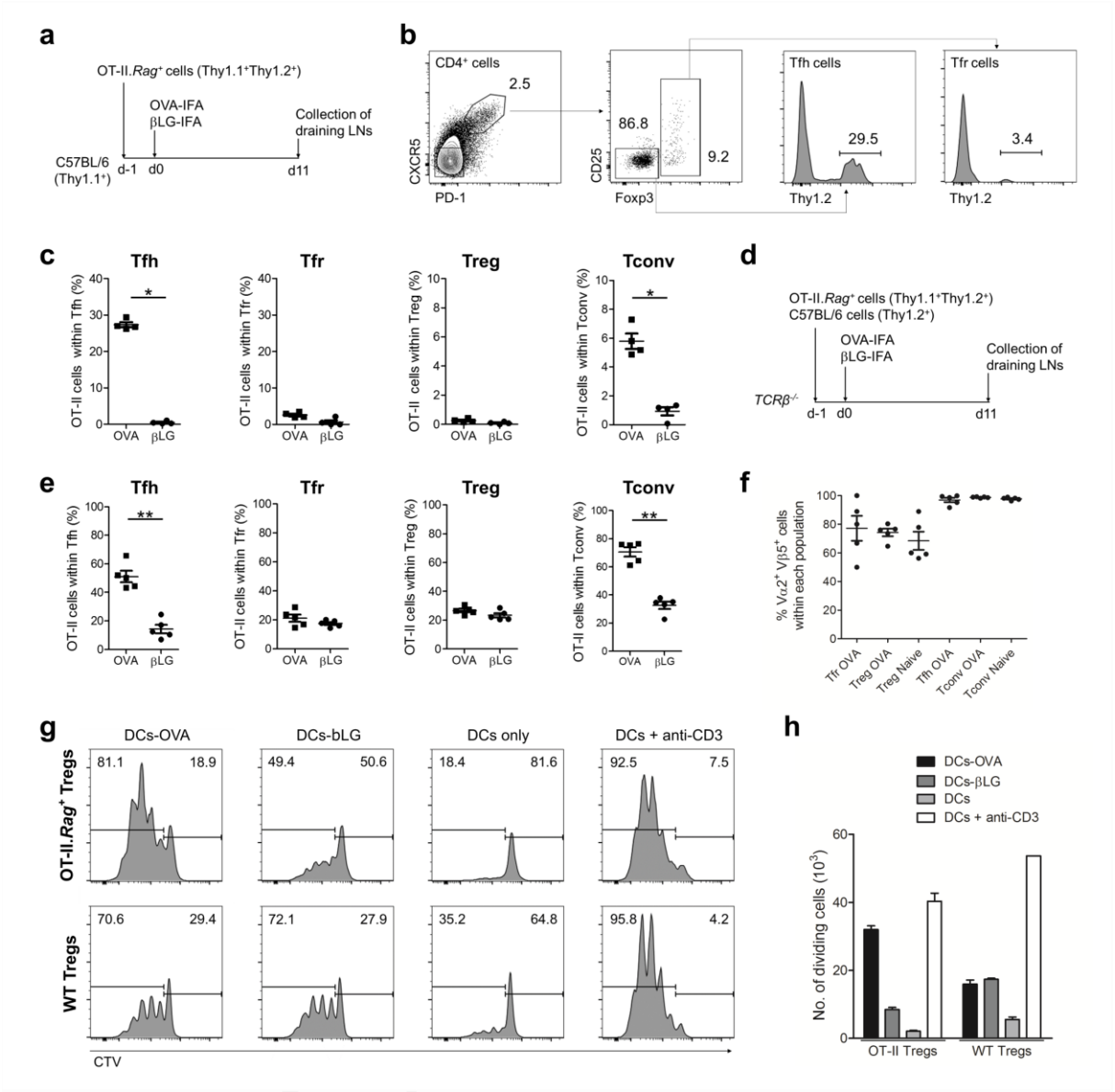


Figure 2. *No preferential accumulation of OVA-specific cells within the Tfr population.* (**a**) 10^7 $CD4^+$ T cells from OT-II.*Rag*$^+$ mice were transferred into C57BL/6 hosts, subsequently immunized with OVA-IFA or βLG-IFA in the footpad. (**b**) Popliteal LNs were analysed for the presence of OT-II.*Rag*$^+$ TCR-transgenic cells within Tfh and Tfr populations based on Thy1.2 staining. Tfh cells were defined as $CD4^+CXCR5^+PD\text{-}1^+Foxp3^-$ and Tfr cells as $CD4^+CXCR5^+PD\text{-}1^+Foxp3^+$. The same gating strategy was applied on Treg ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^+$) and Tconv ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^-$) cells to determine the percentage of $Thy1.2^+$ OT-II.*Rag*$^+$ TCR-transgenic T cells within those two populations. (**c**) OVA-specific TCR-transgenic cells were over-represented within Tfh and Tconv populations in mice immunized with OVA (* p<0.05 using two-tailed nonparametric Mann–Whitney U tests). Within Tfr and

Treg cells there was no significant increase in OVA-specific cells in mice immunized with OVA, compared to βLG immunized animals. (**d**) An equal number (10^7) of $CD4^+$ T cells from OT-II.*Rag*$^+$ and C57BL/6 mice were transferred into T cell deficient *TCRβ*$^{-/-}$ mice, subsequently immunized as described above. (**e**) Under these conditions there was an even greater representation of OVA-specific cells within Tfh and Tconv populations (* $p<0.05$ and ** $p<0.01$ using two-tailed nonparametric Mann–Whitney U tests). Although ~20% of Tfr cells derived from the OVA-specific TCR-transgenic population, that frequency remained similar in mice immunized with OVA or βLG. Similar results were obtained in two additional independent experiments, all with n=5. (**f**) Frequency of Vα2 Vβ5 double-positive cells within Tfr, Treg, Tfh, and Tconv populations from naïve or OVA-immunized mice. (**g**) Fluorescence-activated cell sorting-purified C57BL/6 and OT-II.*Rag*$^+$ Treg ($CD4^+CD25^+GITR^+$) cells were labelled with CellTrace Violet (CTV) and cultured for 3 days in presence of IL-2 and bone marrow DCs loaded with OVA or βLG. In control groups T cells were cultured with unloaded DCs with or without soluble anti-CD3. Histograms are representative of Treg ($CD4^+TCR\beta^+CD25^+Foxp3^+$) cell proliferation at the end of the culture. (**h**) Quantification of the number of proliferating cells. Culture triplicates are presented on the histogram and are representative of three independent experiments. Mean ± SEM are presented in all graphs.

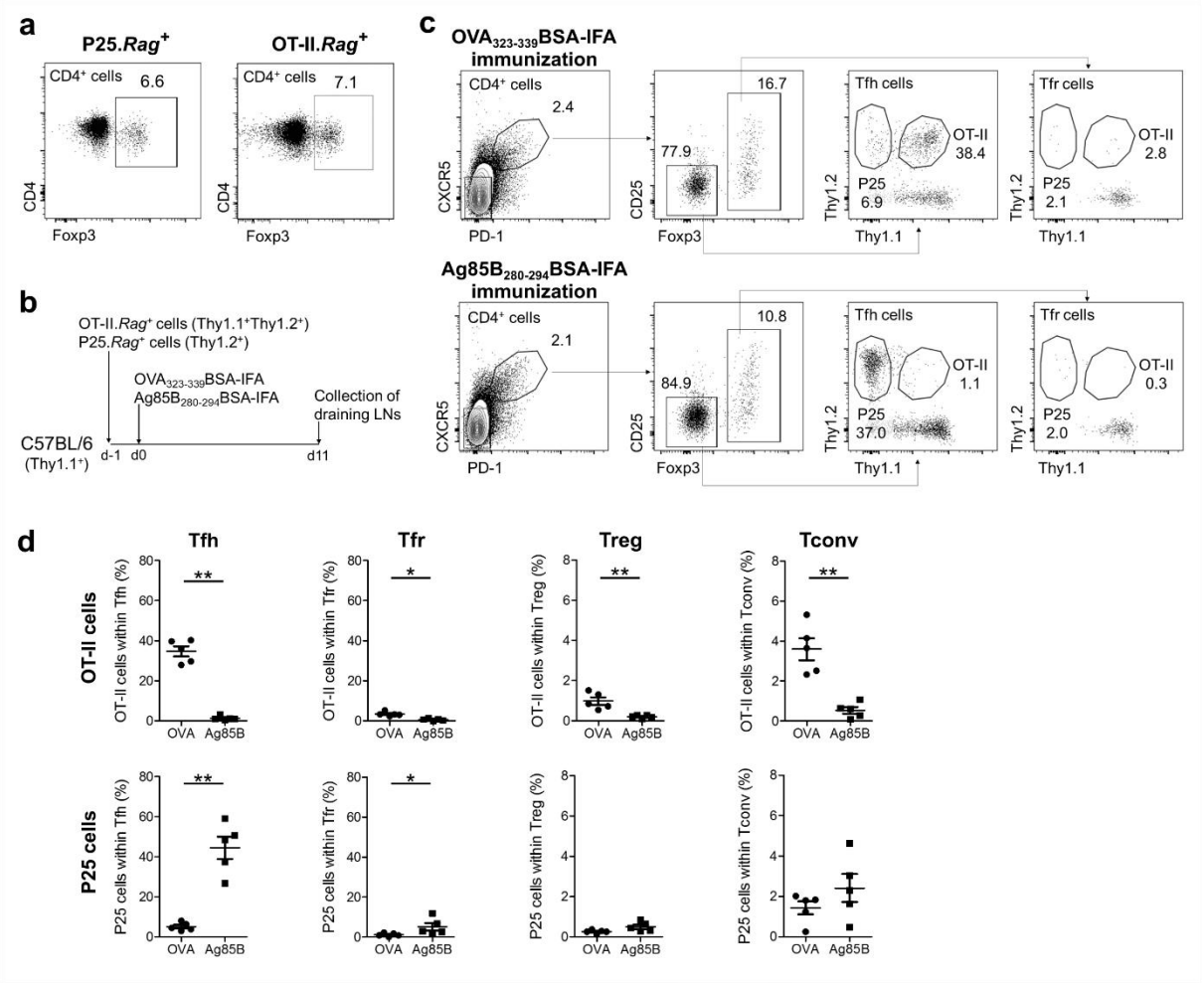


Figure 3. *P25 cells are not preferentially recruited into the Tfr pool.* (**a**) P25.*Rag*$^+$ mice have similar frequency of thymic Treg cells (6.0 ± 0.9%) as OT-II.*Rag*$^+$ mice (6.4 ± 0.7%). (**b**) C57BL/6 mice were transferred simultaneously with 10^7 CD4$^+$ T cells from OT-II.*Rag*$^+$ and P25.*Rag*$^+$ mice and subsequently immunized with either $OVA_{323-339}$BSA-IFA or $Ag85B_{280-294}$BSA-IFA in the footpad. (**c**) Gating strategy to determine the percentage of OT-II.*Rag*$^+$ and P25.*Rag*$^+$ cells within Tfh and Tfr populations in mice immunized with $OVA_{323-339}$BSA-IFA (upper panel) or $Ag85B_{280-294}$BSA-IFA (bottom panel). (**d**) T cell subsets from draining LNs show that mice immunized with $OVA_{323-339}$ have a large accumulation of OVA-specific cells within the Tfh and Tconv populations while, conversely, $Ag85B_{280-294}$-immunized mice accumulate P25-specific T cells among Tfh cells (* p<0.05 and ** p<0.01 using two-tailed nonparametric Mann–Whitney U tests). We observed a very small increase of T cells specific

for the immunizing antigen among the Tfr population. In all graphs, Mean ± SEM are presented. Data are representative of three independent experiments, each with n=5.

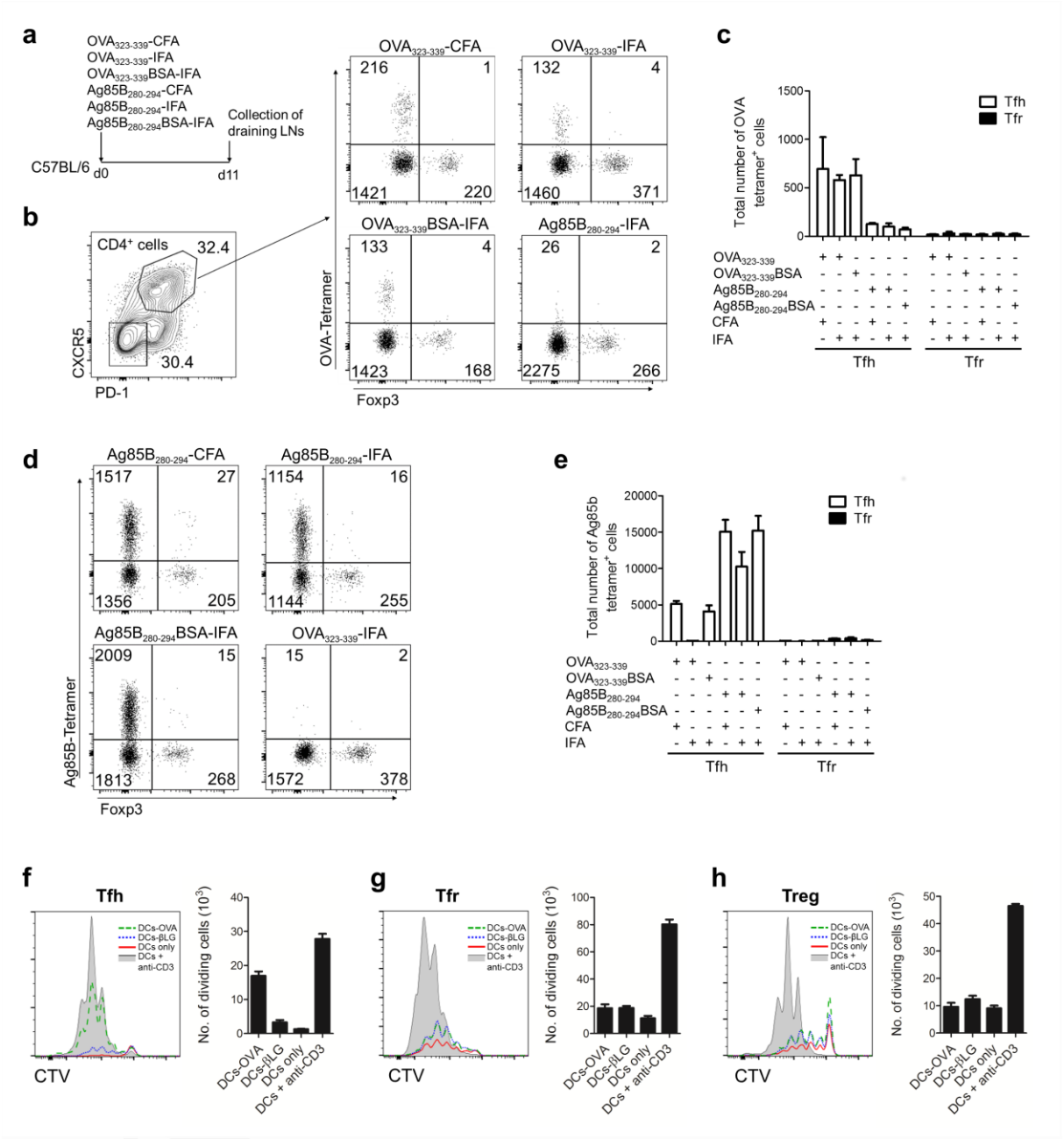


Figure 4. *Tfr cells neither bind antigen-specific tetramers nor proliferate in vitro upon restimulation*. (**a**) C57BL/6 mice were immunized with four different antigens combined with two different adjuvants on a total of 6 different immunizations. On day 11, draining LNs were collected for tetramer binding cells detection. (**b**) Gating strategy to identify OVA-tetramer$^+$ Tfh and Tfr cells within CD4$^+$ T cells. Relative percentages for the two gates are presented on the contour plot on the left, while event counts for each quadrant are presented on the four scatter plots on the right. The Tfh population from mice immunized with the three conditions containing $OVA_{323-339}$ was enriched on cells with TCRs capable of binding the OVA-tetramer. Such OVA-

tetramer$^+$ cells were almost absent in the Tfr population in all immunizations. Scatter plots are representative of the results obtained for each of the immunizations with $OVA_{323-339}$ and of the controls immunized with different formulations of $Ag85B_{280-294}$ (**c**) Total number of OVA-tetramer$^+$ cells in the draining LNs. (**d**) Scatter plots of Ag85B-tetramer$^+$ Tfh and Tfr cells within follicular CD4$^+$ T cells; the numbers represent the event counts for each quadrant. As observed for the OVA-tetramer, a large population of tetramer$^+$ Tfh cells was observed on immunizations containing $Ag85B_{280-294}$ peptide, while low numbers of tetramer$^+$ Tfr cells were found in all immunizations. Scatter plots are representative of the results obtained for each of the immunizations with $Ag85B_{280-294}$ and of the controls immunized with $OVA_{323-339}$ (**e**) Total number of Ag85B-tetramer$^+$ cells in the draining LNs. Mean ± SEM are presented. Data are representative of two independent experiments, each with n=5. (**f-h**) Histograms of proliferation and bar graphs of total cell numbers of sorted Tfh (CD4$^+$CXCR5$^+$PD-1$^+$Foxp3$^-$) (**f**), Tfr (CD4$^+$CXCR5$^+$PD-1$^+$Foxp3$^+$) (**g**), and Treg (CD4$^+$CXCR5$^-$PD-1$^-$Foxp3$^+$) cells (**h**), from OVA-immunized *Foxp3*^{hCD2} mice cultured for 3 days with DCs loaded with OVA or βLG or unloaded DCs with or without anti-CD3 and in the presence of IL-2. Only Tfh cells show higher proliferation and total numbers on OVA-pulsed DCs cultures compared to βLG-pulsed ones. Two independent experiments were performed: Mean ± SEM of one experiment triplicates are presented on the histograms.

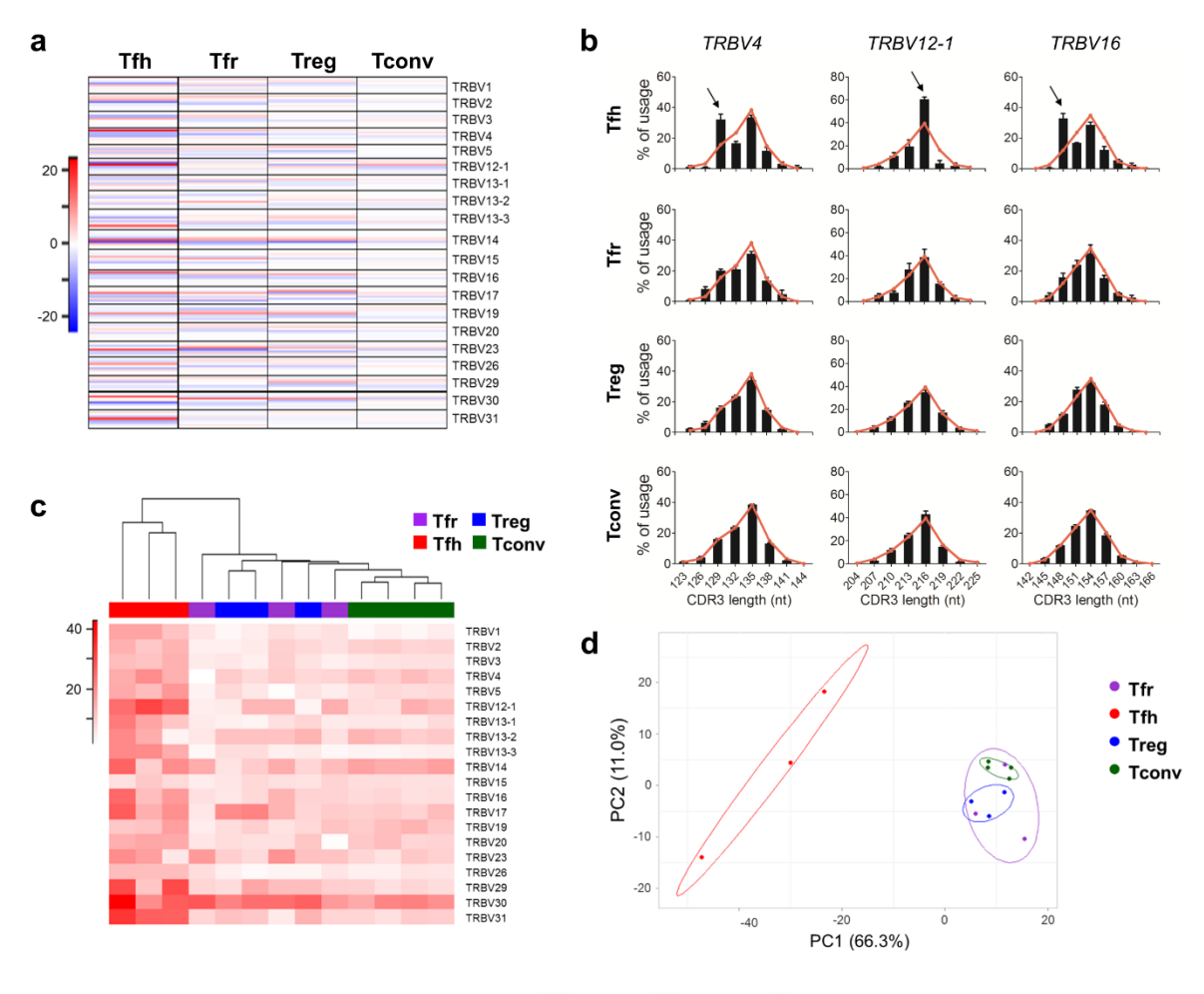


Figure 5. *Tfh cells display antigen-induced oligoclonal proliferation that is absent from Tfr cells*. (**a**) Heatmap showing the differences between the percentage of usage for each CDR3 length of each *TRBV* of Tfh, Tfr, Treg and Tconv populations compared to the Gaussian-like distribution of CD4⁺ naïve T cells (used as a control population). Similar *TRBV* CDR3 length frequencies (compared to control population) are displayed in white, while higher/lower frequencies of specific CDR3 lengths are represented, respectively, in red and blue. The heatmap is representative of, at least, three independent experiments per population. (**b**) Distribution of CDR3-length usage for three representative *TRBV* segments where greatest variation is observed. Bars represent CDR3-length usage distribution for indicated populations, with the reference values (naïve CD4⁺ T cells) superimposed in red. Arrows indicate over-representation of a specific CDR3 length on Tfh cells, a putative consequence of clonal selection and expansion. Neither Treg nor Tfr cells show similar expansions (bar graphs

below). Mean ± SEM are represented on the bar graphs. (**c**) Hierarchical clustering of the samples from the four populations based on their *TRBV* perturbation scores, calculated using the Tfr group average as reference. Heatmap colour code indicates variations of *TRBV* scores between each sample and the average of Tfr group, while the dendrogram shows distance between sample populations. (**d**) Replicates from different T cell subsets were projected according to the first two PCA components. Tfh samples are apart from all the other subsets. In all panels, for each T cell population, we used at least three independent replicates, each one with cells sorted from 10 – 15 mice.

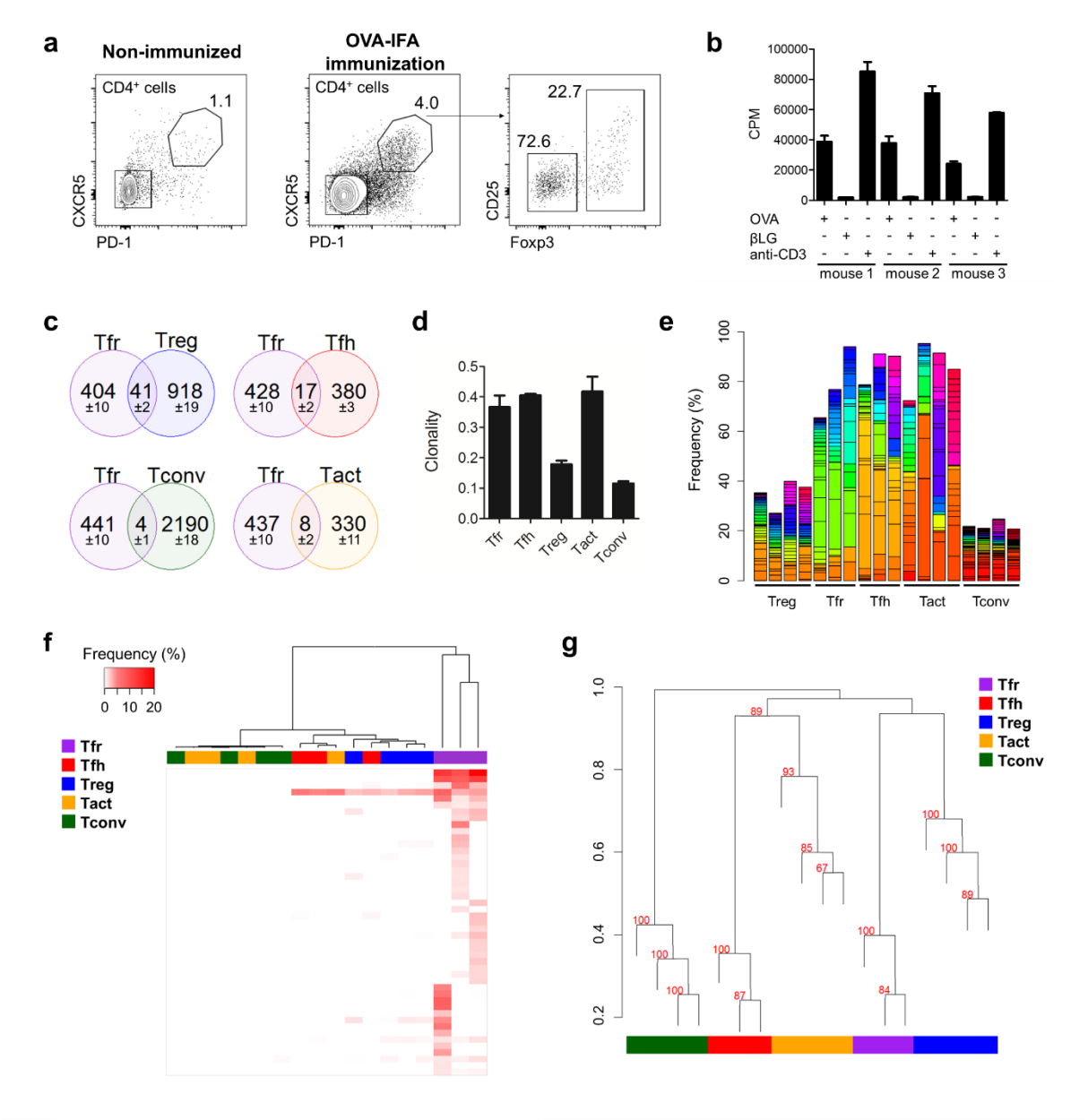
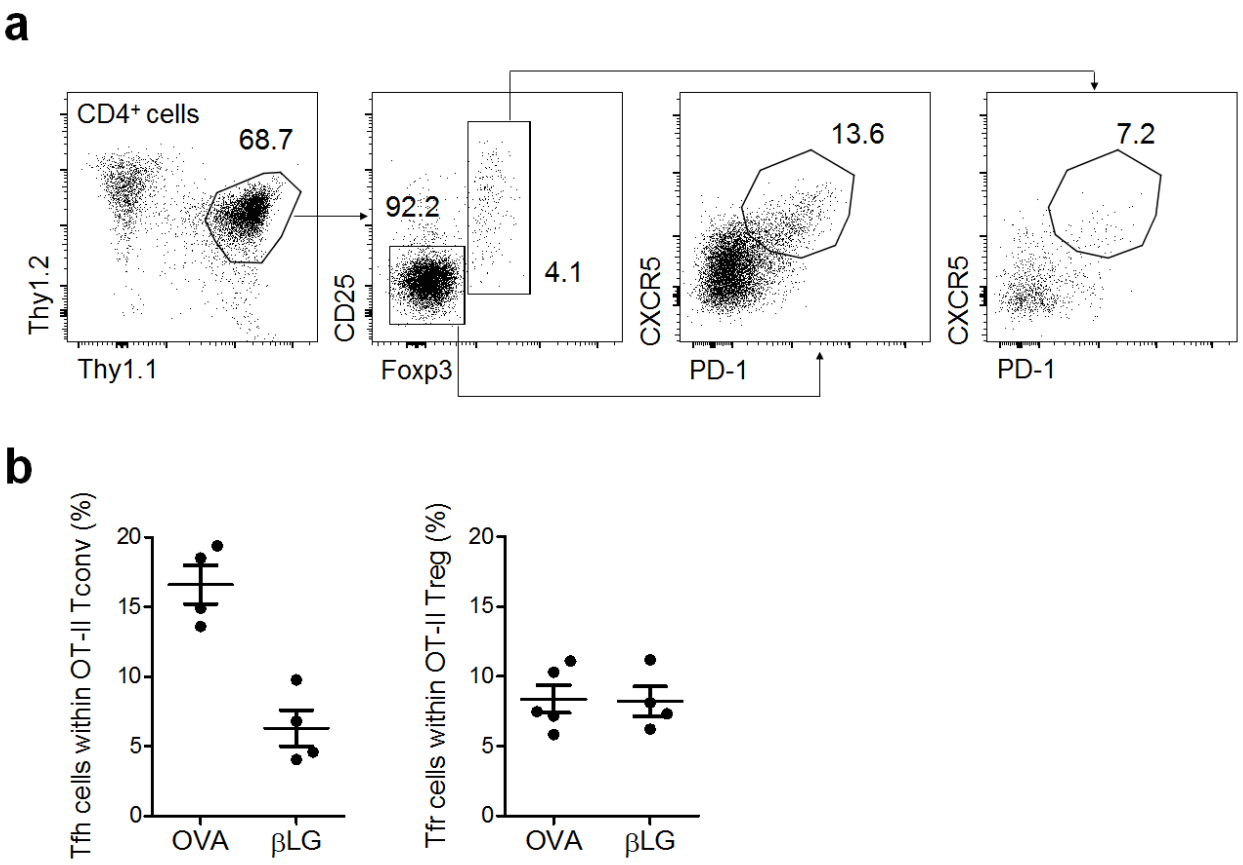


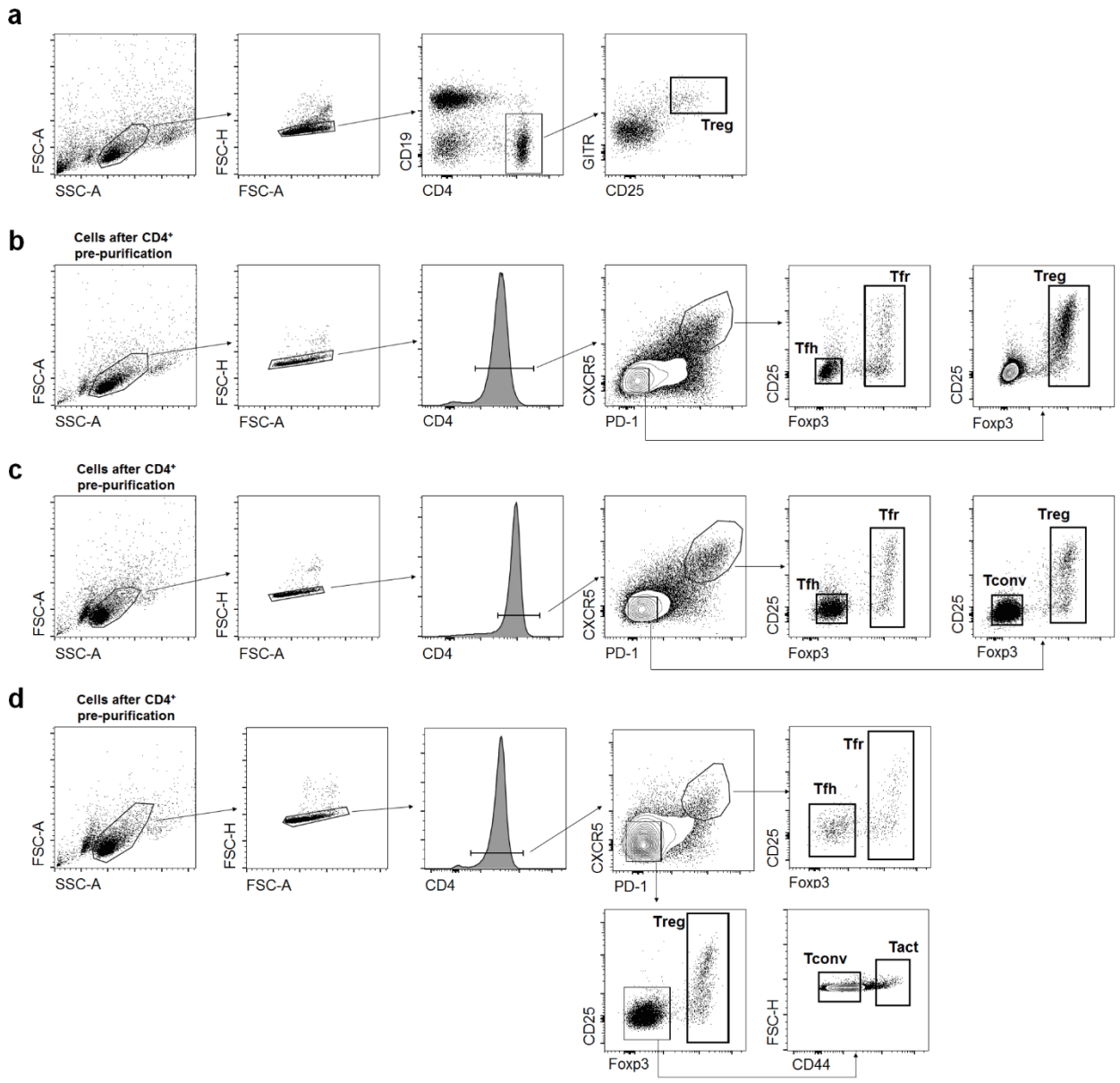
Figure 6. *Deep sequencing analysis unveils a different repertoire between Tfr and Tfh cells.* (**a**) Frequency of follicular $CD4^+CXCR5^+PD\text{-}1^+$ T cells on popliteal LNs of 1D2β mice before (left), and 11 days after OVA-IFA immunization (middle), when both Tfr and Tfh cells are present (right). (**b**) Proliferation of Tfh cells from OVA-IFA immunized 1D2β mice cultured with DCs pulsed with OVA or βLG (anti-CD3 was used as positive control). Cell proliferation was measured by 3H-thymidine incorporation. Mean ± SEM of culture triplicates are presented and are representative of two independent experiments. (**c**) Venn diagrams showing the number of shared clonotypes between Tfr cells and the other four populations for mouse 1. Numbers are the Mean ± SD of clonotypes identified after 100 iterations of the sampling process. Tfr

cells have more common clonotypes with Treg cells than any other cell population. (**d**) Clonality score for the five populations. (**e**) Histogram of cumulative frequency across all samples of the union of the 20 most predominant clonotypes for each sample. Each colour corresponds to a unique clonotype. (**f**) Heatmap and hierarchical clustering of the 20 most predominant clonotypes of Tfr replicates across all samples. Tfr most predominant clonotypes are mostly shared with Treg samples, with the exception of one sequence that is common with Treg and Tfh samples. (**g**) Dendrogram showing the overall relation of all sequenced samples using Horn-Morisita index distance method. Bootstrap resampling was performed to calculate approximately unbiased (AU, in red) p-values for each cluster. In the case of the Tfr and Treg cluster, AU = 96% thus the existence of the cluster is strongly supported by the data. For sequencing data, histograms present Mean ± SEM for n=4, except for Tfh and Tfr where n=3.

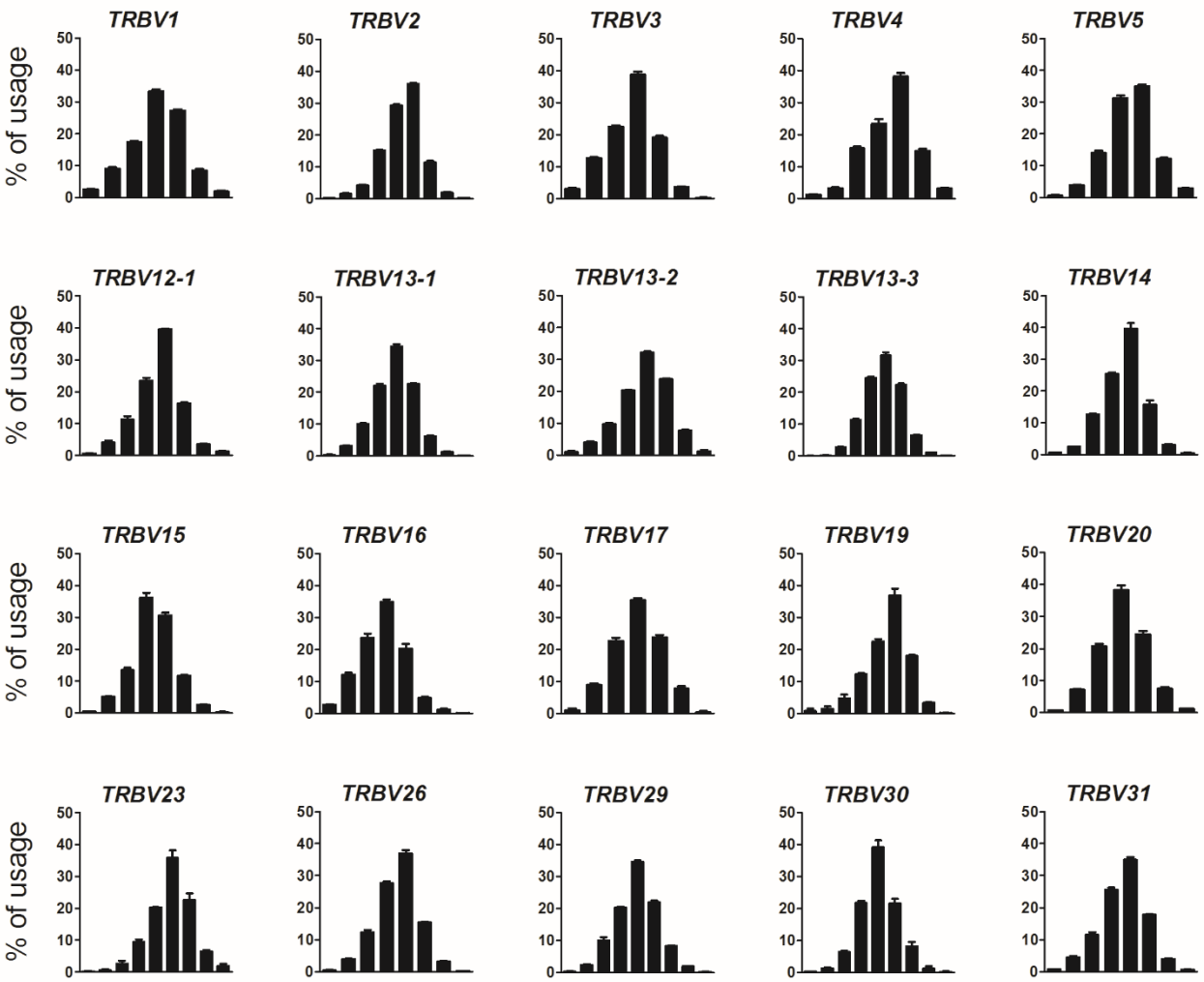
Supplementary Figures



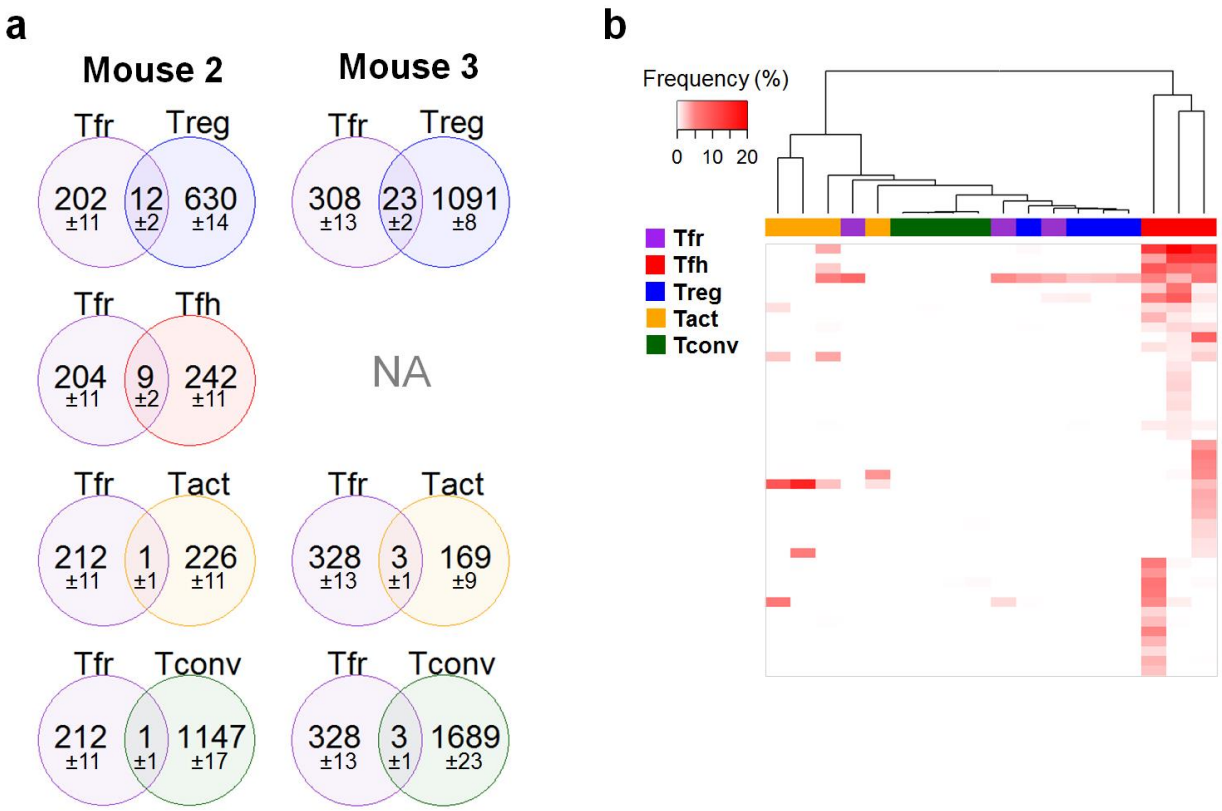
Supplementary Figure 1. *OT-II.Rag^{+}Treg cells differentiate into Tfr cells independently of the immunization and keep Vα2 and Vβ5 expression.* (**a**) Gating strategy to determine the percentage of OT-II Treg and Tconv cells that differentiate into Tfr and Tfh cells, respectively. (**b**) While the percentage of OT-II Tconv that differentiate into Tfh is higher in mice immunized with OVA than βLG (left), the same percentage of OT-II Treg originates Tfr cells in both immunizations (right). Mean ± SEM presented for n=5.



Supplementary Figure 2. *Gating strategies used for cell sorting.* (**a**) Gating strategy to sort Treg ($CD4^+CD25^+GITR^+$) cells from OT-II.*Rag*$^+$ and C57BL/6 WT mice for *in vitro* cultures presented on Fig. 2g,h. (**b**) Gating strategy to sort Tfh ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^-$), Tfr ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^+$), and Treg ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^+$) cells from *Foxp3*^{hCD2} mice used on the *in vitro* proliferation assay presented on Fig. 4f-h. (**c**) Gating strategy to sort Tfh ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^-$), Tfr ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^+$), Treg ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^+$), and Tconv ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^-$) cells from *Foxp3*^{gfp} mice for *TRBV* CDR3 spectratyping/Immunoscope analysis (Fig. 5). (**d)** Gating strategy to sort Tfh ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^-$), Tfr ($CD4^+CXCR5^+PD\text{-}1^+Foxp3^+$), Treg ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^+$), Tact ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^-CD44^+$), and Tconv ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^-CD44^-$) cells from 1D2β mice for *TRA* sequencing analysis (Fig. 6c-g) The same strategy was used to sort Tfh cells for the *in vitro* proliferation assay presented on Fig. 6b.



Supplementary Figure 3. *CDR3 spectratypes obtained from naïve CD4⁺ T cells.* CDR3-length usage distribution of 20 *TRBV* segments from CD4⁺ T cells from naïve mice that present a Gaussian-like distribution. Bar graphs present Mean ± SEM of four samples.



Supplementary Figure 4. *Common clonotypes for Tfr cells and 20 most predominant clonotypes of Tfh samples*. (**a**) Venn diagrams of the shared clonotypes between Tfr and other populations for mouse 2 and 3. Numbers presented are the Mean ± SD of clonotypes identified after 100 iterations of the sampling process. As it was observed on Fig. 6, Tfr cells share more clonotypes with Treg cells. Sequencing results for Tfh sample of mouse 3 are not available (NA). (**b**) Heatmap and hierarchical clustering of the 20 most predominant clonotypes for each Tfh sample. The predominant clonotypes are mainly shared between Tfh samples and Tact samples.

Supplementary Tables

Supplementary Table 1. Pairwise Multiple Comparison Analysis with Holm-Bonferroni Correction between Samples TRBV Perturbation Scores.

	Tconv1	Tconv2	Tconv3	Tconv4	Tfh1	Tfh2	Tfh3	Tfr1	Tfr2	Tfr3	Treg1	Treg2
Tconv2	1	-	-	-	-	-	-	-	-	-	-	-
Tconv3	1	1	-	-	-	-	-	-	-	-	-	-
Tconv4	1	1	1	-	-	-	-	-	-	-	-	-
Tfh1	0,014 *	0,015 *	0,033 *	0,016 *	-	-	-	-	-	-	-	-
Tfh2	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,673	-	-	-	-	-	-	-
Tfh3	0,007 **	0,009 **	0,012 *	0,009 **	1	0,473	-	-	-	-	-	-
Tfr1	1	1	1	1	0,017 *	0,000 ***	0,007 **	-	-	-	-	-
Tfr2	1	1	1	1	0,162	0,000 ***	0,101	0,495	-	-	-	-
Tfr3	1	1	1	1	0,002 **	0,000 ***	0,002 **	1	1	-	-	-
Treg1	1	1	1	1	0,046 *	0,000 ***	0,004 **	1	1	1	-	-
Treg2	1	1	1	1	0,037 *	0,000 ***	0,018 *	1	1	1	1	-
Treg3	1	1	1	1	0,007 **	0,000 ***	0,007 **	1	1	1	1	1

p<0.05 *, p<0.01 **, p<0.001 ***

Supplementary Table 2. List of Primers Used on This Study.

Sequence	Target gene/region	Orientation	Application
TCACTGATACGGAGCTGAGGC	*TRBV1*	Forward	Spectratyping
GCCTCAAGTCGCTTCCAACCTC	*TRBV2*	Forward	Spectratyping
CACTCTGAAAATCCAACCCAC	*TRBV3*	Forward	Spectratyping
ATCAAGTCTGTAGAGCCGGAGGA	*TRBV4*	Forward	Spectratyping
CTGAATGCCCAGACAGCTCCAAGC	*TRBV5*	Forward	Spectratyping
AAGGTGGAGAGAGACAAAGGATTC	*TRBV12-1*	Forward	Spectratyping
CATTATGATAAAATGGAGAGAGAT	*TRBV12-2*	Forward	Spectratyping
TGCTGGCAACCTTCGAATAGGA	*TRBV13-1*	Forward	Spectratyping
CATTATTCATATGGTGCTGGC	*TRBV13-2*	Forward	Spectratyping
CATTACTCATATGTCGCTGAC	*TRBV13-3*	Forward	Spectratyping
AGGCCTAAAGGAACTAACTCCAC	*TRBV14*	Forward	Spectratyping
GATGGTGGGGCTTTCAAGGATC	*TRBV15*	Forward	Spectratyping
GCACTCAACTCTGAAGATCCAGAGC	*TRBV16*	Forward	Spectratyping
TCTCTCTACATTGGCTCTGCAGGC	*TRBV17*	Forward	Spectratyping
CTCTCACTGTGACATCTGCCC	*TRBV19*	Forward	Spectratyping
CCCATCAGTCATCCCAACTTATCC	*TRBV20*	Forward	Spectratyping
CTGCTAAGAAACCATGTACCA	*TRBV21*	Forward	Spectratyping
TCTGCAGCCTGGGAATCAGAA	*TRBV23*	Forward	Spectratyping
AGTGTTCCTCGAACTCACAG	*TRBV24*	Forward	Spectratyping
CCTTGCAGCCTAGAAATTCAGT	*TRBV26*	Forward	Spectratyping
TACAGGGTCTCACGGAAGAAGC	*TRBV29*	Forward	Spectratyping
CAGCCGGCCAAACCTAACATTCTC	*TRBV30*	Forward	Spectratyping
ACGACCAATTCATCCTAAGCAC	*TRBV31*	Forward	Spectratyping
GCCCATGGAACTGCACTTGGC	*TRBC*(1)	Reverse	Spectratyping
FAM-CTTGGGTGGAGTCACATTTCTC	*TRBC*(2) (spectratyping run-off)	Reverse	Spectratyping
CAACGCAGAGTGGCCATTAC	Mint-2 universal adapter	Forward	Sequencing
GCAGGTGAAGCTTGTCTGGT	*TRAC*	Reverse	Sequencing

Chapter 12

Identification of Foxp3$^+$ T Follicular Regulatory (Tfr) Cells by Flow Cytometry

Ana Raquel Maceiras and Luis Graca

Abstract

Flow cytometry is a technology that allows multiparametric analysis of individual cells. As a consequence, it is among the most commonly used tools for the study of immune cells. It is useful both for the study of ex vivo cell populations isolated from experimental animals or human tissue and for characterizing the phenotype of cultured cells. The phenotypic analysis is based on antibodies associated to different fluorophores that specifically bind to key molecules. Genetically modified mouse strains that express a reporter gene under the control of a promoter of interest offer an important alternative for the staining of intracellular molecules without the need to permeabilize the cell membrane. In this chapter, we describe how Foxp3$^+$ follicular regulatory T (Tfr) cells, a population of regulatory T (Treg) cells related to T follicular helper (Tfh) cells and involved in the regulation of germinal centers (GC), can be identified by flow cytometry.

Key words Flow cytometry, Antibodies, Foxp3, Bcl6, CXCR5, PD-1, CD4 T cells, T follicular helper cells (Tfh), Germinal center

1 Introduction

T follicular regulatory (Tfr) cells constitute a population of follicular CD4 T cells that have been recently described [1–3]. Tfr cells are located within the lymphoid tissue, and a circulating counterpart has also been described [4]. Tfr cells are involved in the regulation of the germinal center (GC) response, for example, it has been shown that Tfr cells can limit the production of antibodies specific for an immunizing self-antigen, such as chromatin antigens [5].

Tfr cells share phenotypic characteristics of regulatory T cells (Treg) and of T follicular helper (Tfh) cells: Tfr cells express characteristic Tfh markers such as CXCR5, PD-1, and Bcl-6, but also Treg-associated molecules including Foxp3, CD25, GITR, and CD103. As a consequence, Tfr cells are commonly described as CD4$^+$CXCR5$^+$PD-1$^+$CD25$^+$Foxp3$^+$ cells, although CD25$^-$ Tfr cells have also been identified [1–3].

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Flow cytometry is one of the most commonly used techniques in immunology. Its multiparametric analysis allows an in-depth study of the physical characteristics of cells and also the phenotypic analysis of different proteins expressed by single cells. Flow cytometry utilizes monoclonal antibodies that are specific to different proteins expressed by cells and are often directly conjugated to fluorophores, allowing the direct identification of molecules of interest in cells being analyzed. In addition, the level of expression of a given molecule can be inferred from the intensity of the staining. As a consequence, the staining pattern obtained from a combination of markers allows the identification of cell populations on sample, as well as the quantification of the relative abundance of these populations.

It is also possible to use flow cytometry to isolate cell populations, or single cells, based on their phenotype for subsequent studies. It should be noted, however, that functional studies that require the isolation of live cells are incompatible with the use of monoclonal antibodies to stain intracellular molecules. This limitation is a consequence of the need to permeabilize cells in order to gain access to their intracellular components, with permeabilization leading to cell death. This is a critical issue for the study of Tfr cells as one key marker for the identification of this cell population is Foxp3, a nuclear transcription factor. Mouse strains expressing reporter genes under the control of the *Foxp3* promoter offer an alternative strategy to identify and sort viable Foxp3$^+$ cells (namely, Tfr) based on flow cytometry.

In summary, flow cytometry is a useful tool for the identification of Tfr cells, based on their phenotypic characteristics, allowing subsequent functional studies of Tfr role in the regulation of antibody-mediated immune responses.

2 Materials

All solutions should be prepared using distilled water and analytical grade reagents. Solutions may be prepared and stored prior to utilization.

When flow sorting Tfr cells for functional assays, it is necessary to use strategies that overcome the need for intracellular staining (as it requires fixation and permeabilization of cells). As an alternative Foxp3 reporter mice should be used (*see* Table 1).

In some experiments, in particular following in vitro assays, it may be important to use a cell viability test, using a fluorescent reactive dye that allows discrimination of live and dead cells (live/dead staining). The reagents and extra steps for the live/dead staining are marked as optional.

Table 1
List of Foxp3 reporter mice available

Mouse strain	MGI designation	Reporter molecule	Reference
Foxp3-GFP	$Foxp3^{tm2Ayr}$	GFP	Fontenot et al. [6]
Foxp3-EGFP	$Foxp3^{tm2Tch}$	EGFP	Haribhai et al. [7]
Foxp3-IRES-GFP	$Foxp3^{tm1Kuch}$	GFP	Bettelli et al. [8]
Foxp3-IRES-mRFP	$Foxp3^{tm1Flv}$	mRFP	Wan et al. [9]
Foxp3-hCD2	$Foxp3^{tm1(CD2/CD52)Shori}$	Human CD2	Komatsu et al. [10]

2.1 Surface Staining Reagents

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 M KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2. Store at room temperature.
2. Fetal calf serum. Store at –20 °C.
3. Staining buffer: PBS with 2 % FCS. Store at 4 °C.
4. ACK lysing buffer: 0.15 M NH_4Cl, 10.0 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2. Store at room temperature.
5. Surface antibodies: Anti-mouse CXCR5 biotin (clone 2G8), anti-mouse CD4 APC-eFluor® 780 (clone RM4-5), anti-mouse CD279 (PD-1) PE (clone J43), and anti-mouse CD25 PE Cyanine7 (clone PC61.5) (*see* **Note 1**). Store at 4 °C.
6. Streptavidin: Streptavidin PerCP-Cyanine5.5 (*see* **Note 1**). Store at 4 °C.

2.2 Foxp3 Reporter Mice

1. Several Foxp3 reporter mouse strains have been generated and may be available for use (Table 1), such as Foxp3-GFP [6], Foxp3-EGFP [7], Foxp3-IRES-GFP [8], Foxp3-IRES-mRFP [9], and Foxp3-hCD2 [10]. It was claimed that reporter mice expressing a Foxp3-GFP fusion molecule have impaired Treg induction and function when compared with alternative Foxp3 reporter mouse strains, such as Foxp3-IRES-GFP [11, 12].

2.3 Viability Staining (Optional)

1. Fixable viability staining (*see* **Note 1**).

2.4 Intracellular Staining

1. Intracellular fixation/permeabilization buffer (IFP buffer): Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (eBioscience). Store at 4 °C.
2. Permeabilization buffer: 10× permeabilization buffer (eBioscience). Store at 4 °C.
3. Fc region blocking (clone 24G2 or 93).
4. Intracellular antibody: Anti-mouse/rat Foxp3 APC (clone FKJ-16 s) (*see* **Note 1**). Store at 4 °C.

3 Methods

3.1 Surface Staining

1. Single cell suspensions should be obtained prior to cell staining procedures (except for cell samples from in vitro cultures). This may be accomplished by mechanical dissociation such as mesh sieving or Dounce homogenization.
2. When using splenocytes, red blood cell lysis should be performed before starting the following steps.
3. Make sure the splenocytes are in a tube of at least 15 ml capacity. Centrifuge the cells at 300 × *g* for the appropriate time (depending on the volume) for the cells to form a pellet. Discard the supernatant.
4. Resuspend in 1 ml per spleen of ACK lysing buffer and incubate for 5 min at 4 °C.
5. Add directly 9 ml of staining buffer and mix well. Centrifuge the cells for 5 min at 300 × *g*. Discard the supernatant. When performing red blood cell lysis, wash on step number 7 may be skipped.
6. Count the cells. Do not forget that flow cytometry will only give relative proportions of the different populations, and it is likely that the staining procedure will lead to some cell loss. The starting number of cells is, therefore, essential to calculate the cell number of each subsequently identified population. Cells can be counted manually using a hemocytometer. Alternatively, counting beads can be added to the mixture allowing cell number quantification by flow cytometry.
7. Wash cells in staining buffer. Resuspend cells in an appropriate volume to obtain approximately 2×10^7 cells/ml.
8. Transfer 100 µl of each sample to a well of a U-bottom 96-well plate. Centrifuge the plate for 3 min at 300 × *g*. Discard the supernatant (*see* **Note 2**).
9. Prepare the necessary amount of CXCR5 mix by diluting CXCR5 antibody 50× in staining buffer, in order to obtain at least 20 µl for each sample (*see* **Notes 3–5**).
10. Resuspend each cell sample in 20 µl of CXCR5 mix, and incubate at 37 °C for 10 min followed by 20 min at room temperature (*see* **Note 6**).
11. All the following steps should be performed protected from light and at 4 °C (or on ice). Keep the centrifuge at 4 °C.
12. (optional) Wash cells with PBS: Add 200 µl of PBS to each well and mix; centrifuge the plate for 3 min at 300 × *g*. Discard the supernatant.
13. (optional) Reconstitute the fluorescent reactive dye by adding 50 µl of DMSO to each vial of the kit—dye stock solution (*see* **Note 7**).

14. (optional) Prepare the appropriate volume of the viability staining mix by diluting the previous dye stock solution 100× in PBS.
15. (optional) Resuspend each cell sample in 20 μl of the previous viability staining mix, and incubate for 30 min.
16. Wash cells with PBS (if the viability staining was performed) or staining buffer.
17. Prepare the appropriate volume of the second mix containing the remaining surface antibodies and the streptavidin. The mix must contain all antibodies and the streptavidin diluted 100× in staining buffer (*see* **Notes 3–5**).
18. Resuspend each cell sample in 20 μl of the previous mix and incubate for 30 min.
19. Wash cells with staining buffer.

3.2 Foxp3 Reporter Mice

1. If a Foxp3 fluorescent reporter mouse is used, the staining is complete. In this case, resuspend in 200 μl of staining buffer and transfer to an appropriate plate/tube for acquisition on a flow cytometer. To avoid blockages on flow cytometers, cell samples may be filtered at this step using a 70 μm pore filter.
2. If the reporter protein is not fluorescent, an antibody specific for the reporter protein conjugated to a fluorophore should be used together with the remaining antibodies during the surface staining **steps 17** and **18**. For example, in the case of Foxp3-hCD2, an antihuman CD2 conjugated to a fluorophore (e.g., antihuman CD2 APC, eBioscience) should be added to the mix prepared at step 17 of the surface staining part of the protocol.
3. Samples are ready to be acquired in a flow cytometer.

3.3 Intracellular Staining

1. Prepare the appropriate volume of IFP buffer by mixing 1 volume of the concentrate solution with 3 volumes of the diluent solution.
2. Resuspend each sample in 100 μl IFP buffer. Incubate for 30 min (*see* **Note 8**).
3. Centrifuge the plate for 3 min at 300×*g*. Discard the supernatant.
4. Prepare the permeabilization buffer by diluting the 10× solution to 1× in distilled water.
5. Wash cells with permeabilization buffer.
6. Prepare the Fc blocking solution by diluting the Fc block 200× in permeabilization buffer.
7. Resuspend each sample in 20 μl Fc blocking solution. Incubate for 15 min.

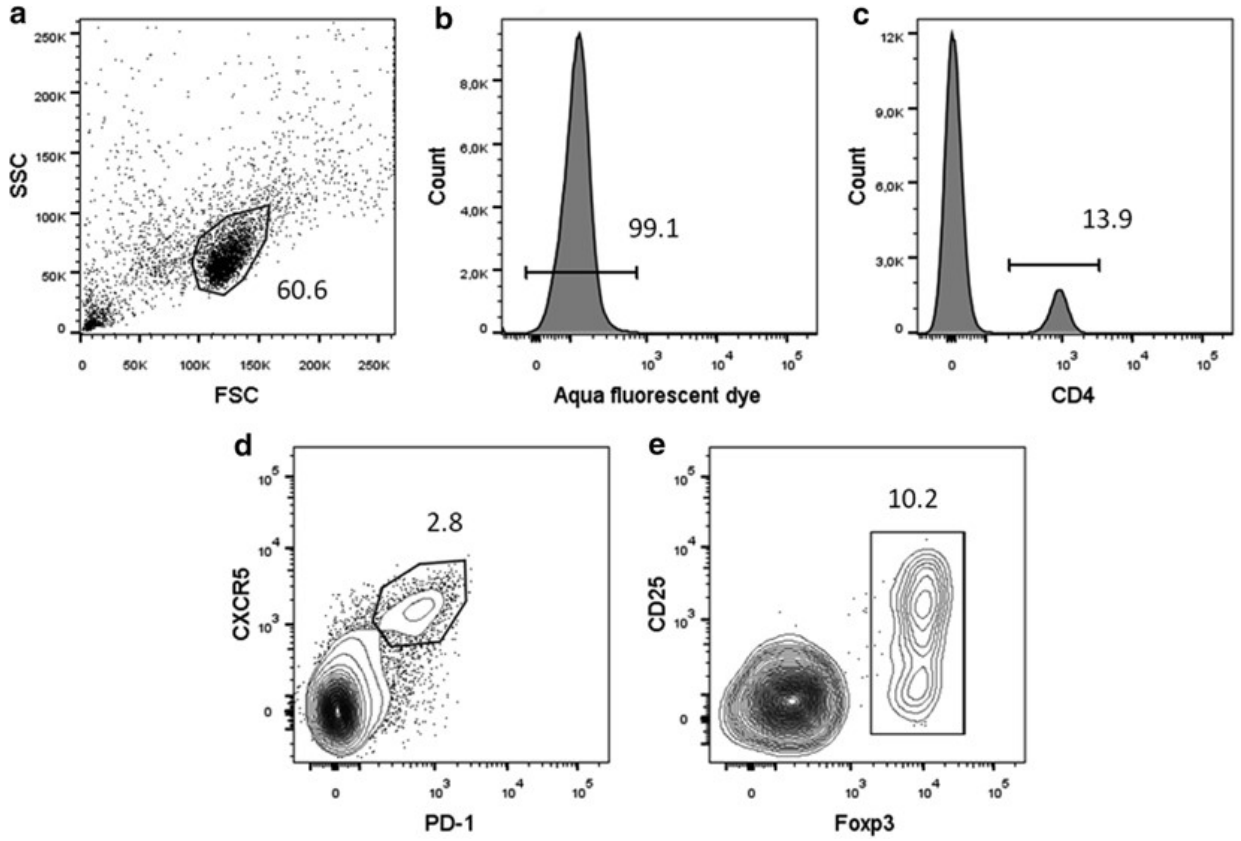

Fig. 1 *Tfr cells sequential gating strategy*. (**a**) Selection of cell based on the forward scatter (FSC) and side scatter (SSC) profiles. (**b**) Live cells are negative for the fluorescent reactive dye, so the negative population is selected. (**c–e**) Sequential selection of known Tfr markers: (**c**) $CD4^+$ cells, (**d**) $PD\text{-}1^+CXCR5^+$ cells, and (**e**) $CD25^+Foxp3^+$ cells

8. Prepare the Foxp3 mix by diluting the Foxp3 APC antibody 20× in permeabilization buffer (*see* **Notes 3–5** and **9**).
9. Add directly 5 μl of Foxp3 mix to each well containing the Fc blocking solution. Incubate for another 30 min.
10. Wash cells with permeabilization buffer.
11. Resuspend in 200 μl of staining buffer and transfer to an appropriate plate/tube for acquisition on a flow cytometer. To avoid clogs on flow cytometers, cell samples may be filtered at this step using a 70 μm pore filter.
12. Samples are ready to be acquired on a flow cytometer.

3.4 Gating Strategy

See Fig. 1.

4 Notes

1. The list of antibodies provided in this protocol is merely an example as other antibodies and conjugations to other fluorophores may be used as pleased. The same applies to the streptavidin used and the fixable cell viability dye, as streptavidin

conjugated to other fluorophores and different fluorescent reactive dyes may be used.

2. We found it easier to perform the following steps using 96-well plates, which also allows the use of smaller amounts of reagents. However, it may be performed in larger tubes, upscaling the volumes used accordingly.
3. An excess of all mixes should always be prepared, as pipetting errors may lead to insufficient volume for the last samples.
4. The antibody dilution stated may be used for the listed antibodies. However, the experimenter should test the optimal antibody working dilution.
5. To help define the negative populations, extra antibody mixes should be prepared so that each antibody is substituted by the corresponding isotype control.
6. In order to obtain a good staining for chemokine receptors, cells should be incubated for at least 10 min at 37 °C.
7. After reconstitution in DMSO, the dye stock solution may be stored at −20 °C and used later. If lower amounts are predicted to be used, store the solution in adequate aliquots in order to avoid repeated thawing and freezing cycles.
8. This incubation should preferentially be performed on ice.
9. Although the Foxp3 APC antibody is only diluted 20× in the Foxp3 mix, the final usage dilution will be 100× since the antibody is further diluted when added directly to the samples already in Fc blocking solution.

Acknowledgments

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References

1. Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, Graca L (2011) Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. J Immunol 187(9):4553–4560. doi:10.4049/jimmunol.1101328
2. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, Srivastava M, Divekar DP, Beaton L, Hogan JJ, Fagarasan S, Liston A, Smith KG, Vinuesa CG (2011) Foxp3+ follicular regulatory T cells control the germinal center response. Nat Med 17(8):975–982. doi:10.1038/nm.2425
3. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, Wang YH, Lim H, Reynolds JM, Zhou XH, Fan HM, Liu ZM, Neelapu SS, Dong C (2011) Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat Med 17(8):983–988. doi:10.1038/nm.2426
4. Sage PT, Francisco LM, Carman CV, Sharpe AH (2013) The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. Nat Immunol 14(2):152–161. doi:10.1038/ni.2496

5. 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(3):545–561. doi:10.1084/jem.20130604
6. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY (2005) Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 22(3):329–341. doi:10.1016/j.immuni.2005.01.016
7. Haribhai D, Lin W, Relland LM, Truong N, Williams CB, Chatila TA (2007) Regulatory T cells dynamically control the primary immune response to foreign antigen. J Immunol 178(5):2961–2972. doi:10.4049/jimmunol.178.5.2961
8. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441(7090):235–238. doi:10.1038/nature04753
9. Wan YY, Flavell RA (2005) Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl Acad Sci U S A 102(14):5126–5131. doi:10.1073/pnas.0501701102
10. Komatsu N, Mariotti-Ferrandiz ME, 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(6):1903–1908. doi:10.1073/pnas.0811556106
11. Bettini ML, Pan F, Bettini M, Finkelstein D, Rehg JE, Floess S, Bell BD, Ziegler SF, Huehn J, Pardoll DM, Vignali DA (2012) Loss of epigenetic modification driven by the Foxp3 transcription factor leads to regulatory T cell insufficiency. Immunity 36(5):717–730. doi:10.1016/j.immuni.2012.03.020
12. Darce J, Rudra D, Li L, Nishio J, Cipolletta D, Rudensky AY, Mathis D, Benoist C (2012) An N-terminal mutation of the Foxp3 transcription factor alleviates arthritis but exacerbates diabetes. Immunity 36(5):731–741. doi:10.1016/j.immuni.2012.04.007