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DIVERSITY OF THE HUMAN MEMORY T CELL REPERTOIRE TO PATHOGENS AND VACCINES

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presented by

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2. General Summary

2.1. Summary (English)

Human $CD4^+$ T cells are key players in orchestrating the response to pathogens and vaccines. Upon first antigen encounter, naïve T cells get activated and clonally expand, meanwhile acquiring effector functions to control the threat and later becoming memory T cells to assure appropriate response in case of re-challenge. Different classes of pathogens are known to induce distinct polarized T cell subsets, each characterized by specific homing and functional properties, that can mount the most efficient response tailored to the challenging pathogen. Thus, viruses and intracellular bacteria induce IFN-γ-producing Th1 cells while helmints induce IL-4-producing Th2 and fungi and extracellular bacteria induce IL-17-producing Th17 cells. Once considered terminally differentiated stages, polarized T cells can show remarkable flexibility and have been proven to undergo phenotypic switch or divergent differentiation in several experimental systems. Understanding the generation of diversity in the human T cell memory compartment would have enormous implications both on a biological and on a clinical level.

Using cell sorting of human memory Th1, Th2, and Th17 cells followed by CFSE labeling, antigenic stimulation, and next generation TCR Vβ sequencing, we were able to demonstrate that memory T cells specific for pathogens such as *Candida albicans* and *Mycobacterium tuberculosis* or tetanus toxoid vaccine could be present in all subsets, albeit at different frequencies. Interestingly, several clonotypes were present in more than one subset and, in some cases, even in all subsets, while other clonotypes were restricted to one particular subset. By cloning antigen-specific T cells from memory subsets we were able to isolate several T cell clones from Th1, Th2, and Th17 subsets and show that they share the same TCR but display different transcription factors, cytokine production and chemokine receptor expression, characteristic of the subset from which they were isolated. Collectively, these results indicate that the T cell response to pathogens and vaccines can comprise T cell clones that in spite of identical TCR, display different, sometimes divergent types of effector functions and provide for the first time demonstration of intraclonal diversification in the human T cell response.

2.2 Riassunto (Italiano)

I linfociti T CD4$^+$ umani rivestono un ruolo fondamentale nel dirigere la risposta a patogeni e vaccini. In seguito all'iniziale riconoscimento dell'antigene, le cellule T naive si attivano e vanno incontro ad espansione clonale, acquisendo allo stesso tempo specifiche proprieta' funzionali. Queste consentono di controllare il pericolo nell'immediato e, venendo ereditate da cellule T della memoria, anche di garantire una risposta appropriata in caso di successivo incontro col medesimo antigene. Classi diverse di patogeni inducono diversi sottotipi di cellule T, ognuno caratterizzato da specifiche capacita' migratorie e proprieta' funzionali, tramite le quali puo' rispondere efficacemente al microbo sollecitante. In particolare, virus e batteri intracellulari promuovono lo sviluppo di cellule Th1 che producono IFN-γ, gli elminti quello di Th2 che producono IL-4, funghi e batteri extracellulari quello di cellule Th17 che producono IL-17. Sebbene i subset di linfociti T siano stati per molto tempo considerati entita' irreversibilmente differenziate e' oggi chiaro che queste cellule presentano una notevole plasticita', e la loro capacita' di modificare il proprio fenotipo o di differenziare in maniera divergente in seguito al riconoscimento dell'antigene e' stata dimostrata in molti modelli sperimentali.

Abbiamo utilizzato un protocollo che prevede l'ottenimento di cellule della memoria di tipi diversi (Th1, Th2, Th17) tramite citofluorimetria a flusso, seguito da marcatura con CFSE, stimolazione antigenica, e sequenziamento di ultima generazione della regione Vβ del TCR delle cellule proliferanti. Tramite questo approccio abbiamo potuto dimostrare che le cellule della memoria specifiche per patogeni quali *Candida albicans*, *Mycobacterium tuberculosis* o il tossoide tetanico (vaccino) sono presenti in tutti i sottotipi di linfociti T, sebbene con frequenze differenti. Inaspettatamente, abbiamo identificato vari clonotipi presenti in piu' compartimenti, talvolta addirittura in tutti quelli analizzati, mentre altri sono risultati selettivamente appartenere ad uno specifico gruppo. Attraverso clonaggio abbiamo isolato alcune di queste cellule presenti allo stesso tempo nei compartimenti Th1, Th2 e Th17, e confermato che possiedono un identico TCR, ma differiscono per l'espressione di fattori di trascrizione, citochine, e recettori chemochinici, in accordo con la classe di appartenenza. In definitiva, questi risultati dimostrano che l'insieme di cellule T che rispondono ad un patogeno o ad un vaccino comprende cloni che, nonostante un identico recettore per l'antigene, mostrano divergenti, talvolta contrapposte, proprieta' funzionali, e rappresentano percio' la prima dimostrazione di differenziamento intra-clonale nella risposta dei linfociti T nell'uomo.

3. Table of Abbreviations

AIRE Autoimmune regulator gene

APC Antigen-presenting cell

BCG Bacillus Calmette-Guerin

BCR B cell receptor

CA *Candida albicans*

CCR C-C chemokine receptor

CD Cluster of differentiation

CDR Complementary determining region

CKR Chemokine receptor

CLR C-type lectin receptor

CMC Chronic mucocutaneous candidiasis

CMV Cytomegalovirus

cTEC Cortical thymic epithelial cell

CTL Cytotoxic T cell

CWE Cell wall extract

CXCR CXC-chemokine receptor

DC Dendritic cell

DN Double negative

DP Double positive

EC50 Half maximal effective concentration

GATA3 GATA binding protein 3

GM-CSF Granulocyte macrophage colony-stimulating factor

HSC Hematopoetic stem cell

ICAM Intracellular adhesion molecule

ICOS Inducible costimulator

IFN Interferon

Ig Immunoglobulin

IL Interleukin

ILC Innate lymphoid cell

iNKT Invariant natural killer T cell

Iono Ionomycin

ITAM Immunoreceptor tyrosine based activation motif

GC Germinal center

Lm *Listeria monocytogenes*

LN Lymph node

LPS Lipopolysaccaride

MHC Major histocompatability complex

MoDC Monocyte derived dendritic cell

MTB *Mycobacterium tuberculosis*

mTEC Medulary thymic epithelial cell

NK Natural killer

NLR Nod-like receptor

PAMP Pathogen associated molecular pattern

PBMC Peripheral blood mononuclear cell

PMA Phorbol 12-myristate 13-acetate

P:MHC Peptide:MHC

PRR Pattern recognition receptor

RAG Recombination-activating gene

SP Single positive

STAT Signal transducer and activator of transcription

T-bet T-cell-specific T-box

TCR T cell receptor

T_{CM} T central memory cell

TdT Terminal deoxynucleotidyl transferase

T_E T effector cell

T_{EM} T effector memory cell

T_{FH} T follicular helper cell

TGF-β Transforming growth factor beta

Th T helper

TLR Toll-like receptor

TNF Tumor necrosis factor

Treg T regulatory cell

TSLP Thymic stromal lymphopoietin

TT Tetanus toxoid

T_{SCM} T memory stem cell

V(D)J Variable, diversity, joining

4. Introduction

4.1. CD4$^+$ T cells: antigen receptor, subsets, and role in the immune response

4.1.1. Generation of CD4$^+$ T lymphocytes

T lymphocyte development is a process occurring early in life in the thymus, a mediastinal organ that enlarges during childhood and undergoes atrophy at puberty. Thymus has a bi-lobar structure, each lobe being composed by multiple lobules organized in an outer cortical and an inner medullary region (Abbas et al., 2012; Murphy, 2011). Multipotent lymphoid progenitors continuously reach the thymus coming from the bone marrow, where they are generated from hematopoietic stem cell precursors, and undergo a complex sequence of events that lead to the generation of a large pool of mature T lymphocytes expressing each a unique T cell receptor (TCR) that can recognize a specific peptide bound to major histocompatibilty complex (MHC) molecules displayed on the surface of antigen presenting cells (APCs) (Takahama, 2006). In mature T lymphocytes, the TCR associates with a co-receptor molecule, either CD4 or CD8, that selectively binds to MHC class II or class I molecules, respectively. As a result, TCRs associated with CD4 can recognize peptides bound on MHC class II molecules, which are expressed on the surface of professional APCs, such as B cells, dendritic cells (DCs), macrophages, and monocytes, while TCRs associated with CD8 can recognize peptides bound to MHC class I molecules, which are expressed by virtually all nucleated cells in the body. Peptides derived from extracellular antigens are generally presented on MHC class II molecules, while MHC class I molecules mainly accommodate peptides derived from antigens from intracellular compartments (Abbas et al., 2012; Murphy, 2011).

The process of T cell development and selection is very restrictive, and only 2-4% of cells entering the thymus will make egress from the organ. Once in the thymus, the lymphoid progenitors become CD4$^-$CD8$^-$ double negative (DN) thymocytes losing the potential to differentiate into B or NK cells (Rothenberg et al., 2008) (Figure 1). Based on the expression of CD44 and CD25, these cells differentiate through 4 distinct stages (DN1 to DN4) in the outer cortex of the organ (Godfrey et al., 1993). At the stage of DN3, cells can produce a TCR β chain upon somatic rearrangement of the dedicated locus on chromosome 7, mediated by the enzymes recombination-activating gene (RAG) 1 and 2 (Figure 1). The TCR β chain pairs on the cell surface with a pre-TCR α chain (pT-α), transcribed instead from a genetic locus that does not undergo rearrangement, and this definitely precludes the possibility to develop

along the pathway of γδ T cells (Saint-Ruf et al., 1994, 2009; Takahama, 2006). The pre TCR αβ can signal downstream thanks to the association with the CD3/ζ complex, allowing further maturation of the T cell precursor to the DN4 stage, and finally recombination of the α chain and surface expression of a mature αβ TCR (Pang et al., 2010). Co-receptors start to be expressed at this stage, generally CD8 first, followed by CD4: the resulting population is indeed composed by $CD4^{+}CD8^{+}$ double positive (DP) cells. Most of these thymocytes will die by neglect, as their TCRs bind too weakly to the endogenous peptides presented in the cortex by thymyc epithelial cells (TECs), and they do not upregulate TCR-signaling associated pro-survival factors. Subsequently, T cells lose the expression of one of the two co-receptors and are instructed to become either $CD4^{+}$ or $CD8^{+}$ T lymphocytes that migrate into the medulla (Germain, 2002; Rothenberg et al., 2008). There is no consensus yet on what signals are precisely required to make this fate decision. Two models have been proposed: in the first one, known as the *selection model*, the choice of which co-receptor has to be maintained is made randomly, and T cells are then selected based on their capacity to recognize antigens on the resulting MHC molecule of choice, most of them dying as a consequence of a non functional TCR-co-receptor coupling. In the second, referred to as the *instruction model*, the signals that the T cell receives during thymic antigen presentation drive the selection of the fittest co-receptor (Germain, 2002). Variations in strength and duration of the TCR signaling have been proposed to be key factors in the lineage decision process. Notch-Notch ligand interaction was reported to be crucial in this process, Notch-1 activation being associated with CD8 SP T cell development (Germain, 2002).

T cells need to be selected by a minimal strength of self recognition, to ensure functionality and capability to recognize a non-self antigen when presented on MHC molecules. This process, called *positive selection*, takes place in the cortex of the thymus, and is mediated by specialized cortical thymic epithelial cells (cTECs) (Klein et al., 2014). cTECs express high amounts of MHC class II molecules on their surface and are endowed with a distinguishing antigen processing machinery. In fact, they possess a unique catalytic subunit of the proteasome, called β5t, and lysosomal proteases (cathepsin L and thymus-specific serine protease, TSSP) that determine their unique array of MHC-displayed peptides. Furthermore, these cells are characterized by constitutive high levels of macroautophagy, a process through which self proteins from endogenous cell compartments can be presented on MHC class II molecules (Klein et al., 2014). Positively selected T cells then migrate to the medulla, where approximately 5% die by apoptosis as a consequence of very strong interaction between the TCR and self peptide-MHC complexes. This process, known as *negative selection*, is essential to purge the T cell repertoire from potentially autoreactive clonotypes. Negative selection is primarily carried out by medullary thymic epithelial cells (mTECs); through the

action of AIRE gene, mTECs can ectopically express a broad set of tissue restricted antigens (TRAs), and therefore present self antigens which would not otherwise be available for screening of auto-reactive T cells (Anderson et al., 2002; Liston et al., 2003). These antigens can be presented directly by mTECs, or handed over to neighbouring APCs. Recent work has provided evidence that negative selection starts already in the cortex, where is mediated by specific subsets of DCs (Daley et al., 2013; Stritesky et al., 2013).

In summary, during thymic development, T cell precursors undergo a process of selection that is consistent with an affinity model, whereby affinity refers to the strength of interaction between TCR and self peptide-MHC complexes. Weak but over-threshold interactions are required to spare thymocytes from death by neglect; strong interactions lead to negative selection and apoptosis. Cells whose TCRs interact with intermediate strength will develop either as non-self responding mature naïve T cells or as tolerized natural T regulatory cells (nTregs) and exit the thymus to relocate in the periphery (Klein et al., 2014).

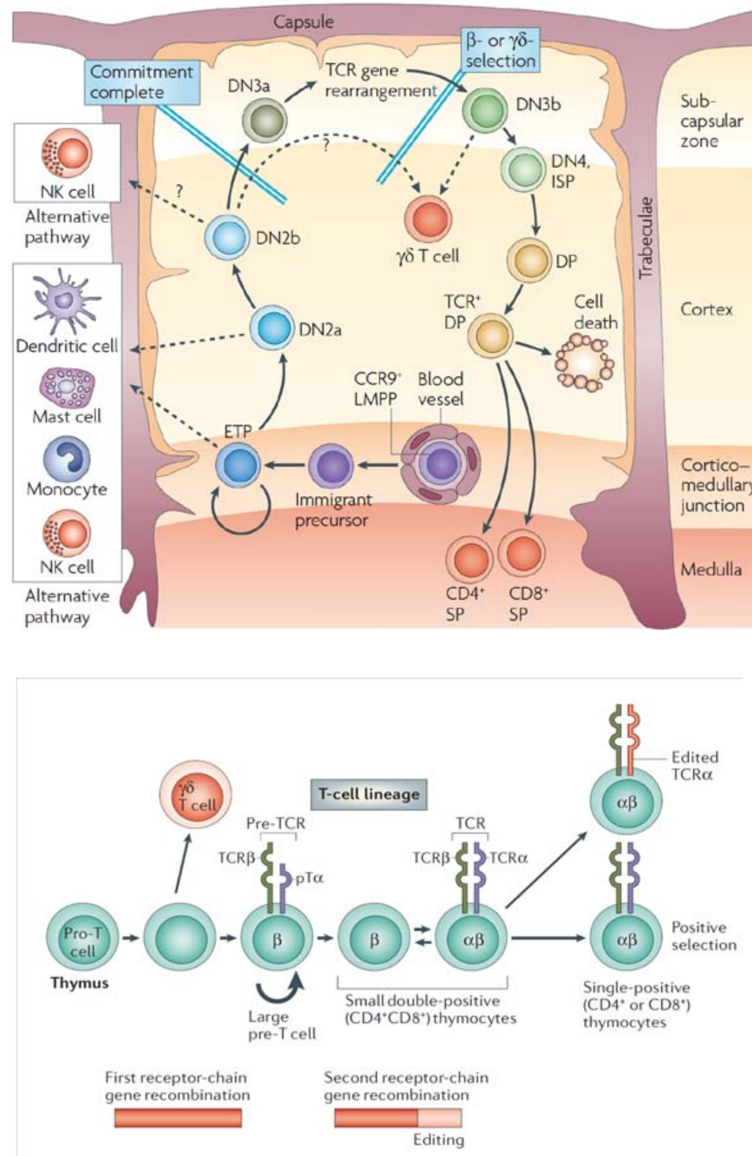


Figure 1. T cell developmental pathway. Upper panel: Immigrant precursors initially enter the thymus through blood vessels and subsequently migrate, meanwhile differentiating from double negative (DN) to double positive (DP) to single positive (SP) stages. DN2 begin their migration from the site of entry deep within the cortex to the outer rim of the cortex. β-selection occurs during the accumulation of the DN3 T cells in the subcapsular zone. A reversal migration back across the cortex towards the medulla occurs for the later stages of thymocyte development (adapted from (Rothenberg et al., 2008)). Lower panel: Common lymphoid progenitors (CLPs) give rise to both B cell and T cell lineages in the thymus. In progenitor (pro)-T cells, the first antigen-receptor chain locus undergoes V(D)J recombination, generating the β-chain of the T-cell receptor (TCRβ) in αβ T cells. In addition, the genes that encode TCRδ and TCRγ recombine in pro-T cells, sometimes giving rise to γδ T cells. The first receptor chain then associates with the (pre)-TCR α-chain (pTα) in pre-T cells, yielding pre-TCR complex, signaling through which mediates proliferation and developmental progression. Next, lymphocytes stop dividing and recombine the genes that encode the second receptor chain, generating αβ TCR in T cells. In the main pathway of αβ T cell development, continued rearrangement at the TCRA locus often occurs because of autoreactivity or lack of positive selection, creating either a non-functional rearrangement (denoted by the reverse arrow) or an edited receptor with a new TCRα. Positive selection ultimately stops gene rearrangements and promotes the loss of either CD4 or CD8 (adapted from (Nemazee, 2006)).

4.1.2. αβ T cell receptor: genetically encoded diversity of the immune repertoire

T cells must face an enormous diversity of challenging pathogens, providing response to an hypothetically infinite spectrum of antigens. The amount of genetic information necessary to encode for a proportional variety of receptors would likely be incompatible with the size of the genome. T cells, as well as B cells, have therefore developed a very efficient system to ensure sufficient diversity of their repertoire by undergoing somatic rearrangement on the genetic loci that encode for their antigen-specific receptor.

Each germline TCR locus comprises different groups of genes called Variability (V), Diversity (D), Joining (J) and Constant (C) segments, respectively. A specific promoter and a leader sequence precedes each V segment (Figure 2). The β chain locus, on chromosome 7, hosts 77 contiguous V genes (of which 48 functional, the rest being pseudogenes whose function has been impaired by occurred mutations), 2 D, each followed by 7 of 14 J (13 functional) and 1 out of two C genes. The α chain locus, on chromosome 14, is composed by 54 contiguous V (45 functional), 61 contiguous J (50 functional), and 1 C (The International Immunogenetics Information System, 2014). D segments are absent from this locus; instead, this region hosts the genetic locus encoding for the δ chain, that is usually co-expressed with a γ chain to give rise to T cells carrying a different type of TCR called γδ. γδ T cell migration from the thymus precedes αβ T cell egress (Dunon et al., 1997).

The process through which the TCR genetic segments are joined and generate a functional chain of the TCR is called *V(D)J recombination*. Particular sequences, called recombination signal sequences (RSSs) are located at the 3' of each V gene segment, 5' of each J segment, and on both sides of each D segment. These sequences consist of a conserved stretch of 7 nucleotides (heptamer, 5'CACACTG3') contiguous to the coding sequence and followed by a non-conserved spacer of either 12 or 23 non-conserved basis, in turn followed by a second conserved set of nine nucleotides (nonamer, rich in AT) (Schatz and Spanopoulou, 2005; Schlissel, 2003). Acting on those particular sequences, a series of enzymes mediate recombination among the gene segments so to obtain a linear gene containing 1 V, 1 D and 1 J for the β chain, or 1 V and 1 J in the case of the α chain, joined together. In the case of the β chain, D and J segments are rearranged first, and the V segment is coupled afterwards. The main enzymes catalyzing this reaction are Rag1 and 2, they are expressed selectively in lymphocytes and multimerize to form a tetramer (called Rag1-2 recombinase). The reaction begins with the recognition of spacer sequences and cleavage at the side of each coding segment; *de novo* addition of nucleotides at the junctions and end joining between the two adjacent segments takes place subsequently, both reaction being mediated by other enzymes

(Schatz and Spanopoulou, 2005; Schlissel, 2003). Importantly, Rag1 and Rag2 can only join segments flanked by RSS of different spacer length (*i.e.* a gene flanked by a 12 bp spacer RSS with one adjacent to a 23 bp spacer RSS) (Kavaler et al., 1984; Kurosawa et al., 1981; Sakano et al., 1981). Due to the spatial organization of RSS within the locus, this makes recombination possible only between V-D and D-J segments (or V-J in the case of α-chain). It is believed that the reason for this "12-23 rule" lies on the structure that nucleotidic stretches of these particular lengths can achieve, most likely 12 nt corresponding to one turn and 23 nt to two turns of the DNA helix; this would allow proper spatial positioning of the segments to be recombined. If the joined segments have the same orientation, the process generates circular products of DNA excision called TRECs (T Cell Receptor Excision Circles), operatively used to identify recently thymus-emigrated cells (Kong et al., 1999). Terminal deoxynucleotidyl transferase (TdT) can add *de novo* nucleotides to the ends of the cleaved strands before the joining is completed, and this greatly enhances the diversity generated through somatic recombination (Cabaniols et al., 2001); added nucleotides are classified as P- or N-, based on the consequent formation of palyndromic sequences or the absence of an encoding template, respectively. Finally, a series of ubiquitous DNA-modifying enzymes are recruited and activated that mediate ligation of the ends of the broken DNA strands (Ku70, Ku80, DNA-PK, Artemis, DNAligaseIV), thus generating a linear sequence of DNA encoding for the whole TCR chain. While CDR1 and CDR2 are entirely encoded by the V segment, the region encoded by the VDJ (or VJ) joining corresponds to the CDR3 on the mature chain (Schatz and Spanopoulou, 2005; Schlissel, 2003).

From a structural point of view each chain of the αβ TCR is composed of two extracellular immunoglobulin-like domains (one being constant and the other variable in sequence), a short hinge region, a transmembrane segment and an extremely reduced cytoplasmic tail, that is not endowed with any signal-transducing domain (Figure 3). The α and β chains are covalently linked by a disulfide bond at the level of the hinge region. Each variable domain contains three loops where the aminoacidic variability is concentrated to allow antigen discrimination, and are therefore called complementary determining regions (CDRs) 1, 2 and 3 (Murphy, 2011) (Figure 3). CDR1s and CDR2s mainly interact with the terminal parts of the peptide lying on the MHC cleft and with the MHC complex itself respectively, while the two CDR3s (one belonging to the α and one to the β chain), bearing the highest level of diversity, directly interact with the peptide from above, allowing fine discrimination of even a single aminoacidic difference (Garcia and Adams, 2005). It must be noted that despite such fine specificity, the affinity of TCR for its cognate antigen is low when compared to antibodies (Kd $10^{-5}/10^{-7}$ M vs $10^{-7}/10^{-11}$ M) (Huppa and Davis, 2013). This has been postulated to allow rapid screening of a multitude of peptide:MHC (p:MHC) complexes. The strength of the

signaling upon antigen recognition by TCR is ensured by several mechanisms, such as the formation of a tight immunological synapse between the T cell and the APC, the presence of co-stimulatory molecules that amplify the signals, and and the capacity of p:MHC complexes to serially engage a multitude of TCRs in a reduced amount of time (Valitutti et al., 1995; Viola and Lanzavecchia, 1996; Viola et al., 1999). A fourth CDR region is present in the β chain that appears to be selectively engaged by some bacterial molecules capable of triggering TCRs in an aspecific manner, referred to as *superantigens* (Murphy, 2011).

As mentioned above, expression of the TCR is a multistep process that takes places in the thymus and contributes to thymocyte development into mature naïve T cells. Pro T cells are precursors that lack any form of antigen receptor and rely on IL-7 of stromal origin for survival. At the stage of DN3, they express a pre-TCR, composed by TCR β chain and an invariant pT α chain (Saint-Ruf et al., 1994). A productive rearrangemement of the β chain is necessary to ensure survival of thymocytes. Signals through the pre-TCR are required to induce allelic exclusion through inactivation of recombining enzymes, thus ensuring that no further TCR recombination will occur on the remaining chromosome for the second possible β chain (Pang et al., 2010). Furthermore, this signaling cascade induces strong proliferation in the thymocyte, now at the stage named DN4. Next, expression of both CD4 and CD8 co-receptors (DP stage) and α chain locus recombination take place, leading to expression on the membrane of a complete TCR (Nemazee, 2006; Rothenberg et al., 2008). Nonproductive rearrangements of both β and α chains can be rescued by subsequent attempts; α-chain in particular can undergo successive rearrangements, until positive selection or cell death intervene (Petrie et al., 1993). Notably, unlike for β chain, α locus recombination does not induce allelic exclusion unless the receptor is positively selected, and as a result roughly up to 1 out of 3 αβ T cells express two functional TCRs on their surface (Padovan et al., 1993). At this stage, signals received thorugh the TCR, as previously discussed, induce T cells to differentiate toward a defined lineage, by losing expression of one of the two co-receptors and becoming single positive (SP) thymocytes. In the meantime the expression of CD3 molecules is upregulated and the TCR complex appears in its mature form on the cell surface. Naïve T cells bearing such receptor complex are now fully mature and can migrate into the circulation. Only roughly one in three developing T lymphocytes will make in frame rearrangements of their αβ chains and undergo further selection (Klein et al., 2014).

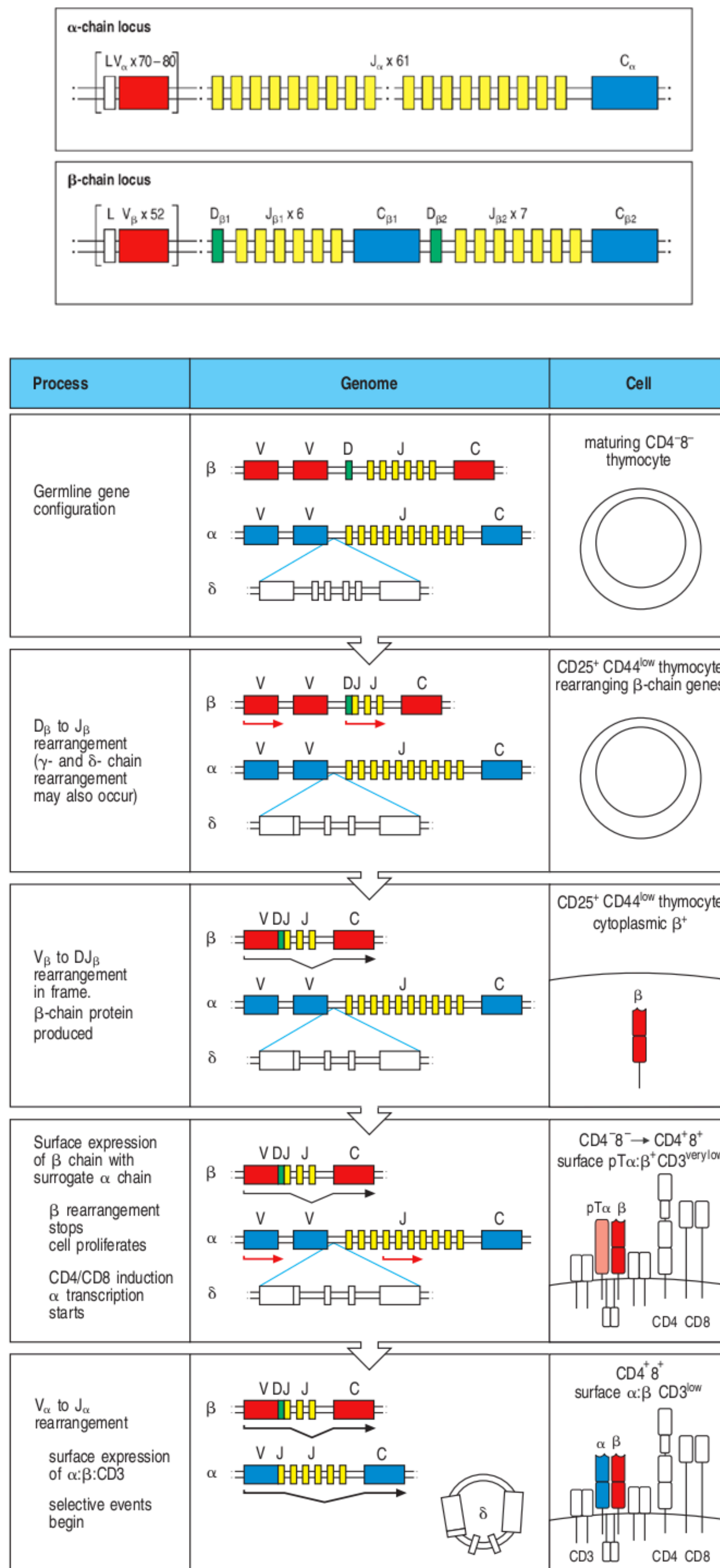


Figure 2. Germline organization of TCR α and β genetic loci and V(D)J recombination. Upper panel: Structure of the α and β genetic loci on chromosome 14 and 7, respectively. Updated genetic segment numbers are reported in the text. Lower panel: schematic representation of stages of gene rearrangement in αβ T cells; of note, J segments downstream the rearrangement site are spliced out from mRNA (lower panel) (from Murphy, 2011).

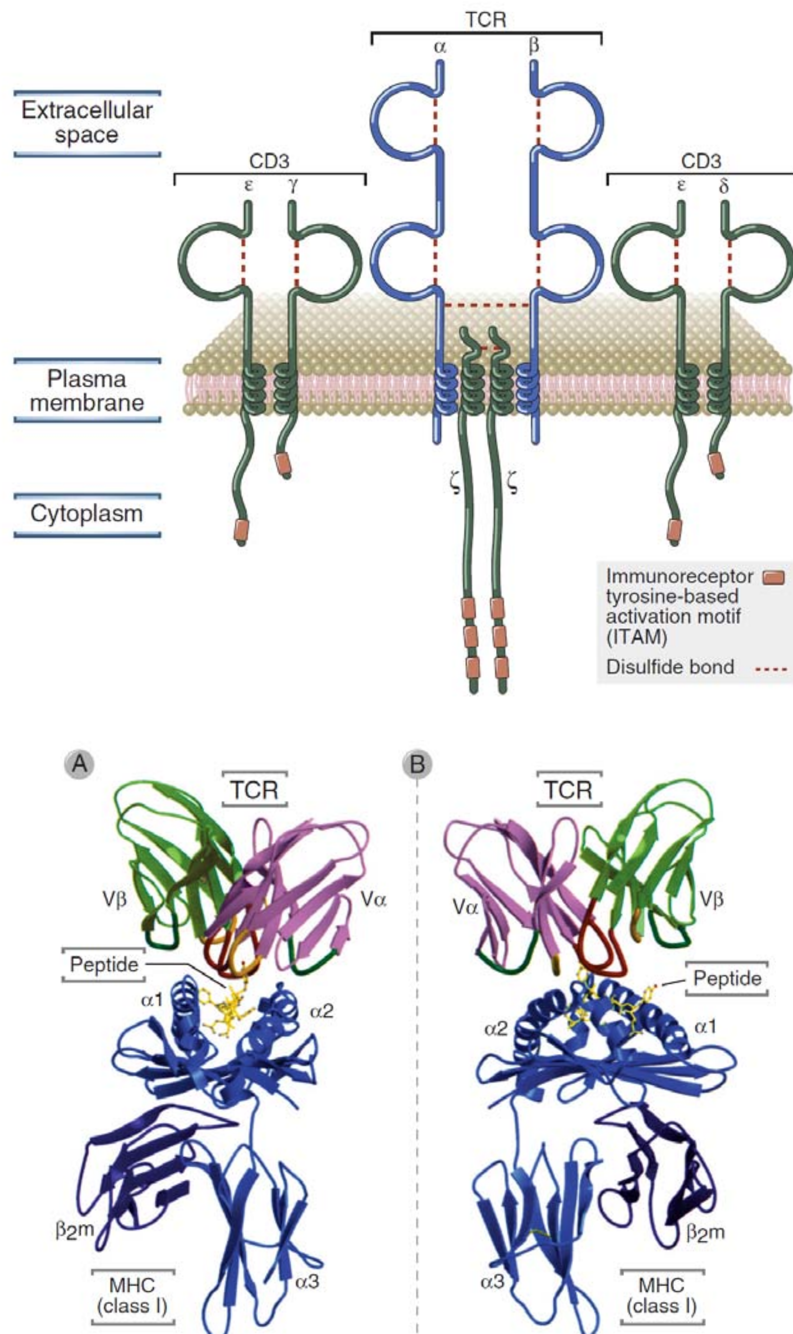


Figure 3. T cell receptor and the TCR complex. Upper panel: schematic representation of the TCR complex. Each complex is composed by 1 αβ TCR molecule, 2 CD3 dimers and 1 ζ chain dimer. Hemi-circles represent Ig-domain. Oppositely charged residues in the transmembrane region (not shown) are responsible for association of the complex. Lower panel: front and side view (A and B, respectively) of the V domains of a αβ TCR recognizing a peptide (yellow) presented on MHC class I molecule. CDR loops are represented in color (red for the CDR3) (from Abbas et al., 2012).

4.1.3. TCR-mediated signaling and T cell activation by dendritic cells

When the T cell receptor is triggered, a downstream cascade leads to activation of transcription factors such as NF-κB and AP-1 and, ultimately, to proliferation and functional response of the T cell. The TCR intracellular region is not endowed with any signaling domain, and transduction of the extracellular event into a cytosolic cascade is mediated by other molecules within the TCR complex. The complex is composed by the αβ TCR, two CD3 molecules, each consisting of two chains, εδ and εγ respectively, and a homodimer of ζ chains (Figure 2). These invariant components associate with the TCR through electrostatic interactions between oppositely charged residues in the transmembrane region (Figure 2) (Call et al., 2002). All these invariant dimers possess immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracellular portion, one in each chain of the CD3 chains, 3 in each ζ chain. ITAM domains contain two tyrosine residues that, upon phosphorylation, provide a site for recruitment of Src-homology 2 (SH2) domain-bearing enzymes (Irving and Weiss, 1991; Letourneur and Klausner, 1992).

The first event upon antigen recognition and TCR triggering is the activation of Lck, a Src-family kinase, associated both with the CD3/ζ complex and with the cytoplasmic tail of CD4 or CD8 (Li et al., 2004). Lck phosphorylates ITAM motifs on CD3 and ζ chains, thus recruiting SH2-domain bearing kinase ZAP70 (ζ-chain Associated 70 KD kinase), which is in turn phosphorylated by Lck (Chan et al., 1995). Lck also activates other proteins, among which the adapter protein LAT (Linker for Activation of T cells). This, through the activation of Phospholypase C-γ (PLC-γ) leads to increase in Ca^{2+} concentration into the cytosol and activation of PKCθ. Ca^{2+} influx promotes activation of transcription factor NFAT through Calcineurin activity; PKCθ activates NF-κB and AP-1 transcription factors, the latter via MAPK cascade triggering. As a result, the cell begins to proliferate, produces effector molecules, such as cytokines, and expresses membrane receptors (Lin and Weiss, 2001; Smith-Garvin et al., 2009).

When a naïve $CD4^+$ T cell encounters a DC bearing the cognate peptide-MHC class II complex on its surface, an immunological synapse is formed, where interactions between a set of integrins on both cellular sides (such as LFA1/ICAM1) contribute to stabilize complexes between the two cells. p:MHC complexes, TCRs and costimulatory molecules are in fact constrained toward the center of an integrin ring (Lanzavecchia and Sallusto, 2001). Importantly, synapse formation increases the sensitivity of TCR for the cognate antigen, thus lowering of about 100-fold the threshold for activation (Fooksman et al., 2010). During this interaction, DCs deliver three different types of signals to the T cells: they present the antigen on their surface MHC molecules (1st signal), provide costimulation (2nd signal) through

surface receptors, and produce cytokines (3rd signal). The strength and type of such signals, together with other environmental cues, instruct proliferating T cells toward specific effector and memory fates, acquiring functions that are believed to be optimal for the clearance of the eliciting pathogen (Lanzavecchia, 1999).

Signal 1 provides the main trigger for T cell activation and induces proliferation; upon TCR triggering, naïve T cells enter the G1 phase, start producing IL-2 and synthesizing CD25, the α subunit of the IL-2 receptor, which greatly increases their sensitivity to the cytokine. These last steps however require to some extent the presence of costimulatory signals (signal 2) (Acuto and Michel, 2003). Only 10 p:MHC complexes are required to fully activate a $CD4^+$ T cell, and even less (1-3) to induce effector functions (Irvine et al., 2002). The concept of progressive threshold for the acquisition of different effector functions was firstly proven on $CD8^+$ T cells, where cytotoxicity is achieved with low levels of stimulation, while proliferation and cytokine secretion are induced upon stronger TCR signal (Valitutti et al., 1996). The fact that very few p:MHC can efficiently activate T cells is due to the very slow off-rate of dissociation between peptide and MHC, which are almost irreversibly bound, as well as to the ability of p:MHC complexes to subsequently engage multiple TCRs: it has been calculated that within 5h a single p:MHC complex can trigger as many as 200 TCRs (Lanzavecchia et al., 1992; Valitutti et al., 1995). Accordingly, in the presence of costimulatory molecules, as few as 300-1500 TCRs need to be triggered to activate a T cell (Viola and Lanzavecchia, 1996; Wei et al., 1999). Very low levels of TCR stimulation is sufficient to induce naïve T cells to differentiate into effector/memory cells, but fails to generate a large progeny of memory cells (Zehn et al., 2009).

A second signal is indeed necessary for the functional expansion of naïve T cell clones: antigen presentation in the absence of costimulatory pathway activation leads either to an unresponsive state, defined as anergy, in which the T cell is refractory to any further stimulation, or to switch to a regulatory (tolerogenic) phenotype (Schwartz, 2003). Fully stimulated T cells proliferate and originate effector and memory progenies; at this stage the presence of costimulation is not anymore required in case of antigen re-encounter (Schweitzer and Sharpe, 1998).

The most important and better characterized costimulatory molecules expressed on DCs (and, in general, on APCs) are CD80 (also known as B7.1) and CD86 (B7.2), that interact with CD28 which is constitutively expressed on naïve T cells; ICOS ligand (Inducible T cell Costimulator ligand, ICOSL), whose receptor on T cells is ICOS; CD40 on B cells and DCs whose receptor on T cells is CD40L. Both ICOS and CD40L are not expressed by naïve T cells and are upregulated upon TCR stimulation. CD28 triggering by B7.1/B7.2 molecules

increases the production of IL-2 through several mechanisms, acting both at transcriptional and translational level, and recruits lipid rafts and Lck to the synapse, thus amplifying and stabilizing the overall amount of TCR complex activation (Acuto and Michel, 2003; Viola et al., 1999). IL-2 binds to the IL-2 receptor, promoting survival and expansion of T cells through the induction of anti-apoptotic factors, such as Bcl-x_L (Boise et al., 1995). Unlike CD28, ICOS enhances proliferation and effector functions on T cells in an IL-2-independent fashion (Greenwald et al., 2005; Hutloff et al., 1999). The CD40-CD40L pair signals in both directions, making T cells able to enhance the antigen presentation properties of B cells and DCs (antigen processing, B7 molecule expression, cytokine production) and therefore indirectly promoting T cell proliferation (Grewal et al., 1997). Through CD40L-CD40 interaction, $CD4^+$ T cells can "license" DCs for priming of $CD8^+$ T cytotoxic responses (Lanzavecchia, 1998).

CTLA-4 is a molecule expressed on T cells that displays high sequence similarity to CD28; this molecule, however, has an inhibitory effect on T cell proliferation, possibly through competition with CD28 for binding to B7 molecules (of note, CTLA-4 affinity for those receptors is about 20 times higher). This is considered an internal control mechanism whose aim is to limit proliferation of activated T cells, thus impeding tissue damage (Greenwald et al., 2005).

The effect of third signal (cytokines) during priming will be analyzed in detail in a subsequent section. It is just important to point out that different cytokines can imprint expanding T cells with specific phenotypes which are thought to be optimal to fight the eliciting pathogen and generate a protective memory pool (Sallusto and Lanzavecchia, 2009).

4.1.4. Naïve T cell priming and dynamics of T cell response

T cells reside in most human tissues, where they can be activated by recognition of foreign antigens presented by APCs. However, the major site for T cell retention and initiation of the immune response is the lymph node. Lymph nodes are highly organized organs of reduced size (ranging from a few mm to 1-2 cm in steady state), bean shaped, and present at very high and variable numbers in mammals, several hundreds in a human being. They are located along a system of vessels referred to as lymphatic, through which a complex mixture of drained interstitial fluids, immune cells, proteins and particulate antigens - called lymph - flows. Several afferent and one efferent vessels connect lymph nodes throughout the body in a very articulated net; the role of such apparatus is to constantly filter lymph in order to rapidly detect any invading pathogen (Abbas et al., 2012; Murphy, 2011).

Lymph nodes are surrounded by a capsule, and are internally structured into a cortical, a para-cortical and a medullary region, each hosting different cell types. T cells, in particular, segregate into the paracortical region (also called T cell zone). Here, both naïve T cells -that have never encountered their cognate antigens- and memory -antigen experienced- T cells reside and recirculate, randomly screening APCs in search of an activating p:MHC complex. Particular blood vessel terminations named high endothelial venules, HEVs, reach the paracortical region and are endowed with a series of unique adhesion molecules that allow extravasation of blood circulating leukocytes. The paracortical localization of HEVs facilitates the encounter between lymph borne DCs and T cells and therefore provides an optimal environment for cellular interaction and initiation of the immune response (Abbas et al., 2012; Murphy, 2011).

Naïve T cells continuously recirculate through lymphoid organs thanks to the expression of surface molecules such as CCR7 and CD62L, among others, which mediate extravasation upon binding of their ligands on HEVs, CCL21 and CD34/GlyCAM-1 respectively. By continuously moving, every single naïve T cell can screen thousands of antigen presenting DCs in a few hours, thus greatly increasing the chance to encounter its cognate antigen, an event whose probability would *per se* be extremely low. Once inside the lymph node, in fact, naïve T cells are driven and kept in the T cell zone by a gradient of CCL18-CCL19-CCL21, produced by stromal cells as well as by DCs. If not activated, T cells leave the lymph node following upregulation of sphingosine 1-phosphate receptor-1 (S1P1), re-enter the circulation and resume their screening activity elsewhere (Rot and von Andrian, 2004).

The main APCs involved in the priming process are DCs, cells of myeloid origin that reside all over organs and tissues, and are endowed with a broad array of innate receptors. DCs reach

very high density at any interface with the outer environment (skin, gut, lungs) and thus with the invading pathogens, as well as in lymphoid organs. Discovered in 1973 by Steinman and Cohn, they owe their name to their tree-like morphology characterized by numerous dendrites, which allow efficient antigen sampling and phagocytosis from the extracellular space (Steinman and Cohn, 1973). In the peripheral tissue, resident DCs can sense and take up pathogens as well as soluble antigens, as they can efficiently exert phagocytosis (Reis e Sousa et al., 1993), fluid phase and receptor mediated pinocytosis and macropinocytosis (Sallusto et al., 1995; Sallusto and Lanzavecchia, 1994), the latter being active even in steady state at very high rate. The sensing and uptake are made possible by the impressive variety of phagocytic and pathogen-sensing receptors these cells are endowed with, such as C-type lectin receptors (CLRs), toll-like receptors (TLRs), NOD-like receptors (NLR), scavenger receptors (Osorio and Reis e Sousa, 2011). Following antigen recognition and uptake, DCs undergo a process known as activation or maturation, that fully enables them for stimulation of naïve T cells, as they undergo major changes in their biological activities (Reis e Sousa, 2006). First, antigen uptake is diminished in favor of an improved antigen-presentation capacity (lysosome acidification, increased proteolysis, augmented MHC class II molecules expression and half life) (Cella et al., 1997; Reis e Sousa, 2006). Concomitantly, DCs downregulate tissue-adhesive molecules and enter the lymphatics, reach the subcapsular sinus of a lymph node and directly migrate to the paracortical T region to interact with T lymphocytes. This migratory behavior is mediated by the upregulation of the chemokine receptor CCR7, that selectively drives DCs through a gradient of chemo-attractant factor produced in the T cell area, mainly CCL19 and CCL21 (Sallusto et al., 1998). Importantly, while migrating, DCs also upregulate costimulatory molecules such as CD80 and CD86, and initiate production of cytokines. In this way, tissue resident DCs transport antigen to the draining lymph nodes. Alternatively, the antigen can be delivered to the lymph node directly via the lymph, where it is taken up by resident DCs that become activated *in situ.* Lymph node-resident DCs can also present antigens handed over by tissue-derived migratory DCs (Allan et al., 2006).

The process through which naive $CD4^+$ T cells are firstly stimulated in the lymph node by their cognate antigen is known as “priming”. Three main temporal phases have been identified in priming, that take place subsequently (Mempel et al., 2004). Firstly, DCs and T cells display high motility, and perform a great number of interactions in search for a high affinity p:MHC complex recognition. As a second step, when the naïve T cell TCR matches its cognate p:MHC complex on a DC, both cells arrest and stably interact for 12-24h, during which the T cell starts producing IL-2. Finally, the T cell dissociates from the DC, the latter moving away in search of other potential interaction candidates, the former launching an intensive proliferative activity (Mempel et al., 2004). The time of interaction between DC-T

cell during the priming phase appears to be crucial for the magnitude of the expansion (even though does not significantly impact on the phenotype of the T cell population); this finding underlies the short term requirements to imprint a certain fate, at least in the mouse system, and with differences between $CD4^+$ and $CD8^+$ cells, as $CD4^+$ T cells require longer periods of antigen exposure to be fully activated (Lees and Farber, 2010).

The clonal expansion that ensue priming peaks around day 7-8 in mice and day 14 in humans, and can bring a single naïve T cell through more than 15 cell divisions, generating a population of more than 50,000 daughter cells (Zehn et al., 2012). While proliferating, these cells differentiate and acquire specific functional properties. This process generates both effector T cells, that are short lived and readily deal with the invading agent, and memory T cells, that confer efficient protection in case of pathogen re-attack. The effector phase of the T cell response is followed by a 1-2 week long contraction phase, during which most of the clonally expanded cells undergo apoptosis, so that only about 5% of the progeny enter the memory compartment and remain to confer protection to a second encounter with the same pathogen (Pepper and Jenkins, 2011).

4.1.5. Generation of effector and memory $CD4^+$ T cells

Following acute phase response and pathogen eradication, the majority (90–95%) of effector T cells undergo apoptosis, leaving behind a heterogeneous pool of memory cells. These cells are responsible for systemic immune surveillance and rapid response to re-challenge by the eliciting threat in virtue of an increased frequency (100-1000 fold) with respect to their naïve counterpart, decreased activation threshold and high functional capacity (Lees and Farber, 2010; Pepper and Jenkins, 2011).

In 1999, Sallusto et al. identified two distinct subsets of circulating memory T cells on the basis of effector function, proliferative capacity, and migratory potential (Sallusto et al., 1999). The authors distinguished, both in the $CD4^+$ and in the $CD8^+$ compartment, central memory T cells (T_{CM}), expressing CCR7 and CD62L (L-selectin) and therefore endowed with the capacity to circulate through or reside in lymph nodes, and effector memory T cells (T_{EM}), lacking those receptors, and expressing receptors for migration to peripheral non-lymphoid tissues. Upon antigenic stimulation, T_{CM} cells produced interleukin (IL)-2 but very little effector cytokines, such as IFN-γ or IL-4, and proliferated extensively generating more differentiated effector T cells (T_E) and T_{EM} cells. In contrast, T_{EM} had a lower threshold of activation compared to T_{CM}, were endowed with effector functions, such as production of cytokines and perforin, but had limited proliferative capacity (Sallusto et al., 1999). Those subsets, initially identified in humans, were soon after identified also in the mouse system (Masopust et al., 2001; Reinhardt et al., 2001). Recently, a third population of memory T cells, designated as resident memory T cells (T_{RM}), has been described that permanently resides in peripheral tissues after clearance of infection; however these cells are to date poorly characterized (Gebhardt et al., 2009; Masopust et al., 2010; Mueller et al., 2013).

All memory T cells, both those residing in lymphoid organs and those sitting in peripheral tissues, are maintained viable and proliferate at slow rate (homeostatic proliferation) by the effect of cytokines such as IL-7 and IL-15. Both T_{CM} and T_{EM} cells proliferate in response to such cytokines, even in the absence of TCR triggering, but T_{EM} fail to expand due to the high levels of apoptosis, while T_{CM} can self renew and further differentiate (Geginat et al., 2003; Lees and Farber, 2010). Different requirements for maintenance seem to exist however for $CD8^+$ and $CD4^+$ cells, the latter showing more stringent need for tonic TCR stimulation by self antigens and less for IL-7/IL-15 signaling. In particular, tonic signaling was shown to be necessary for both survival and functionality of memory $CD4^+$ T cells (Kassiotis et al., 2002; Seddon et al., 2003). $CD4^+$ memory T cells can be relocated to reside in the bone marrow in niches constituted by IL-7 producing stromal cells (Tokoyoda et al., 2009).

How T_E, T_{CM} and T_{EM} are generated following antigen encounter and naïve T cell priming remain to be fully elucidated. Studies on $CD8^+$ T cells in a mouse model of viral infection showed that IL-$7R^{hi}$ memory precursors (memory precursors of effector cells, MPECs) are already present at the peak of the primary response, while the vast majority of cells expressing high levels of KLRG1 represent precursors for short-lived effector cells (SLEC) (Kaech et al., 2003). These finding would predict that memory or effector fates are programmed early at priming. Importantly, different inflammatory conditions and, more in general, different types of infection, can induce extremely different ratios between SLEC/MPEC. Such precursors however have not been identified for $CD4^+$ cells (Lees and Farber, 2010).

Given the irreversible differentiation from T_{CM} to T_{EM} upon antigenic stimulation, a developmental model was initially proposed according to which T_{CM} - retaining proliferative capacity - would generate more terminally differentiated T_{EM}. Effector T cells would stand at the end of this irreversible one-way linear pathway of differentiation (Sallusto et al., 1999). A few years later, a study challenged this view based on *in vivo* experiments, proposing that instead a linear differentiation pathway Tn->T_E->T_{EM}->T_{CM} would take place upon infection (Wherry et al., 2003). However, recent work based on the transfer of single precursors in naïve hosts and subsequent infection, confirmed the early prediction and provided mathematical models for the progressive developing pathway. Accordingly, naïve T cells would generate T_{CM} precursor which in turn would give rise to T_{EM} precursors; in approximately 10% of cases, naïve T cells would also directly generate T_{EM} precursors. These precursors would then terminally differentiate into short lived T_E. Each precursor in this linear chain of development would be thus enabled to self-maintain as well as to generate a progeny of more differentiated and rapidly proliferating cells. In this scenario, T_{CM} would be endowed with characteristics of stemness in order to maintain and replenish the memory pool (Buchholz et al., 2013; Gerlach et al., 2010; Stemberger et al., 2007). This is consistent with the fact that transfer of T_{CM} into naïve hosts confers long-term protection, while T_{EM} have only limited reconstitution capacity (Gattinoni et al., 2005). The above mentioned findings have now been confirmed also by single cell transfer experiments on up to three generations of T_{CM} derived by *in vivo* primed naïve T cells and appear therefore extremely robust (Graef et al., 2014). Recently, also a small population with stem cell characteristics was detected within the T cell memory pool and proposed to account for memory propagation; however there is no consensus yet on the importance of such subset (Gattinoni et al., 2011).

With respect to $CD4^+$ T cells, potential precursors for T_{CM} and T_{EM} have been identified in a Th1 model response (*Listeria monocytogenes* infection) based on the expression of T-bet and CCR7 (Pepper et al., 2010). It has also been proposed that while $CD4^+$ T_{EM} would derive from an early precursor giving rise also to T_E cells, T_{CM} would be generated from T_{FH} cells or from

a common early precursor in response to ICOSL-mediated signals from B cells (Pepper and Jenkins, 2011). Even though more careful investigations are required with respect to Th2 and Th17 subsets regarding the $T_{CM}/T_{EM}/T_{E}$ differentiation, it has been postulated that memory formation should not be influenced by the functional fate of the cells, as memory T cells can be generated from cells producing any or none of the lineage specifying cytokines (Lees and Farber, 2010). In this regard it is worth to note that the T_{CM} pool of humans has been found to comprise both uncommitted populations, characterized by high self renewing capacity, and T cells that are already partially committed to differentiation toward defined subsets (Rivino et al., 2004), strongly suggesting that the model of linear differentiation and pool replenishment would hold true for any specific $CD4^{+}$ T cell lineage.

4.1.6. CD4$^+$ T cell subsets: origin, phenotype and function

CD4$^+$ T cells play central roles in shaping the immune response by regulating the function of many other immune and non-immune cell types (Sallusto and Lanzavecchia, 2009). They help B cells to produce antibody, regulate the activity of phagocytes and APCs, sustain and enhance CD8$^+$ T cell response and memory formation, induce a responsive state in peripheral tissues. Importantly, they also regulate the magnitude and persistence of such responses to prevent tissue damage. Their role in inducing or enhancing functions in other cells earned them the title of T helper (Th) cells.

The response to different pathogens has to be accurately tailored, as the effector mechanisms of clearance differ from one microbe another. To achieve such a multiplicity of functions, CD4$^+$ T cells are endowed with a surprising capacity of differentiation toward phenotypically and functionally distinct effector lineages. These lineages, or subsets, are defined as "*cell populations in which a change in cytokine production is promoted by polarizing signals and stably imprinted by a lineage-specifying transcription factor through epigenetic mechanisms"* (Sallusto and Lanzavecchia, 2009). Importantly, also chemokine receptors, that allow preferential homing of effector and memory cells to the site of entry of the pathogen eliciting response, and other surface effector molecules, are coordinately expressed together with lineage-specifying cytokines in each subset. Thereby this compartment can be described in terms of functional modules that promote a tailored response (Sallusto and Lanzavecchia, 2009).

Many different factors influence the outcome of the priming process; however, the main driver of T cell differentiation is considered to be the cytokine milieu produced during activation, which is mostly determined by DCs. Different pathogens, acting on distinct innate pathways on DCs, ultimately lead to generation of defined priming microenvironments (Pulendran, 2005). Cytokines, acting on T cell cytokine receptors, modulate activation of intracellular cascades that eventually lead to the phosphorylation of a given signaling transducer and activator of transcription (STAT) protein. As already mentioned, costimulatory signals and physical parameters of the stimulation, such as strength and duration of the TCR signaling, influence this process. Once phosphorylated, STAT proteins dimerize and translocate into the nucleus, where they promote transcription of a series of lineage-specifying molecules, among which "master" regulator transcription factors. STATs and master regulators together orchestrate the coordinate expression of a variety of molecules, including effector cytokines and tissue homing chemokine receptors, that define the identity and thus the fate of the proliferating T cells. The principal subsets to date identified for CD4$^+$ T cells are Th1, Th2, Th17, Th22, Th9, Tfh, and Tregs (Figure 4). Each of these subsets is optimally

equipped for the response to a specific range of pathogens (O'Shea and Paul, 2010; Zhu and Paul, 2010; Zhu et al., 2010).

Th1. DC-derived IL-12 acts on the IL-12R to activate expression of STAT-4, determining acquisition of a Th1 phenotype (Hsieh et al., 1993). Th1 cells are characterized by production of IFN-γ (together with TNF-α and other inflammatory cytokines) and expression of inflamed tissue homing receptors, such as CXCR3 and CCR5. On a functional level, through the production of IFN-γ and other pro-inflammatory cytokines, Th1 are the main players in response to viruses and intracellular bacteria, as they efficiently activate macrophages and cytotoxic $CD8^+$ T cells, and act on non-immune cells by inducing an anti-viral state and upregulation of MHC molecules (Abbas et al., 2012; Murphy, 2011). Importantly, IFN-γ can act in an autocrine manner, as well as in a paracrine fashion if produced by IL-12-activated NK cells, and promote expression of STAT-1 via T-bet activation; this generates a positive feedback signal that strengthen the acquisition of the Th1 fate. Beside activating the Th1 program, STAT-4 and T-bet also repress other polarizing programs by inhibiting the respective master regulator, as it is the case for GATA3 that would lead to Th2 fate (Yamane and Paul, 2012; Zielinski et al., 2011). Cytokine milieu is not the only determinant of such fate acquisition: antigen dose has been shown to play a major role in the induction of Th1 phenotype. High doses of cognate peptide induce in fact strong and prolonged ERK signaling that represses GATA3 activation and makes cells transiently insensitive to IL-2, avoiding STAT5 activation, thus instructing T cells towards Th1 fate (van Panhuys et al., 2014; Yamane et al., 2005).

Th2. IL-4 of various origin (innate lymphoid cells, ILCs, are thought to be the main sources) induces instead activation of STAT-6 and consequently of GATA3. GATA3 is the master regulator of Th2 cells, characterized by expression of chemokine receptors such as CCR4, CCR3 and CRTh2, and by production of IL-4, IL-5, and IL-13. IL-2 signaling has been shown to act synergistically with IL-4 in the induction of a Th2 fate through the activation of STAT-5 (Le Gros et al., 1990; Yamane and Paul, 2012). Th2 can be obtained also in the absence of IL-4 or STAT-6 but strictly depend on GATA3 (Dent et al., 1998; Jankovic et al., 2000); weak TCR signaling activates weakly and transiently ERK and upregulates GATA3 in an IL-4 independent manner, and this cooperates with STAT-5 induced by endogenous IL-2 for production of IL-4 (induction phase); IL-4 acts on IL-4R to induce STAT-6 that further activates GATA3 (polarization phase) (Yamane et al., 2005). A recent report underlined antigen dose as the main factor influencing Th1/Th2 fate decision, concluding that the cytokine milieu acts only as an adjuvant in strengthening this decision (van Panhuys et al., 2014). Th2 cells are efficient inducers of a response characterized by eosinophils and basophils infiltration, mucus production, smooth muscle contraction, and production of mast-

cell-activating IgE; all these effector mechanisms are required to counteract invasion from multicellular parasites, such as hemints, protect from venom, but also provoke onset of allergic diseases (Allen and Sutherland, 2014).

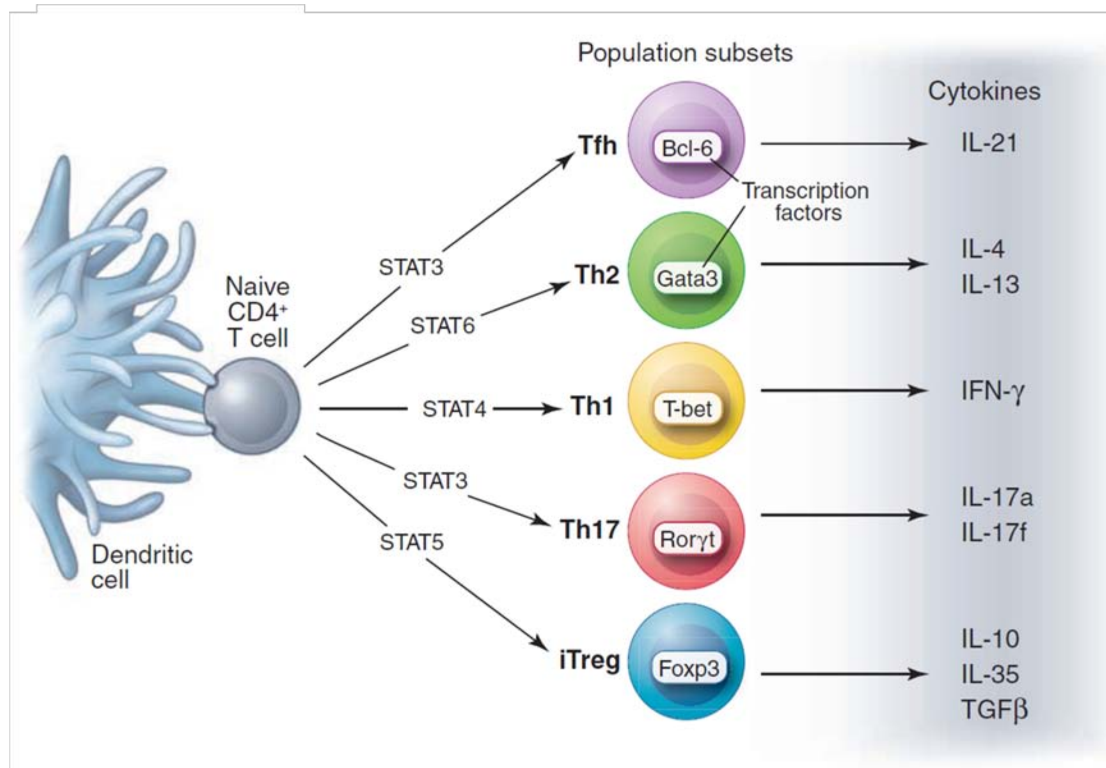
Th17. TGF-β, IL-6 and IL-1β (with a few discrepancies observed between the human and the mouse system) induce STAT-3 (Acosta-Rodriguez et al., 2007a; Bettelli et al., 2006; Chung et al., 2009; Manel et al., 2008; Mangan et al., 2006; Veldhoen et al., 2006), and ultimately RORγt (Ivanov et al., 2006), a transcription factor that specifies for the Th17 lineage (Harrington et al., 2005; Park et al., 2005). IL-21 and IL-23 have been shown to generate an amplification loop that promotes more efficient Th17 development (Korn et al., 2007; Nurieva et al., 2007; Volpe et al., 2008; Yang et al., 2008; Zhou et al., 2007). Th17 are characterized by production of IL17A/IL-17F, IL-22, and GM-CSF and express the mucosal, CNS, and skin-homing markers CCR6 and CCR4; they have also been reported to express the NK marker CD161, already at the level of Th17 precursors in the cord blood (Acosta-Rodriguez et al., 2007a; Acosta-Rodriguez et al., 2007b; Annunziato et al., 2007; Zielinski et al., 2011). Through the production of IL-17 and IL-22, Th17 promote the recruitment and activation of neutrophils on the one hand, and act on epithelial cells to induce anti-microbial peptides, on the other. Th17 are therefore required in case of infection from fungi or extracellular bacteria (Hernandez-Santos and Gaffen, 2012; LeibundGut-Landmann et al., 2012).

Th22. A population characterized by the expression of the chemokine receptor CCR6, as well as skin homing molecules CCR4 and CCR10, and by the capacity to produce IL-22, but not IL-17, has been recently identified and operatively defined Th22 (Duhen et al., 2009; Trifari et al., 2009). Polarizing cytokines in this case appear to be IL-6 and TNF, and a fundamental transcription factor is the xenobiotic receptor aryl-hydrocarbon receptor (AHR). The functional role of these cells remains to be defined, even though the effect of IL-22 in the induction of anti-microbial peptides by epithelial cells suggests a role in pathogen control at the body surface. Interestingly, Th22 have also been shown to contain cells specific for CD1a-restricted self-antigens (de Jong et al., 2010).

Th9. Cells producing exclusively IL-9, a generally Th2-associated molecule, have also been identified and defined as Th9. In this case a necessary, though not sufficient, transcription factor appears to be PU.1, under the control of STAT-6, while defining chemokine receptors have not been characterized yet (Goswami et al., 2012; Veldhoen et al., 2008). These cells have been associated to several functions, mainly helminth clearance, and they have been linked to atopic reactions, tumors, and recently response to skin microbes (Schlapbach et al., 2014).

T_{FH}. Induction of the transcription factor Bcl-6, probably but not certainly by IL-6 and IL-21, promotes differentiation of T cells toward T_{FH} fate (Johnston et al., 2009). These cells express high levels of PD-1, ICOS, and CXCR5, produce IL-21 and are instrumental for mounting an efficient and high affinity antibody response, as they direct the germinal center reaction (Crotty, 2011). However, whether this represents a distinct lineage parallel to Th1/2/17, or rather a functional state of cells belonging to different subsets, remains to be definitively established (Zhu et al., 2010).

Tregs. Finally, a subset of T cell exist whose role is to dampen the immune response, protecting from excessive tissue damage during inflammation, as well as from autoimmunity. These cells have been named regulatory T cells (Tregs), and can be originated through two distinct pathways. Natural Tregs (nTregs) originate in the thymus, where the driving element is thought to be the strength of self peptide recognition upon antigen presentation by TECs. In particular such interaction has been postulated to happen with a strength that is below threshold for negative selection, but higher than that for non-self specific T lymphocytes. This would induce tolerization in potentially autoreactive cells and prevent autoimmunity (Klein et al., 2014). Alternatively, Treg phenotype can be induced in the periphery (iTregs) by antigen presentation in the absence of costimulatory molecules and inflammatory cytokines (Chen et al., 2003). Cytokines such as TGF-β and IL-2 have been shown to play an important role in driving this fate decision by inducing the specifying transcription factors Foxp3 (Fontenot et al., 2003; Hori et al., 2003). Tregs can dampen the immune response acting through both release of soluble factors such as TGF-beta, and expression of surface immune-inhibitory receptors such as CTLA4. It must be acknowledged that to date human *in vitro*-differentiated iTregs have failed to show any regulatory activity (Zhu and Paul, 2010).



T CELL TYPE	DIFFERENTIATION FACTORS		EFFECTOR MOLECULES		FUNCTION
Th1	IL-12, IFN	T-bet	IFN-γ	CXCR3, CCR5	Bacteria *Autoimmunity*
Th2	IL-4	GATA-3	IL-4,-5,-13	CCR4,3, CRTh2	Parasites *Allergy*
Th17	IL-6,-1β,TGF-β	ROR-γt	IL-17,-22	CCR6+CCR4	Fungi *Autoimmunity*
Th9	IL-4, TGF-β	?	IL-9	?	Helminths
Th22	IL-6, TNF	AHR	IL-22	CCR10+CCR6	Skin pathogens? *Skin inflammation*
T_{FH}	(IL-21)	Bcl-6	IL-21	CXCR5	B cell help
Treg	(TGF-β)	FOXP3	TGF-β	CCR7, CCR6	Regulation

Figure 4. CD4$^+$ T helper subsets. Upper panel: schematic representation of the T cell priming process: different polarizing cues are delivered by the DC to the naive T cell during antigen presentation, that lead to activation of distinct STAT molecules. These in turn induce expression of lineage-specifying transcription factors (master regulators) and result in acquisition of specific functional properties such as cytokine production. Depicted subsets are widely characterized in the literature (O'Shea and Paul, 2010). Lower panel: updated list containing recently proposed T cell subsets and relative role in immune response as well as in immunopathology (Adapted from (Sallusto and Lanzavecchia, 2009)).

4.1.7. Balancing subsets: when pathology arises from an inappropriate Th response

Not only the magnitude but also the “flavor” of the response determines the outcome of the battle between host and pathogen. Striking examples can be found in the literature showing the importance of an appropriate type of response to reach sterilizing immunity. Most of these works have proven such concept with respect to the Th1-Th2 dichotomy, as for nearly two decades these subsets were the only ones known to exist.

A first example is represented by *Leishmania major* infection. While C57BL/6 mice that are prone to mount vigorous Th1 response are protected from the infection (through the production of IFN-γ and the activation of macrophages), BALB/c mice succumb due to their genetic propensity to generate an IL-4 driven Th2 response (Heinzel et al., 1989). Importantly, IL-4$^{-/-}$ or IL-4R$^{-/-}$ animals on the same background are largely rescued from susceptibility to *L. major* infection, demonstrating that decision on the fate that pathogen-specific T cells should undertake, regardless of their activation, is of fundamental importance in terms of response outcome (Noben-Trauth et al., 1999).

On the same line, it was shown that in humans infection with *Mycoacterium leprae* (the causative agent of leprosy) is associated with development of the lepromatous form of disease, which is more aggressive and disseminated, when a Th2 response is predominant. In contrast, individuals that mount appropriate Th1 response can confine the pathogen and develop a generally milder form of disease called tuberculoid leprosy (Sieling and Modlin, 1994; Yamamura et al., 1991). Severity of tuberculosis, a diseases provoked by *Mycobacterium tuberculosis*, has also been reported to be increased in individuals displaying cytokine profile skewed toward the production of IL-4/IL-5 rather than IFN-γ (Lindestam Arlehamn and Sette, 2014).

Early work in different mouse models has shown that susceptibility to *Candida albicans* can be associated to a Th2 type of response; IL-4 blockade, as well as pathogen-specific Th1 transfer prior to infection, greatly improves survival of the animals (Romani, 1999). It should be noted however that these works precede the discovery of the Th17 lineage, and it is thus likely that all the reported conditions that favored Th1 with respect to Th2 development in fact provided advantages also to the non-detected Th17 cells, which are now considered to be the main players in protection against fungi (Hernandez-Santos and Gaffen, 2012).

Human genetic deficiencies offer a unique possibility to study the requirements for protective immune responses. Seminal work on the importance of IL-17-driven response for *C. albicans* clearance or control has been done in the last ten years to understand the immune response of

patients suffering from chronic infection. Patients affected by mutations of different genes can develop a disease known as chronic mucocutaneous candidiasis (CMC) (Eyerich et al., 2010), characterized by uncontrolled growth of the fungus on skin, nails and mucosal surfaces. In particular, loss of function in AIRE (APECED syndrome), IL 17/IL-17R genes, as well as STAT-3 genes (the latter associated with hyper IgE syndrome, HIES) or gain of function STAT-1 (STAT-1 GOF), have been implicated in the onset of CMC (de Beaucoudrey et al., 2008; Liu et al., 2011; Milner et al., 2008; Puel et al., 2011). What all these inborn errors have in common is that they generate an impairment of the IL-17 axis, eventually allowing *C. albicans* overgrowth, and producing a highly invalidating and life-lasting syndrome. It remains however to be established whether the magnitude of the response is still preserved in those individuals, and therefore a T cell response is generated having non protective capabilities (for instance, exaggerated production of IL-4).

The above reported findings underline the importance of regulating not only the magnitude, but also the type of T cell response that is generated. Conceptually, the activation of an unwanted type of T cell lineage results in failure of infection control and leads to tissue pathology.

4.1.8. Flexibility of CD4$^+$ T cells at the clonal and population level

Polarization of effector and memory T cells is considered a remarkably efficient process, as it consistently leads to the generation of a response that optimally counteracts the inducing insult. As a matter of fact, indeed, most human beings successfully deal with an enormous number of potentially harmful microbes every day. However, a growing set of data in the literature is suggesting a certain degree of flexibility in this system (Ahmadzadeh and Farber, 2002; O'Shea and Paul, 2010; Zielinski et al., 2011).

To understand the magnitude of the heterogeneity possibly achieved by the T cell compartment, we can analyze the concerning literature by first targeting the single unit of the immune response, i.e. the clone. Already at this basic level, plenty of possibilities for generating diversity are associated with T cells. Every single naïve T cell upon stimulation can undertake several destinies and give rise to phenotypically mixed progenies (“one cell-multiple fates”), whose averaging accounts for the reproducibility of any pathogen-specific response. Recent studies have in fact ultimately refuted the “one cell-one fate” model, according to which each naïve T cell could only give rise to a progeny with homogeneous characteristics, and the combination of the resulting populations, one for each primed naïve cells, would account for the overall phenotype of the elicited response (Buchholz et al., 2012). As already discussed in the previous section, most of the work that has lead to these conclusions focused on mouse CD8$^+$ lymphocytes, given the narrower range of fates that associate with this cell type and the availability of dedicated tracking technologies as well as transgenic models. It must be noted that the main goal of these works was to define heterogeneity of daughter cells upon priming in terms of effector vs memory development rather than opposite lineages (Buchholz et al., 2012).

Reiner and colleagues (Chang et al., 2007) showed first that CD8$^+$ T cell asymmetric division upon *Listeria monocytogenes* (*Lm*) infection is involved in the generation of both effector and memory cells from a single naïve precursor. In particular, being the interaction between DC and T cell rather prolonged in time, it appeared that, following the first division, one of the two daughter cells would be proximal to the DC, receive stronger stimulation and be instructed towards an effector fate. The distal cell, on the contrary, would acquire memory characteristics. Interestingly, the authors proposed that the mechanism of asymmetric division could be involved in the generation of different subsets (Th1, Th2, Th17) in the context of a CD4$^+$ T priming, an hypothesis that to the best of our knowledge has not been investigated in depth ever since. On the same line, seminal work from Busch and colleagues has followed the development of congenically marked unexperienced single CD8$^+$ T cells upon transfer into a naïve host and challenge with *Lm* (Stemberger et al., 2007). These experiments have shown

that T_E, T_{CM} and T_{EM} cells, as defined by the surface expression of CD127 and CD62L, could be recovered within the progeny of a single $CD8^+$ naïve T cell. Importantly, intra-clonal heterogeneity was also generated according to IFN-γ, TNF-α, IL-2 production and degranulation capacity. Shortly after, using a different approach based on genetic labeling (or barcoding) of individual naïve $CD8^+$ T cells with a retrovirally delivered DNA tag, Schumacher and colleagues independently confirmed these findings (Gerlach et al., 2010).

A recent work from Lefrançois laboratory identified environmental cues (such as inflammatory stimuli) as the main trigger for such intra-clonal differentiation, comparing different infection models, such as VSV and *Lm* (Plumlee et al., 2013). With respect to $CD4^+$ T cells, single cell transfer approach has been undertaken by Jenkins and colleagues to determine the intra-clonal diversification patterns upon infection (Tubo et al., 2013). The conclusions of this study were that different effector fates, such as Th1, T_{FH} and germinal center (gc) T_{FH} cells (identified on the basis of the expression of transcription factors, surface markers, cytokine production), could be generated from each naïve precursor. In this experimental model the relative expansion of each subset was mainly determined by the nature of the cognate antigen and the resulting bio-physical properties of the TCR-p:MHC interaction, such as aggregate time. Based on previous work from the same laboratory, as well as other works showing the importance of signaling strength in the acquisition of T_{FH} phenotype (Fazilleau et al., 2009; Pepper et al., 2010), the authors proposed that asymmetric division at the initial stages of stimulation would harbor cellular population with differential expression of IL-2 receptor. Early signaling through this receptor would induce STAT-5 activation, leading to expression of the transcription factor Blimp-1, that would simultaneously inhibit Bcl-6 expression, thus preventing T_{FH} fate determination, and induce expression of IL-12R, leading to STAT-4 signaling and T-bet activation and, therefore, Th1 differentiation. Lack of IL-2 signaling would instead favor ICOS expression and promote T_{FH} differentiation (Pepper and Jenkins, 2011). However, even if providing valuable insights, this study did not face the challenge to analyze the generation of completely alternative lineages during the course of infection, as T_{FH} are currently considered to be rather a differentiation stage of transient nature, possibly achieved within each subset (Baumjohann et al., 2013; Yamane and Paul, 2013).

All the above works provide proof of concept that it is possible for a single naïve T cell to differentiate towards distinct fates. Asymmetric division, environmental cues, strength of stimulation, number of re-encounters between T cell and DC, have been shown to impact on the yield of effector *vs* memory population, and it is therefore likely that such a variety of factors can modify also the cytokine profile of the primed T cells. Possibly the high efficiency of fate decision process represents a limiting factor to observe secondary products of the

polarization (*i.e.*, in strongly Th1-favoring priming conditions it will be more difficult to retrieve any generated Th2 cells). As a matter of fact, there are no reports available in the literature investigating the possibility that alternative lineages, such as Th1, Th2, or Th17, are generated from a single cell upon infection.

Interestingly, a recent report identifies through TCR sequencing a certain level of clonotypic sharing among different humans $CD4^+$ T cell compartments as determined by the expression of surface chemokine receptors (Wang et al., 2010a). The possibility that heterogeneity takes place in humans at an intra-clonal level is intriguing; however in the above-mentioned study the complete lack of information on the antigenic specificity as well as on the phenotype of the supposedly shared clonotypes do not allow validation for such hypothesis.

Several mechanisms can also be identified that contribute to generate diversity during priming of response to a pathogen at a multi-cellular level, meaning driving forces that will act differentially on distinct clonotypes and produce heterogeneous mixes of homogeneous progenies. First, we identify a primary role for antigen intrinsic properties (sequence, abundance, etc.) in promoting this process. As already mentioned, stimulation by different antigens held different ratios among Th1, T_{FH} and gcT_{FH} (Tubo et al., 2013), based on the aggregate time of TCR stimulation they could promote. This appears to be possible even in the contest of a single microbe. In fact, Newell and colleagues recently showed that human $CD8^+$ T cells primed by a virus (CMV, EBV, or Influenza virus) and having the same fine specificity, as assessed by pMHC-tetramer binding, display enormous differences in terms of phenotype and cytokine production, creating almost a continuum among all possible parameter combinations (Newell et al., 2012). In a consecutive paper, the authors described different phenotype for $CD8^+$ T cells specific to different Rotavirus antigens, that vary in expression of the trafficking receptors integrin β7 and αE (Newell et al., 2013). Antigen dose is also a valuable factor in the induction of Th1 *vs* Th2 fate on T cells (high dose favoring the first), that was recently shown to act upstream the cytokine signaling, and thus proposed to occupy a higher hierarchical position in fate determination. This suggests that mixed Th1-Th2 populations could be generated upon priming depending on the availability of each antigen, as some proteins are produced by the microbe at much higher rate than others (van Panhuys et al., 2014).

On a second level, it is well established that each pathogen bears a multitude of different PAMPs, that can potentially drive differential polarizing programs on DCs (Pulendran, 2005). In the case of *C. albicans*, β-glucans or α-mannans recognition by Dectin-1 and Dectin-2, respectively, activate a Th17-polarizing program (LeibundGut-Landmann et al., 2007; Saijo et al., 2010), TLR4-mediated recognition of *O*-linked mannans is believed to induce Th1

differentiation through production of type I IFNs or TNF-α (Netea et al., 2008), while TLR2 triggering has been linked to Treg/Th2 induction (Netea et al., 2008). Fungal chitin has also been proven to promote Th2-skewing conditions (Van Dyken et al., 2014), underlining the concept that a single microbe can induce multiple polarizing programs in DCs-T cells. The scenario becomes even more complex if considering that *C. albicans*, which is a dimorphic fungus, is differentially recognized by the innate immune system in its yeast vs filamentous form (Acosta-Rodriguez et al., 2007b; d'Ostiani et al., 2000).

Third, the priming milieu is supposed to play a fundamental role in dictating fate-determining signals. Pathogens can be encountered at different locations, each constituted by a peculiar microbiological and immunological environment. The route of infection is indeed well known to influence T cell phenotype, as shown for intravenous *vs* intranasal *Lm* infection, inducing Th1 and Th17 responses respectively (Pepper et al., 2010). Besides, the site of entry is well known to strongly influence the migratory capacities of primed cells (Sallusto and Lanzavecchia, 2009). Fungi such as *C. albicans* are widely spread on the surfaces and mucosal tissues of the human body (LeibundGut-Landmann et al., 2012; Underhill and Iliev, 2014); it is presumable that encounter with the microbe in the oral mucosa, in the vagina, in the gut or in the skin will generate distinct types of response and potentially target different antigens, as the protein profile of this fungus differs at each anatomical sites, based on characteristics of the environment, such as microbiota, pH, and osmolarity (Hube, 2004).

On the same line, the origin of the APC has also to be taken in account when considering T cell fate heterogeneity. Just considering DCs, that are widely recognized as main inducers of T cell responses, many reports have demonstrated such phenomenon. For instance, splenic $CD8\alpha^+$ DCs are far better inducer of Th1 responses than $CD8\alpha^-$ DCs, due to their increased production of IL-12 (Pulendran, 2005); $CD301b^+$ DCs are fundamental for priming of Th2 response *in vivo* (Gao et al., 2013; Kumamoto et al., 2013); human inflammatory DCs and monocytes have been shown to efficiently induce Th17 response *in vitro* (Acosta-Rodriguez et al., 2007a; Segura et al., 2013; Zielinski et al., 2012). The functional status of DCs adds a further variable to the scenario, as monocyte-derived DCs that efficiently prime Th1 responses shortly after maturation, preferentially induce Th2 polarization overtime when exhausted (Langenkamp et al., 2000).

The phenotypic flexibility of the T cell memory pool, both within a population and at a clonal level, is possibly a major factor in generating heterogeneity (Figure 5). This issue becomes particularly relevant in the human system, due to the life span of human beings and the resulting high level of exposure to completely different immunostimulatory conditions - such as infections, allergies, and vaccinations. Plasticity is a feature that has been reported in many

experimental systems, *in vitro* and *in vivo*, for $CD4^+$ T cells, at all stages of their life cycle; yet it is unclear the extent of such phenomenon in the course of a normal response to a pathogen or a vaccine and what its importance could be in generating protective immunity. For example, inducing T cells with a broad migratory capacity to different peripheral tissues could in principle be beneficial to ensure a higher level of protection, and would potentially represent a key factor to design effective vaccines (Lees and Farber, 2010; Yamane and Paul, 2012). Besides, plasticity represents an important adaptive mechanism for memory cells to cross-react to pathogens different from those that elicited their priming, given the high relevance of antigenic sharing between different microbes, and this phenomenon is supposed to play an important role in elderly individuals, whose naïve repertoire can be dramatically reduced (Yamane and Paul, 2012).

Cross-reactivity has recently been described as an extremely common feature of T cell, as underlined by elegant work from Garcia and colleagues (Birnbaum et al., 2014): it is therefore conceivable that most T clones will be stimulated by different antigens (in a different milieu) during the course of their lives. Indeed we found that T cell clones specific *C. albicans* can react to conidia from up to 8 different *Candida* species, each generating a peculiar inflammatory environment (Mele F. et al., unpublished).

Memory T cells can undergo recall response in different conditions upon antigen reencounter, for instance due to co-infection or different anatomical site of entry of the pathogen, and therefore modify their phenotypic characteristics. This issue is particularly relevant for those memory T cells that present a non-polarized phenotype, such as T_{CM} cells (Rivino et al., 2004). Differences in tonic signaling can induce variability among resting memory T cells, as some cells may get more frequent or stronger stimulation than others, leading to phenotypic changes during homeostasis (Lees and Farber, 2010).

Plasticity of effector and memory T cells has been investigated in a variety of experimental models, trying to interconnect all different T cell lineages. IFN-γ production could be acquired *in vivo* by Th2 cells in several mouse models, for instance upon LCMV infection (Hegazy et al., 2010; Krawczyk et al., 2007). Th1-Th2 plasticity has been shown *ex vivo* for human memory cells, upon restimulation under opposite polarizing conditions (Messi et al., 2003): most instructed T cells retain in fact an epigenetic state of accessibility in genes associated with other lineages (cytokines, chemokines, surface receptors) (Wei et al., 2009). Th17 is considered to be an extremely plastic lineage, and Th17 cells easily gained IFN-γ production in a variety of experimental models. Recent fate mapping or cell transfer studies showed that *in vivo* Th17 cells can switch their phenotype to become Th1, or just acquire production of IFN-γ (Bending et al., 2009; Hirota et al., 2011; Lee et al., 2009). The same has

been reported in *ex vivo* studies on human cells, demonstrating that IL-12 promotes conversion of Th17 to Th1 (Annunziato et al., 2007). Interestingly, spectratyping analysis performed in patients suffering from juvenile idiopathic arthritis (JIA) identified Th17-derived $CCR6^{+}CD161^{+}$ Th1 in synovial fluid, thus supporting a model of high plasticity between these compartments, especially under strong inflammatory conditions (Cosmi et al., 2011).

Th17-Th2 plasticity has been reported as well, with the identification of an IL-17/IL-4-double producing T cell population in patients with atopic asthma. The pathogenic potential of these cells was proven *in vivo* in a mouse model of asthma; *in vitro* experiments have suggested that these cells derive from Th17 cells that were restimulated in allergic environment (*i.e.* in the presence of IL-4) (Cosmi et al., 2010; Wang et al., 2010b). The T cell response induced by vaccination, characterized by highly diverse cytokine production and homing receptor display, is a striking example of heterogeneity generated in response to a single protein and under defined conditions (Livingston et al., 2013; Rivino et al., 2004). However, the impact of this variety on the long-lasting protection resulting from vaccination has never been considered, most likely due to the fact that protection relies on humoral immunity.

Overall, the analysis of the literature concerning naïve and memory Th fate decision suggests that the repertoire of T cells specific to a given microbe or vaccine could be highly heterogeneous, and comprise cells endowed with different homing properties and effector functions.

Figure 5. An integrated model for CD4⁺ T cell subset imprinting and flexibility. Shown is a schematic representation of T cell fate decision upon priming. Dashed arrows represent phenotypic switches that have been reported to occur in experimental models (from O'Shea and Paul, 2010).

4.1.9. T cell repertoire: facts and open questions

In total, approximately 10^{11}-10^{12} αβ T cells, of which at least $2.5x10^{11}$ $CD4^+$ and $1x10^{11}$ $CD8^+$, are believed to reside in a human being (Bains et al., 2009a; Clark et al., 1999). Circulating αβ T cell in humans have been estimated to be in the range of 10^{10}, and are generally considered to represent 2% of the total T cell population throughout the literature. The vast majority of T cells (estimated as $2.24x10^{11}$ in 5 donors (Clark et al., 1999)) have been shown to reside in lymphoid organs, but most peripheral organs are widely populated by these lymphocytes; recently, the remarkable amount of $2x10^{10}$ T cells were calculated to be in human skin, which represents twice as much the amount of T cells in the blood (Clark et al., 2012).

The thymic output of naïve cells decreases overtime, being maximal around 1 year after birth ($2x10^9$ CD4 naive/day), and reaching values of about $3x10^8$/day at the age of 20, 10^8/day at the age of 30, and so forth (Bains et al., 2009a; Bains et al., 2009b). Thymic output, however, is not the only factor contributing to the homeostasis of the naïve pool, as in humans this also largely depends on peripheral T cell division; competition for IL-7 and availability of self peptide-MHC providing tonic signals are crucial in this regard (Lees and Farber, 2010). On this topic the literature shows several discrepancies, mainly attributable to differences in the adopted experimental approach and mathematical model. McLean and Michie (McLean and Michie, 1995) suggested that naïve T cells divide on average once every 3.5 years and die after 20 years, which makes an average of 6 post-thymic divisions for each naïve T cell. Others have suggested higher proliferation rates: based on telomere length reduction (estimated in 50-100 bp per cycle), 7-13 division were estimated during the 20 years life of a naïve T cell (Weng et al., 1995); De Boer and colleagues considered instead only 35-70bp to be lost during each division, which would result in 10-19 cycles (De Boer and Noest, 1998). Elsewhere, the rate of cycling naïve T cells was found to approximate 0.8%, which would suggest 1 division every 125 days, for a total of 60 cycles during a T cell life span (Sachsenberg et al., 1998). However, most of these works did not take in consideration the fact, recently reported, that mean interdivision time of naive cells proportionally increases over time, becoming extremely lengthened in elderly individuals (Bains et al., 2009a). Surprisingly, according to this work, T cell proliferation in periphery would contribute more than thymic output to the maintenance of the naïve pool, as the authors calculated that up to the age of 20 only a maximum of 30% of new naïve appearing in the periphery can be derived from thymic output.

The number of T cell receptors potentially generated by V(D)J recombination has been estimated to be between 10^{16}-10^{20} (Zarnitsyna et al., 2013). Even considering as correct the

lower boundary of such estimate, the actual number of different specificities represented by the T cell compartment appears to be necessarily several orders of magnitude smaller, as T cells in the body are 10^{12} at maximum. Arstila and colleagues estimated that 10^6 different β chains would be borne by circulating T cells, of which only 1-2x10^5 carried by memory T cells (Arstila, 1999). Each β chain is believed to be paired with approximately 25 different α chains in the naïve repertoire (given the fact that pre-T cells, after rearrangement of the β locus, expand massively and generate up to 10^3 daughter cells that independently rearrange the α locus, but mostly die afterwards due to neglect), and with only one α chain in the memory compartment. Given these numbers the authors calculated that the repertoire of circulating T cells is composed of at least 2.5 x 10^7 different TCRs.

By generating a novel high throughput approach to in depth sequence αβ TCR, Robins and collaborators (Robins et al., 2009) calculated that 10^7 different Vβ chains are borne by circulating lymphocytes, thus suggesting a repertoire of approximately 2.5 x 10^8 different TCRs if the pairing of each β with 25 α chains holds true. The authors further estimated in 1.4 x 10^6 αβ TCRs the repertoire of memory cells. The level of diversity would then result 4 and 10 times higher than previously thought in the overall and in the memory T cell compartment, respectively. These values were elegantly extrapolated making use of the "unseen species problem" approach, which is commonly used in ecology to estimate the number of total species in large populations from diversity measurements in random finite samples. Interestingly, up to 400 naïve clones bearing an identical Vβ chain were found in a 5 ml blood sample; if we assume a ratio 1:1 between $CD4^+$ and $CD8^+$ lymphocytes in the blood, and an average pairing with 25 different α chains, it can be extrapolated that up to 200/25 = 8 naïve cells bear an identical TCR within the sample, which would imply a single naïve being possibly present in up to 800 copies in the average 5 liters of blood of a human being. A surprisingly similar value can be obtained by analogous extrapolation from Arstila's work: every T cell clone would have an average copy number of 1x10^3 (0.5x10^{11} circulating CD4/2 naïve vs memory/2.5x10^7 αβ TCRs). The fact that several hundreds of naïve T cells bearing an identical TCR can be present at any time in an individual has important implications for the generation of a protective memory pool and could, at least partially, contribute to the production of intra-clonal diversity.

Robins and colleagues further observed that sequences that more closely resemble the germline had higher frequency. This finding was confirmed in a detailed analysis by Venturi and colleagues (Venturi et al., 2011), pinpointing that higher frequency clonotypes are more likely to be shared between naïve and memory pools and among different individuals; the fact that elevated frequencies are associated with high efficiency of V(D)J recombination indicates

that some TCRs are created multiple times during thymic development and ultimately that convergent recombination shapes the TCR repertoire.

How many different cells can recognize an antigen? By making use of the T cell library technique, that allows frequency estimation for T cells specific to naturally processed antigens, 5-170/10^6 circulating naïve $CD4^+$ T cells were found to recognize any given antigen in human beings (Geiger et al., 2009). Memory T cells with the same specificity showed a 10-1,000-fold increase within individual donors. The frequency of specific-$CD4^+$ memory T cell was as high as 10,000/10^6 for complex antigens such as CMV (Geiger et al., 2009). The frequency of T cells specific to a single antigenic protein, such as TT, ranged from 40/10^6 in the naïve compartment to 600-900/10^6 (up to 4,000/10^6) in the memory pool, depending on the donor and the memory compartment considered (Campion et al., 2014; Geiger et al., 2009). Kwok and collaborators, on the same line, calculated the frequency of human naïve T cells specific to different epitopes to be of 2-100/10^5 cells, and increase upon immunization with the same epitope about 100 folds; importantly, following vaccination, epitope specific cells that were already over-represented in the naïve repertoire expanded the most, similarly to what observed by Moon and collaborators in the mouse system (Kwok et al., 2012; Moon et al., 2007).

In mice 5-8x10^5 β chains, each pairing with 2.4 α chains on average, have been calculated, achieving an overall repertoire of 2x10^6 diverse TCRs (Casrouge et al., 2000). Early estimates instead postulated the presence of 10 cells/clone/spleen, and, if these numbers held true, the repertoire would be of 2x10^7 clonotypes, impressively similar to that of human beings, despite the remarkable difference in size between the two organisms. In most mice, however, every single TCR is considered to be found in single copy, and for any given epitope a range of 10-1000 cells per animal have been calculated to be specific using different sampling approaches. The most recent and finest strategy, based on pMHC tetramer-enrichment and sorting, allowed detection of 20-200 epitope-specific $CD4^+$ T cells per mouse, all of which carried a different TCR (Moon et al., 2007). Shortly after, the same approach was used to calculate the number of $CD8^+$ T cells specific to different pMHC I complexes, which were found to be in the range of 80-1200 per mouse, depending on the epitope (Obar et al., 2008). As already mentioned, the frequency of specific naïve precursors was found to be predictive for the expansion of the deriving memory pool.

Having revised the basic quantitative aspects of the human TCR repertoire a question arises: how can $<10^8$ TCRs possibly provide effective immunity against an array of $>10^{15}$ distinct pMHC complexes? Recent reports that have identified cross-reactivity as one of the major – if not the main – factors to allow coverage broadening of the T cell repertoire. Wooldridge and

collaborators generated a library of almost 10^{13} decamer peptides, which were randomly sampled and tested for recognition by a single $CD8^{+}$ T cell clone (Wooldridge et al 2012). Using this approach, the authors could prove that a given TCR can respond to $>10^{6}$ different peptides, many of which trigger activation even more efficiently (up to 100-fold) than the natural ligand (Wooldridge et al., 2012). On the same line, a recent work from Garcia lab (Birnbaum et al., 2014) took advantage of a yeast display library to discover p:MHC ligands for a given human or mouse TCR. Following identification of hundreds of peptides that would bind the studied TCR, the authors generated algorithms to predict the sequence of peptides that would be recognized: production and testing of such ligands proved their predictions to be accurate (94% of predicted ligands triggered T cell activation). Importantly, the authors identified naturally occurring sources for peptides with the predicted characteristics, thus providing compelling evidence that cross-reactivity is not only possible but also likely to be commonly exploited by T cells during immune responses.

4.2. Pathogens and vaccines object of this study

4.2.1. *Candida albicans*

C. albicans is a dimorphic fungus that resides as a commensal organism in the gastrointestinal tract, on the genital mucosa, and on the skin of healthy individuals. Occasionally, overgrowth at such locations results in tissue damage and invasion; *C. albicans* is thus considered an opportunistic pathogen (Conti and Gaffen, 2010).

Infection by *C. albicans* are generally mild and concern mainly the GI tract and vaginal mucosa; vulvo-vaginal (VVC) candidiasis, in particular, affects up to 75% of reproductive-age women at least once, and becomes recurrent (RVVC, with several episodes per year) in 5-10% of them (Cassone, 2013). Importantly *C. albicans* represents a major threat for immunocompromised individuals, such as HIV^+ individuals, transplant recipients, and chemotherapy-treated patients, in which both mucosal and systemic infections are frequent. *Candida* genus ranks among the most common microbial causes of nosocomial bloodstream infections, with a mortality rate of approximately 40%. Although *C. albicans* is the most clinically relevant member of the genus, it is in fact worth to acknowledge that other species diffusely cause pathology, mainly in health care unit patients, in particular *C. glabrata*, *C. krusei*, *C. parasilosis* and *C. tropicalis* (Richardson and Rautemaa, 2009). Virulence factors of the fungus include surface adhesion and biofilm generation, invasion of the tissue through filamentous growth, secretion of hydrolytic enzymes (Richardson and Rautemaa, 2009).

C. albicans has a complex structure and expresses a broad variety of surface molecules, mainly proteins and carbohydrates. This determines a very articulated interaction with the immune system that has been widely characterized and described (LeibundGut-Landmann et al., 2012; Netea et al., 2008). The cell wall, that resides outside the cellular membrane, shaping and protecting the fungal particle, represents the most relevant compartment in terms of immune recognition and activation. It is organized in three main layers: an inner shelf composed by chitin (β-(1,4)-linked polymer of N-acetylglucosamine (GlcNAc)); a core layer of β-(1,3) and, in minor amount, β-(1,6) glucans; an outer layer of highly mannosylated GPI anchored proteins (mannoprotein) (Chaffin et al., 1998; Hernandez-Santos and Gaffen, 2012; Netea et al., 2008).

Recognition of *C. albicans* and activation of the immune system proceeds through different, sometimes synergizing, pathways, and involves all the major known classes of pattern-recognition receptors, as TOLL-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs). Acting on these receptors, different PAMPs have been shown to

induce different types of T helper response, some of which deleterious to the clearance of the fungus (Figure 6).

A variety of TLRs can recognize component of *C. albicans* cell-wall associated molecules: TLR4 recognizes *O*-linked mannan, while TLR2/1 and TLR6/2 heterodimers recognize phospholipomannans and β-glucans. TLR4 is considered the most important TLR involved in *Candida* recognition, and it was shown to induce the production of type I IFNs as well as TNF-α, eventually inducing Th1 responses (LeibundGut-Landmann et al., 2012; Netea et al., 2008). At the intracellular level, *C. albicans* DNA can be recognized by TLR9, which localizes in the phagosomal membrane. Even though most TLR-deficient mice show in general an increased susceptibility to *C. albicans* infection, the fact that patients with MyD88 deficiencies do not display augmented occurrence of fungal diseases, argues against a major role for TLR-mediated signaling in the response to the fungus. Interestingly, TLR2 pathway seems to play a detrimental role in the response to *Candida*, as it has been linked to production of anti-inflammatory cytokines and induction of regulatory (and possibly Th2) phenotype in T cells; consistently, $TLR2^{-/-}$ mice showed increased survival in a model of systemic *Candida* infection (Netea et al., 2004). This finding well exemplifies the complexity and variety of polarizing programs that such a pathogen can activate.

NLRs have also been strongly implicated in anti-*Candida* response, as NLRP3 activation and association with the adaptor protein ASC (to form the scaffold for the NLRP3 inflammasome) is crucial for production and release of the mature form of IL-1β, a key cytokine in response to fungi (Brown, 2011; Zielinski et al., 2012). Accordingly, ASC and NLRP3 knockout animals showed dramatically increased susceptibility to both systemic and mucosal *Candida* infections (Gross et al., 2009; LeibundGut-Landmann et al., 2012).

CLRs are widely considered to be the key receptors in the initiation of the response to *C. albicans*, given their ability to induce T cell polarization toward the protective Th1 and Th17 fates. Dectin-1 and Dectin-2 recognize β-glucans and α-mannans, respectively, while α-mannose is recognized by Mincle, another member of the family (Osorio and Reis e Sousa, 2011). Of note, β-glucans are generally hidden by the mannoprotein layer of the cell wall, and become exposed only on scars that are left on the mother cell after separation from the bud (bud scars) or revealed during the process of hyphal transition (Hernandez-Santos and Gaffen, 2012; Netea et al., 2008). Dectin-1 can transduce signals thanks to the presence of ITAM motifs in its intracellular portion, Dectin-2 and Mincle, instead, activate intracellular cascades by coupling with the FcγR (Osorio and Reis e Sousa, 2011). All these molecules activate the downstream kinases Syk and PKCδ and the adaptor protein CARD9, leading to activation of NFκB, NFAT, and NLRP3 inflammasome; as a result pro-inflammatory cytokines such as IL-

1β, IL-6, IL-23, and TNF-α, are released by DCs, which induce primarily Th17 and Th1 responses (LeibundGut-Landmann et al., 2007; Robinson et al., 2009; Saijo et al., 2010; Strasser et al., 2012). The crucial role that such receptors exert in the immune response to *Candida* is clearly illustrated by studies performed on mouse models, where the non-redundant downstream molecules on these pathways were knocked out (Gross et al., 2006; Gross et al., 2009; LeibundGut-Landmann et al., 2007; Strasser et al., 2012), as well as by epidemiological evidence that patients with mutations in Dectin-1 or CARD9 genes develop invasive mucocutaneous candidiasis (Ferwerda et al., 2009; Glocker et al., 2009).

Mannose receptor has also been reported to recognize α-mannans and induce IL-1β and TNF-α production in macrphages, thus leading to Th17 polarization (van de Veerdonk et al., 2009). Chitin represents a major component of the cell wall of *C. albicans* and can actively be recognized by the immune system. Recently, it was reported that chitin induces IL-10 production in APCs via activation of mannose receptor, NOD2 and TLR9 (Wagener et al., 2014), resulting in dampening of the inflammation. Interestingly, chitin has been linked to induction of allergy and asthma in a variety of models, due to the potent activation of type 2 ILCs via epithelial production of IL-25, IL-33 and TSLP when delivered to the lungs, which counteracts the development of a Th17 response (Van Dyken et al., 2014); these findings further underline the heterogeneity of polarizing signals possibly induced by a complex pathogen such as *C. albicans*.

Protective immunity to *C. albicans* is believed to be mediated mainly by T cell response. Early studies identified Th1 response as protective and Th2 as deleterious for the clearance of the pathogen in a variety of models, such as gastric, oropharingeal and systemic infection (Cenci et al., 1995; Romani, 1999; Romani et al., 1991; Tonnetti et al., 1995). However, it appeared that mice deficient for IL-12 p40 subunit or IL-12R were more susceptible than mice deficient for IFN-γ to *C. albicans* infection. This discrepancy was resolved when IL-23, a cytokine that has a common sub-unit to IL-12 (p40), as well as a common chain in their respective receptors (IL-12Rβ1), was identified. IL-23 plays in fact a fundamental role in the maintenance of Th17 cells (Hernandez-Santos and Gaffen, 2012). Most part of the literature is in agreement with the concept that Th17 cells have a protective role in candidiasis; this has been tested in a variety of models of infection (systemic, oral, cutaneous, vaginal) (Hernandez-Santos and Gaffen, 2012). The fundamental role of the IL-17 axis in *Candida* containment is strongly supported by the association between chronic infections and genetic deficiencies that impair this developmental path in humans. Genetic defects in IL-17 or IL-17 receptor genes, or mutations that impair STAT-1 or STAT-3 function (*e.g.,* STAT-1 GOF), AIRE mutations leading to APECED syndrome with production of anti-IL17 and anti-IL-22 antibodies, as well as defects in Dectin-1 or CARD9, promote the onset of CMC (Ferwerda et

al., 2009; Glocker et al., 2009; Liu et al., 2011; Milner et al., 2008; Puel et al., 2011; Puel et al., 2010; van de Veerdonk et al., 2011). Acute and systemic infections tend instead to be associated with neutropenia or granulocyte-impairing defects (Hernandez-Santos and Gaffen, 2012).

IL-17 acts through several mechanisms to fight fungal infections. Firstly, it promotes granulopoiesis, neutrophil chemotaxis (by inducing CXCL1-2-5-8 and G-CSF production in the tissue) and activation (by enhancing myeloperoxidase and NADPH oxidase activity for ROS production) (Hernandez-Santos and Gaffen, 2012; LeibundGut-Landmann et al., 2012). Neutrophils are believed to be the most effective cell type in fungal clearance, as they are endowed with an extremely potent and dedicated enzymatic machinery and can produce extracellular nets that trap and kill both yeast and hyphal forms of the fungus (Urban et al., 2006). Secondly, IL-17 together with IL-22 induces production of a plethora of antimicrobial peptides, such as S100 and β-defensin 2 (Gorr 2009, Liang 2006). A novel mechanism of action of IL-17 that has been elucidated is the induction of functional competence in NK cells in a model of systemic candidiasis; only upon exposure to IL-17 these cells become capable of producing GM-CSF and activating neutrophils, leading to control of the infection (Bar et al., 2014). It is however not clear what the source of IL-17 is in this and in other experimental models; in this regard, it has to be acknowledged that a growing amount of reports in the literature support the role of cell types other than Th17 in the generation of a robust IL-17 axis against *C. albicans*, such as type 3 ILCs or γδ T cells (Gladiator and LeibundGut-Landmann, 2013).

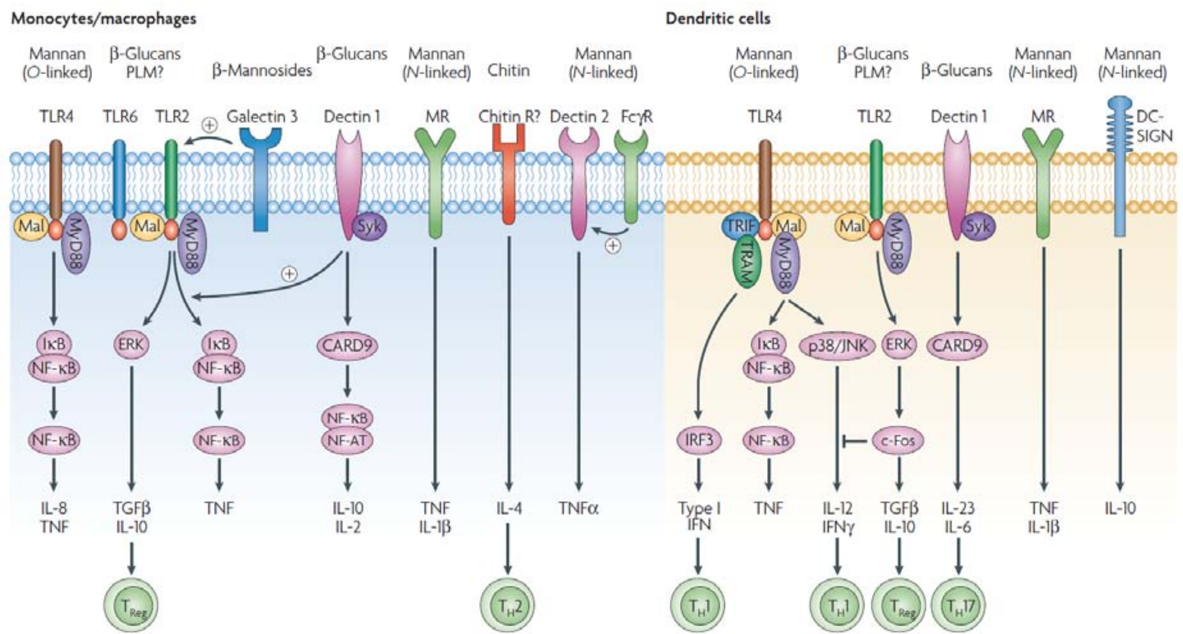


Figure 6. An integrated model for *C. albicans* innate recognition and T cell polarization. Schematic representation of the innate pathways that can be activated upon recognition of *C. albicans* associated PAMPs. T cell phenotypes reported to be generated upon selective triggering of a certain receptor are depicted below. Of note, intracellular TLRs and NLRs are not indicated in the figure, still were shown to be involved in the process of *C. albicans* recognition and immune activation (adapted from (Netea et al., 2008)).

4.2.2. *Mycobacterium tuberculosis*

The genus *Mycobacterium* comprises more than 50 species, most of which are non-pathogenic environmental bacteria closely related to the soil bacteria Streptomyces and Actinomyces (Cosma et al., 2003). Among those, *M. tuberculosis* and a few other species are the etiological agents of tuberculosis (TB), one of the most devastating infectious disease worldwide, accounting for more than 2 million deaths and 9 million new infections every year (Andersen and Kaufmann, 2014).

The pathogen enters through the lungs and is generally taken up by macrophages, where it can survive by preventing fusion of the phagosome with lysosomes. The incapacity to kill the bacterium leads to a containment strategy that implicates the formation of granulomas, compact, organized aggregate of mature macrophages. Overtime, macrophages tend to transform into epithelioid cells, and other cell types, such as neutrophils, DCs, B and T cells, NK cells, and fibroblasts, are recruited to the granuloma (Ramakrishnan, 2012). Through this mechanism most infected individuals can control the spread of the bacterium for a life-long period (a status defined as LTBI, latent TB infection), and only approximately 5-10% fail to control it and ultimately develop TB disease. Disturbances in the granuloma results in exaggerated cell death and causes necrosis, thus leading to the formation of a caseous granuloma that fails to constrain *M. tuberculosis* and, on the contrary, promotes its growth and expansion (Andersen and Kaufmann, 2014).

A vaccine, constituted by the Bacille Calmette–Guérin (BCG) strain, is available since decades and covers nowadays 80% of all infants in the world, making it the overall most widely used vaccine. Unfortunately, this protects against serious forms of disseminated TB, but fails to protect against the most prevalent form of this disease, pulmonary TB, and has therefore a minor impact on TB incidences (Andersen and Doherty, 2005). The BCG is an attenuated strain of *M. bovis*, the pathogen responsible for TB in cattle, and was obtained by continuous *in vitro* passages for 13 years: this process led to loss of virulence factors, such as ESAT6 and CFP10. The protection given by BCG vaccination is not life-long, and most studies have reported that it lasts for 10–20 years (Andersen and Kaufmann, 2014).

As already mentioned the *Mycobacterium* genus comprises many closely related species, mostly non-pathogenic soil saprophytes, referred to as environmental mycobacteria. These bacteria share several features with their pathogenic counterparts, such as a lipid-rich outer cell wall, genomes with a high GC content, and many similar gene families, including some of the major immunodominant antigens (such as the Ag85 complex); similarity of antigens induces frequent cross-reactivity, and it has been postulated that pre-exposure to common

environmental bacteria strongly reduces the efficacy of BCG vaccination and actually represents a cause for its failure (Andersen and Doherty, 2005).

Immunity against *M. tuberculosis* is cell-mediated, with T lymphocytes serving as orchestrators and mononuclear phagocytes as effectors of both protection and pathology. Due to the intracellular lifestyle of *M. tuberculosis*, immunity relies on a successful T cell response against a repertoire of antigenic targets. Th1-type response is believed to be fundamental, as production of IFN-γ and TNF-α promotes recruitment and activation of anti-mycobacterial activities in macrophages; consistent with this notion, individuals with genetic defects in the IFN-γ receptor show an increased susceptibility to mycobacteria (Lindestam Arlehamn et al., 2013; Lindestam Arlehamn and Sette, 2014). Recently, the response to *M. tuberculosis* was shown to be contained in a subset of Th1 cells expressing CCR6 and CXCR3 and producing high quantity of IFN-γ and modest amounts of IL-17 (Lindestam Arlehamn et al., 2013). IL-17, TNF-α, GM-CSF, and IL-1β, together with IFN-γ, have also been found to be important for protection. Instead, several studies indicate that lower IFN-γ/IL-4 or IFN-γ/IL-5 ratios are found in active TB patients compared to LTBI individuals. T cells other than CD4, namely CD8, γδ and CD1-restricted T cells, also play an important role in pathogen containment (Andersen and Kaufmann, 2014; Khader and Cooper, 2008; Lindestam Arlehamn et al., 2013; Lindestam Arlehamn and Sette, 2014).

4.2.3. Tetanus toxoid vaccine

Clostridium tetani is a gram-positive anaerobic bacterium whose spores are widely distributed in soil and in the intestines and feces of many animals, such as of horses, sheep, dogs, cats, and chickens. It usually enters the body through a wound, and in the presence of anaerobic conditions, the spores germinate (Hatheway, 1990). Toxins, tetanolysin and tetanospasmin, are produced and diffused systemically through tissue and lymph. Tetanospasmin is constituted by two proteic subunits linked by a disulfide bond of 100 and 50 KDa, respectively, and is one of the most potent known toxins, with an estimated minimum human lethal dose of 2.5 ng per kg of body weight. It acts on the central nervous system, by blocking the release of inhibitory mediators at the synapse of the spinal cord motoneurons, leading to unopposed muscle contraction and spasm (Hatheway, 1990) (Centers for Disease Control and Prevention, 2014). The World Health Organization (WHO) reported 15,516 worldwide cases of tetanus in 2005, and an estimated 290,000 deaths between 2000 and 2003; most of these cases occurred in newborn infants in developing countries. Nonetheless, vaccination against tetanus represents one of the major successes in the history of active immunization (World Health Organization, 2014).

The vaccine is formulated as tetanus toxoid (TT, the formaldehyde-inactivated preparation of neurotoxin tetanospasmin) adsorbed on alum salts and delivered intramuscularly. In western countries, 3-4 doses of the TT vaccine are recommended at infancy (2/4/6/18 months, generally in combination with the diphtheria/pertussis vaccine), followed by a TT-containing booster at school-entry age (4-7 years), and one in adolescence (12-15 years). A recall shot every approximately 10 years is afterwards required to ensure protection; a complete TT vaccine shot series has a clinical efficacy of virtually 100%. No cases of tetanus were reported in 2013, neither in Switzerland nor in the US (Centers for Disease Control and Prevention, 2014; World Health Organization, 2014).

Several mechanisms have been proposed that account for alum potency as an adjuvant, a topic that has raised many controversies in the literature. A recent work has proven that the most important mechanism of action is the recruitment of inflammatory monocytes to the site of injection (*i.e.* the muscle) following a gradient of CCL2 released by cells of the tissue. Upon antigen uptake, monocytes migrate to the lymph nodes while differentiating into DCs, and efficiently prime T cell response (Kool et al., 2008a; Kool et al., 2008b). Furthermore, enhanced uptake of the particulate antigen and targeting of the antigen to more acidic organelles are also supposed to enhance antigen presentation. It has also been shown that intramuscolar injection of antigen in alum salts results in formation of a depot from which the antigen is slowly released, increasing immunogenicity (Kool et al., 2012).

On a molecular level, the action of alum seems to be dependent mainly on the NLRP3 inflammasome, that is activated both by direct sensing of alum itself, and indirectly, through release of DAMPs such as ATP and uric acid by the necrotic tissue (uptake of salt crystals damages lysosomes and induce cell death) (Kool et al., 2012). The induction of a preferential Th2 response has been linked to production of several factors by APCs as well as to recruitment of eosinophils, an early source of IL-4, to the site of injection (Jordan et al., 2004; Kool et al., 2012).

Vaccination-induced protection relies on antibody-mediated neutralization of the toxin. In fact, protection is estimated using a threshold titer of more than 0.01 IU of anti-tetanus antibodies per milliliter of blood; anti-tetanus antibodies have an estimated average half-life of 11 years (Amanna et al., 2007). Notably, beside promoting humoral immunity, the vaccine induces a very strong T cell response, and TT-specific memory T cells can be isolated from virtually all immune donors. TT has been used as a model antigen in many *in vitro* and *in vivo* studies. Several reports indicate that the phenotype of such T cells greatly differs in terms of chemokine receptor expression and cytokine production, even though these features have never been studied in depth. Most studies detected both Th1 and Th2 responses in healthy donors upon *ex vivo* restimulation with the toxoid (Allen et al., 2006; van Riet et al., 2008). In other studies IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-13, IL-9, but also IL-17 were detected in response to boost immunization (Lenarczyk et al., 2000; Livingston et al., 2013). It has been proposed, though with discrepant conclusions, that the ratio between Th1/Th2/Th17 may change over time following primary or recall vaccination (Livingston et al., 2013; Rowe et al., 2000); from these studies it can be extrapolated that Th2 would be favored at first, and Th1 prevail at later time points. TT-specific cells were found both in T_{EM} and T_{CM} compartment, in populations enriched for differential expression of the chemokine receptors CXCR3, CXCR5 and CCR4 (Rivino et al., 2004), implicating induction of extremely different migratory capacities in T cells primed by the TT-vaccine. Interestingly, also the hepatitis B virus (HBV) vaccine and the staphylococcal enterotoxin B (SEB) vaccine are reported to generate a broad cytokine profile in T cells (De Rosa et al., 2004).

All the above mentioned reports indicate that T cell response to tetanus toxoid, and possibly to any other vaccine antigens, is extremely heterogeneous; this issue needs to be further addressed as in most cases what determines the efficacy of some vaccines with respect to others remains unclear, and the shape of the induced T cell response could be of great relevance.

5. Aims of the Thesis

In response to pathogens or vaccines, $CD4^+$ T cells proliferate and differentiate toward distinct fates (namely Th1, Th2, Th17), each endowed with a peculiar set of homing receptors and specific cytokine production profile. Th1, Th2 and Th17 are considered alternative fates of differentiating T cells, which are instructed by signals from the TCR, costimulatory molecules and innate cytokines. However, T cells can show remarkable flexibility and have been proven to undergo phenotypic switch or divergent differentiation in several experimental systems. Understanding the generation of diversity in the human T cell memory compartment would have enormous implications both on a biological and on a clinical level.

Functional diversity of $CD4^+$ T cells has evolved as a mean to provide the immune system with the capacity to mount appropriate types of defense against different classes of pathogens. Th1 cells producing IFN-γ activate macrophage effector function and protect against viruses and intracellular bacteria, while Th17 cells producing IL-17 and IL-22 activate neutrophils and protect against fungi and extracellular bacteria and Th2 cells producing IL-4, IL-5 and IL-13 activate eosinophils and other innate cells to protect against parasites and venoms. Classical observations in mice and humans with genetic defects in Th1, Th2 and Th17 polarization demonstrate the importance of generating the right type of response against each type of pathogen.

In this work, we set out to finely dissect the human T cell memory response to microbes and vaccines (*C. albicans*, *M. tuberculosis* and TT), taking advantage of a variety of recently developed cellular and molecular high throughput screening methods. Our work was in particular focused on the following questions:

- Do T cells specific to the same pathogen/vaccine display homogeneous properties or rather belong to distinct subsets?
- Is the antigenic fingerprint of T cell subsets the same or does it differ for each distinct subset? Can we retrieve antigens that induce specific types of T helper response?
- Can heterogeneity of the T cell response be involved in pathology?

The main result obtained in the course of these studies demonstrate that human memory $CD4^+$ T cells specific for pathogens and vaccines show an unexpected degree of heterogeneity in terms of polarization, even at the level of single T cell clones.

6. Results

6.1. Functional heterogeneity of human memory T cell clones primed by pathogens and vaccines

6.1.1. Functional heterogeneity and clonal composition of *C. albicans*-specific $CD4^+$ T cells

To analyze the heterogeneity of human memory $CD4^+$ T cells specific for *C. albicans* (CA), we isolated from peripheral blood of healthy donors T helper cell subsets based on chemokine receptor expression. Th1 cells, isolated as $CXCR3^+CCR4^-CCR6^-$, produced IFN-γ and expressed T-bet mRNA, while Th2 cells, isolated as $CCR4^+CXCR3^-CCR6^-$, produced IL-4 and expressed the highest level of GATA3 mRNA and Th17 cells, isolated as $CCR6^+CCR4^+CXCR3^-$, produced IL-17 and expressed ROR-γt (Figure 7). In addition we isolated a $CCR6^+CXCR3^+CCR4^-$ subset, that we previously defined as Th1*, that produced IFN-γ and low levels of IL-17 and expressed both T-bet and ROR-γt mRNA (Figure 7).

Cells from the four subsets were labeled with CFSE and stimulated with *C. albicans* in the presence of autologous monocytes. In the 20 donors analyzed, $CFSE^{lo}$ cells were detected at higher levels in the Th17 and Th1* subsets, but were also present in Th1 and Th2 subsets (Figure 8A,B). The pattern of cytokine produced in *C. albicans*-stimulated cultures was distinct for each subset. Thus, Th17 cells produced IL-17A, IL-22 and IFN-γ, consistent with previous reports (Acosta-Rodriguez et al., 2007b; Zielinski et al., 2012), Th1* cells produced high amounts of IFN-γ and low amounts of IL-17A, Th2 produced IL-4, IL-5 and IL-13, and classical Th1 cells produced mainly IFN-γ (Figure 8C). The above results indicate that human memory CD4 T cells primed by *C. albicans* are functionally heterogeneous in terms of migratory capacities and cytokine production.

To assess the clonotype composition of *C. albicans*-specific memory T cells within each Th subset, we isolated $CFSE^{lo}$ cells from *C. albicans*-stimulated cultures and performed TCRVB CDR3 sequence analysis on genomic DNA. In the 5 donors analyzed, *C. albicans*-responsive T cells were highly polyclonal, with comparable numbers of clonotypes in the 4 subsets (mean values of 976, 595, 830 and 696 clonotypes in Th1, Th2, Th1* and Th17, respectively, Figure 8D). Furthermore, the number of clonotypes in each subset varied from donor to donor

by a factor of approximately 5 fold. For instance, in the Th17 subset there were 290 clonotypes in donor CA-01 and 1,518 clonotypes in donor CA-05 (Table 1). Interestingly, the frequency of individual clonotypes varied widely, ranging from 20% to 0.01%, with similar distribution in the 4 subsets (see example in Figure 8E for donor CA-01). In all donors, 3-4.5% of the clonotypes were present at frequencies >1% and accounted for 34-43% of all reads (Figure 8F,G).

To address whether the most expanded clonotypes found after *in vitro* stimulation correspond to clonotypes that were expanded *in vivo*, we sequenced, in the same donor, total memory CD4 T cells directly *ex vivo* (Figure 8H). From $1.5x10^6$ total memory T cells stimulated *in vitro* with *C. albicans* we isolated from CFSE^{lo} cells 5,075 unique clonotypes, which showed the typical broad frequency distribution. Interestingly, a large proportion of clonotypes and reads (46% and 84%, respectively) expanded *in vitro* by stimulation with *C. albicans* could be detected in total memory CD4 T cells *ex vivo*. In addition, there was a highly significant correlation between *in vitro* and *ex vivo* frequencies. These results indicate that *C. albicans*-specific T cells undergo different degrees of expansion *in vivo,* which is reflected by their frequency found upon *in vitro* stimulation.

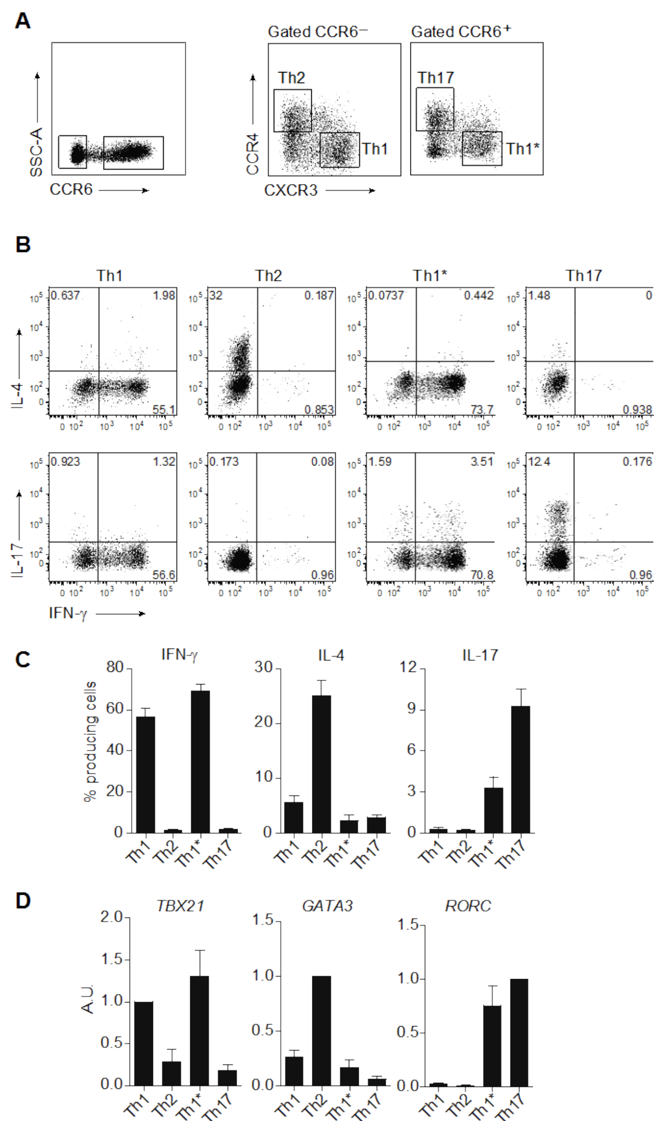


Figure 7. Sorting strategy to identify functional memory T cell subsets in human peripheral blood. (A) Expression of chemokine receptors identifies four subsets of human CD4$^+$ memory T cells. Cells expressing CD45RA, CD25, CD14, CD19 were excluded from the gate. (B, C) Cytokine production by the four subsets after stimulation with PMA and Ionomycin (PI) for 4 hours. Dot plots from a representative donor (B). Mean ± s.e.m. of 4 donors (C). (D) *TBX21*, *GATA3*, and *RORC* mRNA levels in the four subset immediately after sorting. Data are the mean ± s.e.m. of 4 donors.

Figure 1

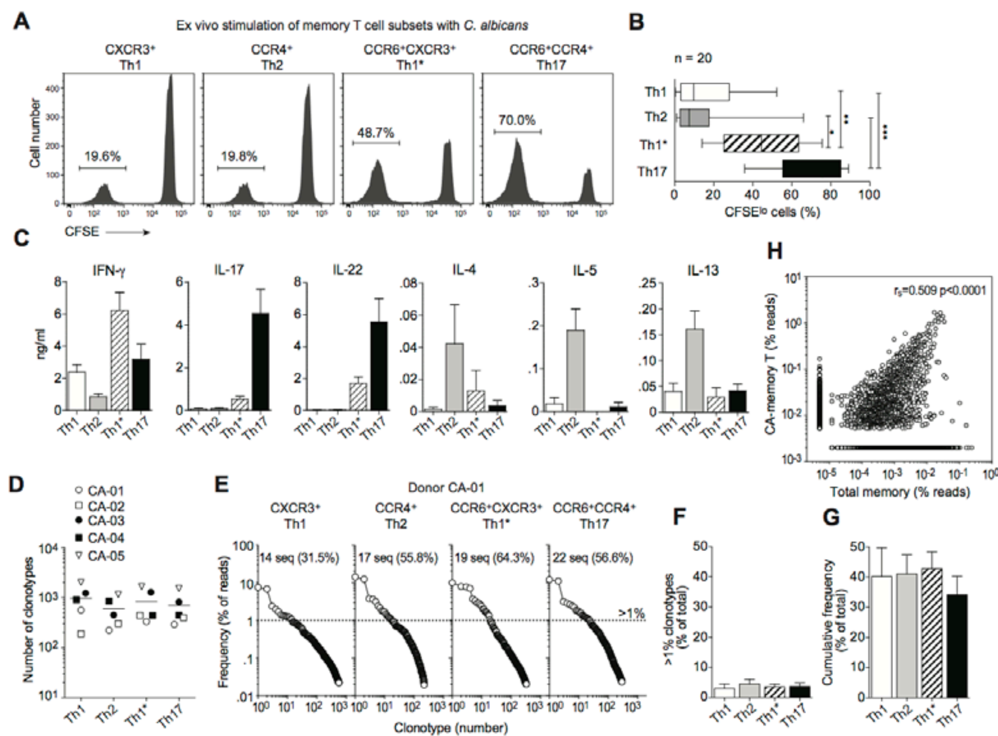


Figure 8. Functional heterogeneity and clonal composition of *C. albicans*-specific CD4 T cells. (A,B) Human memory CD4 T cell subsets were isolated according to the expression of chemokine receptors, labeled with CFSE and stimulated with heat-killed *C. albicans* in the presence of autologous monocytes. Shown are CFSE profiles on day 6 and percentage of $CFSE^{lo}$ proliferating cells in a representative donor (A) and median values with 25 and 75 percentiles in 20 donors with whiskers representing the highest and lowest values in each subset (B). ***$P<0.0001$, ** $P<0.001$, * $P<0.05$ as determined by non parametric Friedman test. (C) Cytokine production measured on day 6 in the supernatants of the *C. albicans*-stimulated cultures. Values represent mean ± s.e.m. (D) Number of TCRVB unique clonotypes detected by sequencing of genomic DNA from *C. albicans*-specific T cells isolated form the 4 memory subsets. Each symbol represents a different donor. Lines represent mean values. (E) Frequency distribution of clonotypes in the four *C. albicans*-specific T cell subsets from a representative donor. Dotted line represents the 1% frequency threshold. Indicated are the number of clonotypes with frequencies >1% and their cumulative frequencies expressed as % of reads (values in parentheses). (F,G) Percent of clonotypes with frequencies >1% (F) and their cumulative frequencies expressed as % of reads (G) in 5 donors (mean ± s.e.m.). (H) Comparison of clonotype frequency distribution of *C. albicans*-specific memory $CD4^+$ T cells (y-axis) and total memory $CD4^+$ T cells sequenced directly *ex vivo* after sorting (x-axis). Shown is the Spearman correlation and the significance analyzed by paired *t* test.

Table 1

Donor	CFSE^{lo} cells (%)				Number of clonotypes					Number of clonotypes with frequency >1%				Cumulative frequency (%) of clonotypes with frequency >1%			
	Th1	Th2	Th1*	Th7	Total	Th1	Th2	Th1*	Th17	Th1	Th2	Th1*	Th17	Th1	Th2	Th1*	Th17
CA-01	10.3	2.8	27.2	55.8	1096	560	223	330	290	14	17	19	22	31.5	55.8	64.3	56.6
CA-02	2.7	2.4	13.8	37.3	1072	188	301	438	395	17	24	18	19	75.9	46.1	41.2	35.7
CA-03	43.5	50.9	68.8	85.8	3177	1222	450	1284	817	12	21	20	13	26.7	43.2	32.7	21.8
CA-04	53.2	83.5	75.6	89.1	2056	903	851	439	445	19	10	23	15	43.4	17.4	37.5	31.4
CA-05	59.7	66.0	74.0	80.6	5188	2005	1150	1661	1518	7	14	18	16	23.6	43.1	38.6	25.5
TT-01	68.2	90.6	38.4	34.8	1386	483	648	858	620	18	12	17	11	38.3	27.3	41.3	23.5
TT-02	84.2	71.3	85.8	78.1	3259	952	1146	999	1140	21	14	16	16	51.1	49.6	45.0	45.2

Table 1. Clonotypic composition of the analyzed cell populations. Indicated are, for each donor, the percentage of CFSE^{lo} cells obtained from the different subsets in response to the antigen of interest, and the parameters defining their clonotypic composition.

6.1.2. Unique and shared clonotypes among *C. albicans*-specific memory T cell subsets

The above results indicate that memory T cells specific for *C. albicans* were present in all subsets analyzed and in each subset there were many *C. albicans*-specific clones that were distributed over a wide range of frequencies. It was therefore important to establish whether this heterogeneous response was due to different clones each polarized to a distinct fate or to clones that had acquired multiple fates through a process of intraclonal diversification. We therefore determined whether and to which extent individual clonotypes could be detected in different subsets. In donor CA-01, out of the 395 clonotypes found in the Th17 subset, 143 were also found in Th1*, while 53 were found in Th2 and only 8 in Th1 (Figure 9A). Interestingly, the most expanded Th17 clonotypes were all found to be present at high frequency also in the Th1* compartment. Among the 143 clonotypes shared between Th17 and Th1*, 27 were also found in Th2, and 3 in Th2 and Th1 subsets (Figure 9B). It should be noted that in all cases, several of the most frequent clonotypes were not found in other subsets, thus excluding the possibility of a cell contamination and suggesting the presence of both promiscuous as well as uniquely polarized clonotypes. Similar patterns of clonotype sharing were found in additional 4 donors analyzed (Figure 10). From the compiled analysis of the 5 donors, the highest sharing of clonotypes and reads was found between Th17 and Th1* and the lowest between Th17 and Th1 (Figure 9C). Surprisingly, there was also a substantial sharing of clonotypes between Th17 and Th2 and between Th1* and Th2.

With regard to the *C. albicans*-specific response, a sharing of clonotypes between Th17 and Th1* could have been predicted on the basis of the common expression of T-bet and RORγt as well as of reported plasticity of Th17 cells. To investigate whether clonotype sharing between Th1* and Th17 is a general property in these two memory subsets, we analyzed the TCR repertoire of T cells specific for *Mycobacterium tuberculosis*. In the two donors analyzed, *M. tuberculosis*-reactive cells could be readily identified in Th1* and Th17 subsets, as well as in a small subset of double positive (DP, $CXCR3^+CCR4^+$) cells, which produce IFN-γ and low amounts of IL-17 (Figure 11A,B). The analysis of *M. tuberculosis*-reactive, $CFSE^{lo}$ CD4 T cells showed a comparable number of clonotypes in the 3 subsets, with a broad range of frequency distributions. Interestingly, in contrast to what observed for *C. albicans*, in the case of *M. tuberculosis*-reactive cells only very few clonotypes were shared between Th17 and Th1*, while several, and in particular the most expanded clonotypes, were shared between the Th1* and DP subsets (Figure 11C,D).

Taken together the above results indicate that there is a substantial sharing of clonotypes among different subsets, but that the pattern of sharing is dependent on the nature of the

antigen. In particular, the large sharing of clonotypes between Th17 and Th1* observed in *C. albicans*-specific memory T cells contrasts with the distinct repertoires of Th17 and Th1* memory cells reactive to *M. tuberculosis*.

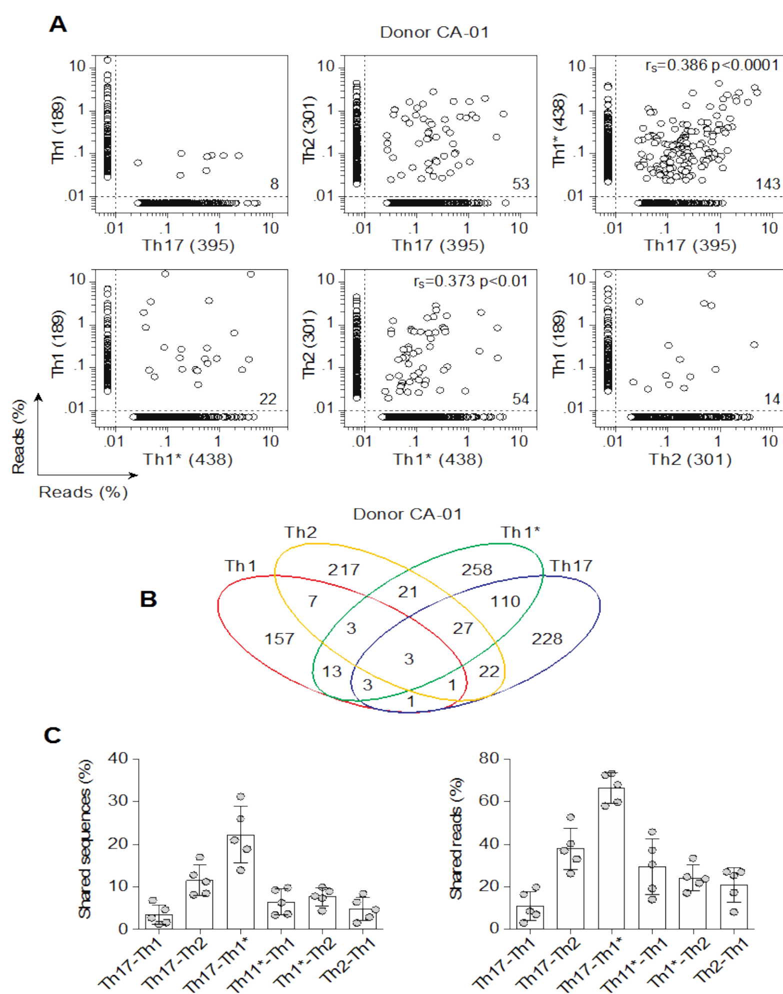


Figure 9. Unique and shared clonotypes among *C. albicans*-specific memory T cell subsets. (A) Comparison of clonotype frequency distribution in samples of T cells isolated from *C. albicans*-stimulated Th1, Th2, Th1* or Th17 subsets from donor CA-01. Frequencies are shown as % of total reads. The total number of clonotypes in each sample is indicated in parenthesis in the x- and y-axis. Values in the lower right corner represent the number of shared clonotypes between the two samples. The Spearman correlation and paired *t* test value are also shown when significant. (B) Venn diagrams showing the number of unique and shared clonotypes in the 4 subsets of donor CA-01. (C) Bar histograms showing for 5 donors the percentage of clonotypes (left) and the percentage of reads (right) that are shared by the indicated subsets.

Supplementary Figure 1

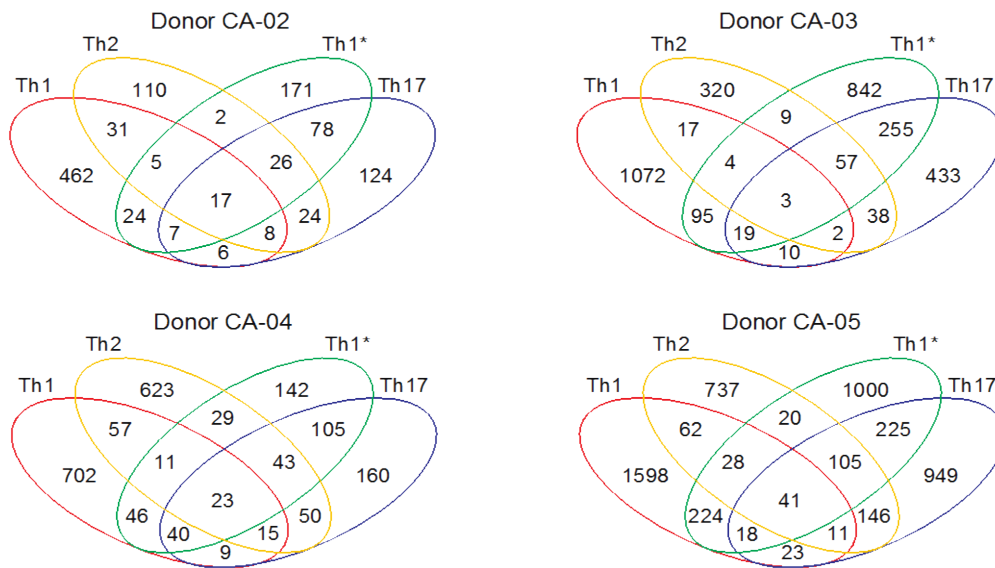


Figure 10. Unique and shared clonotypes among *C. albicans*-specific memory T cell subsets. Venn diagrams showing the number of unique and shared clonotypes in the 4 subsets of 4 additional donors in experiments performed as described for donor CA-01 in Figure 9.

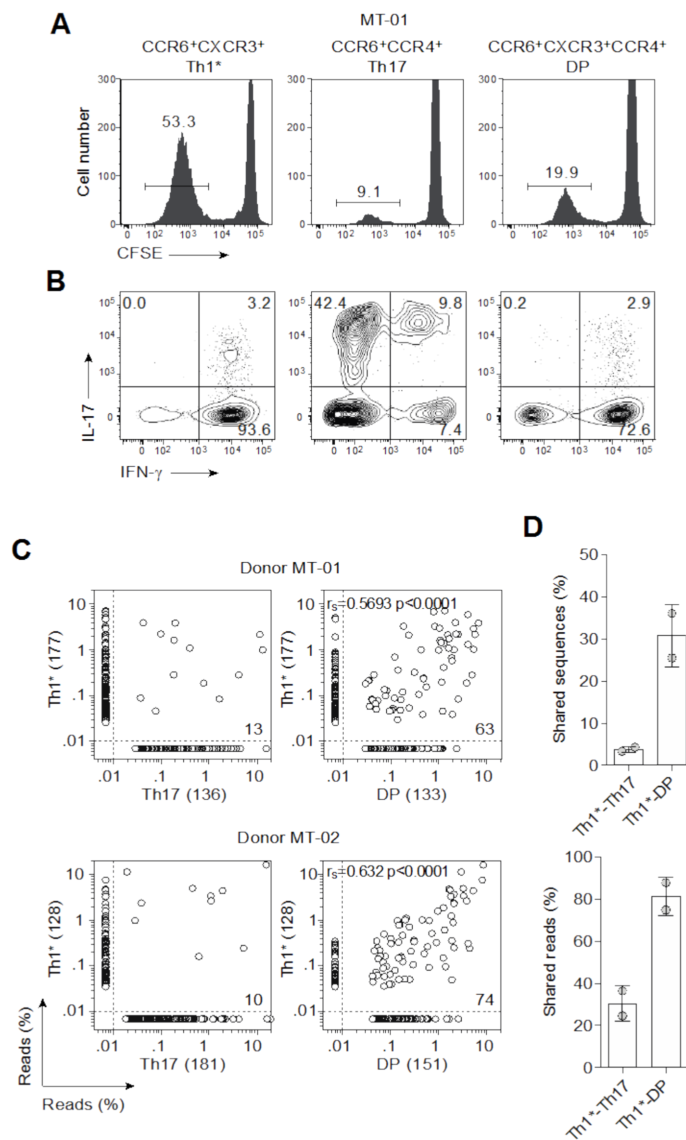


Figure 11. Clonotype analysis of *M. tuberculosis*-reactive memory T cell subsets. (A) Representative CFSE profiles for Th1*, Th17 and CXCR3$^+$CCR4$^+$ double-positive (DP) memory T cell subsets from a healthy donor stimulated with *M. tuberculosis*; analysis was performed at day 6 (B) Cytokine production profile of the CFSE^{lo} populations shown in (A) assessed by intracellular staining (D) Comparison of clonotype frequency distribution in samples of T cells isolated from *M. tuerculosis*-stimulated Th1*, Th17, DP subsets. Frequencies are shown as % of total reads. The total number of clonotypes in each sample is indicated in parenthesis in the x- and y-axis. Shown are the number of clonotypes shared between the two samples and the Spearman correlation and paired *t* test value. (E) Bar histograms show for 2 donors the percentage of clonotypes (up) and the percentage of reads (bottom) that are shared by the indicated subsets.

6.1.3. Extensive clonotype sharing among TT-specific T cell subsets

The heterogeneity of memory T cells observed for *C. albicans* and *M. tuberculosis* could be attributed to the multiplicity of the antigens and microbial products present in the pathogens. To address whether a single protein antigen would induce a more polarized response, we studied memory T cells induced by vaccination with Tetanus Toxoid (TT), which is administered with alum as adjuvant. CFSE-labeled Th1, Th2, Th1* and Th17 cells were stimulated with TT in the presence of autologous monocytes. Surprisingly, in the four donors analyzed proliferation to TT was high and comparable in all four subsets (Figure 12A,B). The pattern of cytokine produced in TT-stimulated cultures was distinct for each subset. The Th1 subset produced IFN-γ, as expected, but also low levels of IL-4, IL-5 and IL-13, while the Th2 subset produced high levels of IL-4, IL-5 and IL-13, as well as some IFN-γ. The Th1* and Th17 produced IL-17 and IL-22. In addition, Th1* also produced high levels of IFN-γ (Figure 12C).

Interestingly, even in the case of a single antigen, the response was highly polyclonal in each subset with a total of 1,386 and 3,259 individual clonotypes in the two donors analyzed (Table 1). Furthermore, within each subset the clonal frequency was widely distributed, with a few dominant clonotypes accounting for a large fraction of total reads (Figure 13). Surprisingly, there was a high level of clonotype sharing among the 4 subsets, in particular between Th2 and Th1 (24.4% of clonotypes and 70.4% of reads) and between Th2 and Th17 (17.1% of clonotypes and 62% of reads). Furthermore 83 out of 1,386 clonotypes (in donor TT-01) and 65 out of 3,259 clonotypes (in donor TT-02) were present in all subsets (Figure 12D and Figure 13). These findings indicate that vaccination with a single protein can prime a large number of specific T cell clones that acquire different phenotypes and functions.

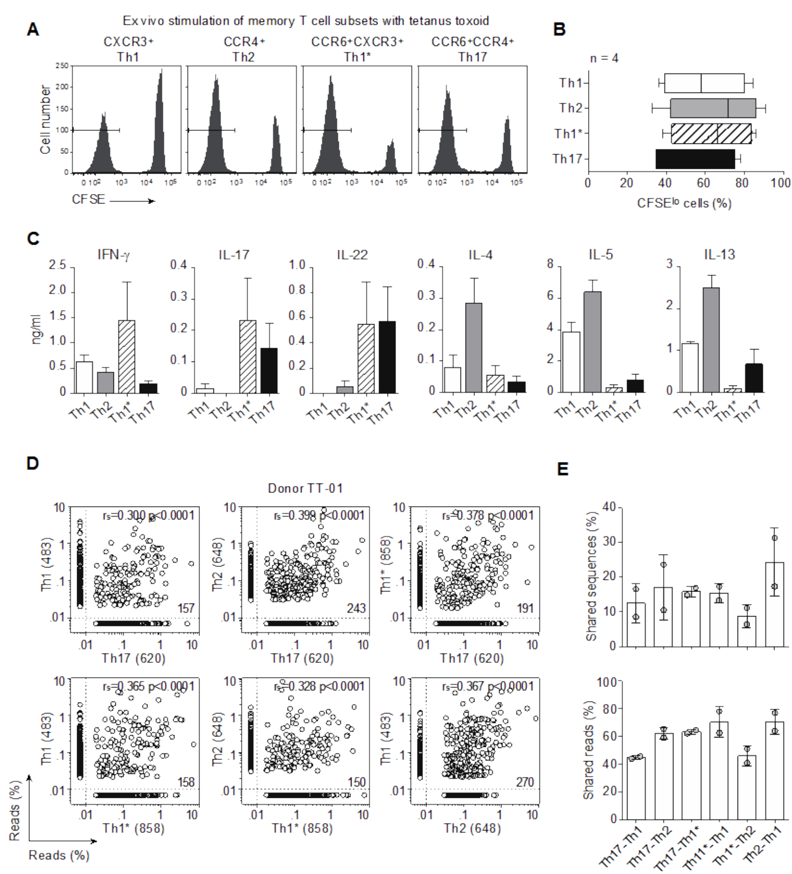


Figure 12. Extensive clonotype sharing among TT-specific CD4 T cell subsets. (A,B) CFSE profiles and percentage of CFSE^{lo} proliferating cells in the indicated 4 memory T cell subsets stimulated with TT in a representative donor (A) and median values with 25 and 75 percentiles in 4 donors, with whiskers representing the highest and lowest values in each subset (B). (C) Cytokine production measured in the day 6 supernatants of the TT-stimulated cultures. Values represent mean ± s.e.m. (D) Comparison of clonotype frequency distribution in samples of T cells isolated from TT-stimulated Th1, Th2, Th1* or Th17 subsets from donor TT-01. Frequencies are shown as % of total reads. The total number of clonotypes in each sample is indicated in parenthesis in the x- and y-axis. Shown are also the number of clonotypes shared between the two samples and the Spearman correlation and paired *t* test value. (E) Bar histograms showing for 2 donors the percentage of clonotypes (upper panel) and the percentage of reads (lower panel) that are shared by the indicated subsets.

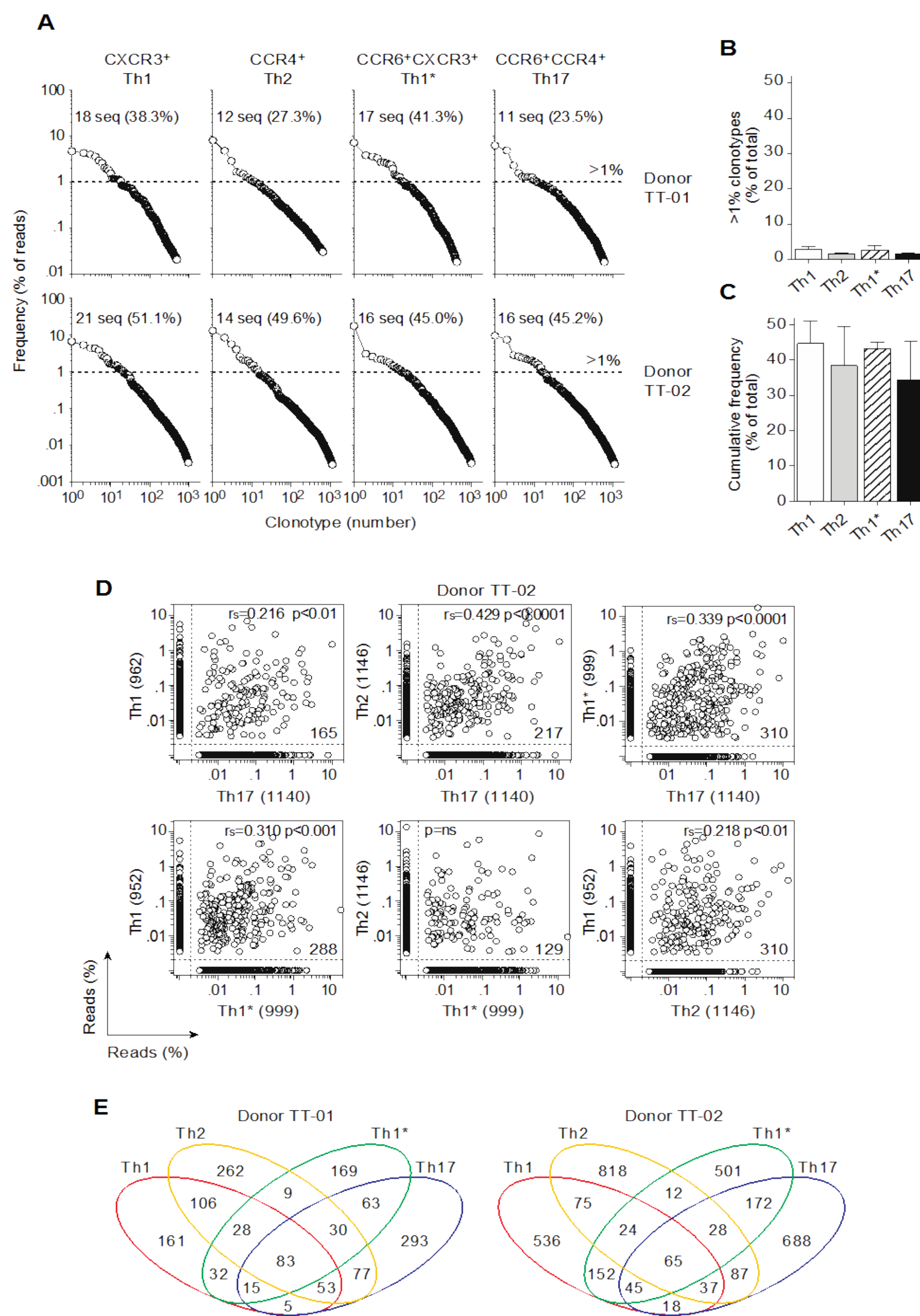


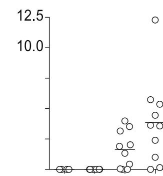
Figure 13. Extensive clonotype sharing among TT-specific CD4 T cell subsets. (A) Frequency distribution of clonotypes in the 4 TT-specific T cell subsets from donor TT-01 and TT-02. Shown are the 1% frequency threshold, the number of clonotypes with frequencies >1% and their cumulative frequencies (% of reads) in parentheses. (B,C) Percent of clonotypes with frequencies >1% (B) and their cumulative frequencies (% of reads) (C) in the 2 donors (mean ± s.e.m.). (D) Comparison of clonotype frequency distribution in samples of T cells isolated from TT-stimulated Th1, Th2, Th1* or Th17 subsets from donor TT-02. Frequencies are shown as % of total reads. The total number of clonotypes in each sample is indicated in parenthesis in the x- and y-axis. Shown are also the number of clonotypes shared between the two samples and the Spearman correlation and paired *t* test value. (E) Venn diagrams showing the number of unique and shared clonotypes in the 4 subsets in the two donors.

6.1.4. Isolation of sister clones from different memory subsets

To provide further evidence that memory T cells expressing the same αβ TCR have different phenotypes and functional properties we isolated a large number of T cell clones from samples of *C. albicans*-specific Th1, Th2, Th1* and Th17 cells that were frozen as a backup. From donor CA-01 we isolated a total of 242 T cell clones, of which 153 were unique since they expressed the same TCRVB sequences (Figure 14A). As expected, several clones with identical TCRVB sequences were found in two subsets (clonotypes 3, 4, 7, 8, 11, 12 13, 17, 18, 64, 75, 77, 82, 206) and in one case in three subsets (clonotype 32). The most frequent sharing was between Th17 and Th1* and between Th17 and Th2. Sequencing of the TCRVA chain (done in collaboration with Dr. Ton Schumacher, NKI, The Netherlands) confirmed that the cells isolated from different subsets carried the same αβ TCR (Figure 14B).

The sister clones isolated form different subsets were functionally characterized for cytokine production and expression of transcription factors and CCR6. With the exception of IFN-γ, which was produced by virtually all clones as a consequence of the prolonged *in vitro* culture, cytokine production was consistent with the origin of the cells: IL-4 was produced at high levels by clones isolated from the Th2 subset, while IL-17 and IL-22 were produced by clone derived from the Th17 and Th1* subsets (Figure 14C). In addition, CCR6 expression and RORγt mRNA were detected only in clones derived from Th17 or Th1* (Figure 14D-E). As an example, two sister clones of clonotype 64, isolated from the Th17 or Th2 subset, showed the characteristic polarized cytokine production and expression of transcription factor (Figure 14F). Another consistent example was provided by two sister clones of clonotype 11 that were isolated from the Th17 or Th1* subset (Figure 14G). We conclude that T cell with the same TCR can be found in different subsets and that their phenotype is stably maintained.

A



F

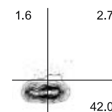
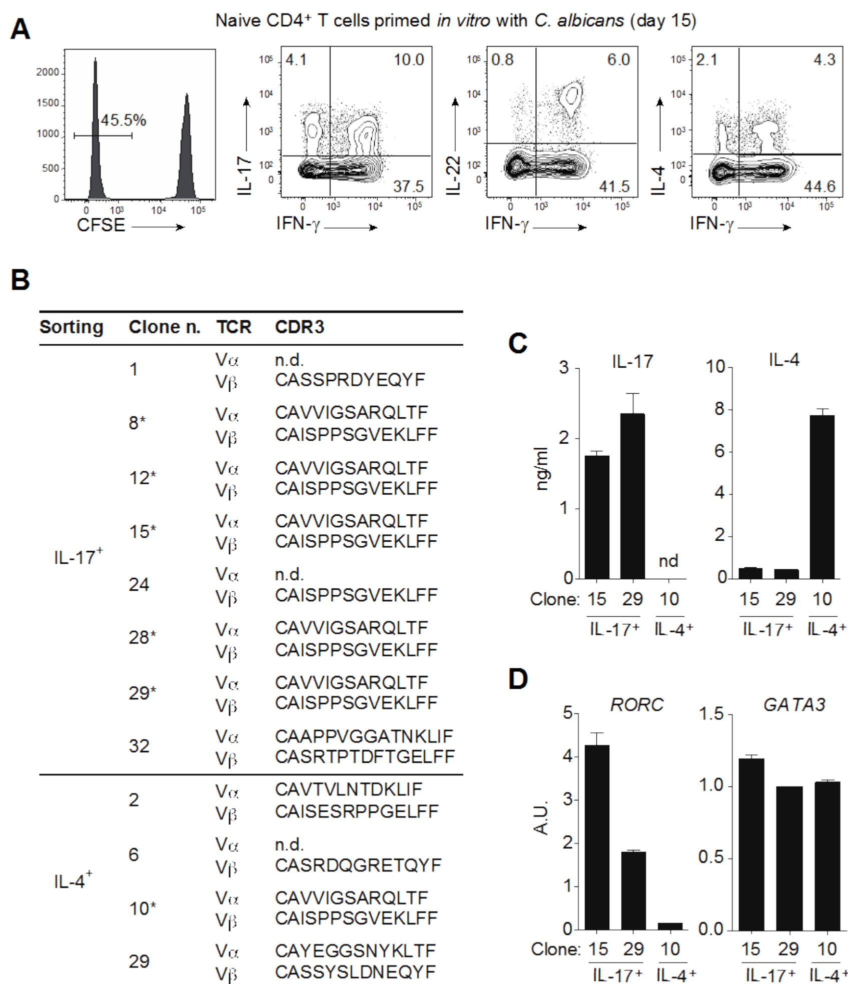


Figure 14. Isolation of sister clones from different memory subsets. T cell clones were isolated from cryopreserved samples of *C. albicans*-stimulated CFSE^{lo} Th1, Th2, Th1* and Th17 cells from donor CA-01 and TCR αβ sequences were obtained by Sanger sequencing on amplified cDNA. For each subset the number of individual clonotypes is indicated. (A) The circles indicate the total number of clones (center), the number of clones unique to that subset (white section) and the number of clones whose TCR was found also in T cell clones isolated from another subset (color coded sections). (B) List of the clonotypes that were found in two or more subsets. (C-E) Characterization of sister clones specific for *C. albicans* isolated from different memory subsets and expressing one of the shared TCR clonotypes. Shown are cytokine production (C), and expression of transcription factors (D) and CCR6 (E). (F,G) Characterization of two pairs of sister clones isolated from Th1* and Th17 subsets (F) and from Th2 and Th17 subsets (G).

6.1.5. Generation of multiple T cell fates by *in vitro* priming of naïve T cells

The above data show that the CD4 T cell response to pathogens and vaccines can be highly heterogeneous, both at the population and at the clonal level. To test whether a single round of stimulation could imprint these heterogeneous fates, we isolated highly pure naïve CD4 T cells, which were labeled with CFSE and stimulated with *C. albicans* in the presence of autologous monocytes (Zielinski et al., 2011). Proliferating, CSFE-negative T cells recovered on day 15 produced IFN-γ, IL-17 and IL-22, consistent with previous findings (Figure 15A). Interestingly, in the same cultures some cells produced IL-4, alone or together with IFN-γ. These findings demonstrate that multiple polarized cell fates can be generated from naïve T cells in a single round of stimulation and even in the same culture.

To ask whether the same clonotype could be found in different functional subsets, we sorted IL-4- and IL-17-secreting cells using the cytokine secretion assay and isolated a number of clones, which were then characterized phenotypically and molecularly for TCR usage. Remarkably, the same TCR αβ pair was found in 5 T cell clones isolated from the IL-17$^+$ cells and in 1 clone isolated from IL-4$^+$ cells. Importantly, the clones isolated from IL-17$^+$ cells, produced IL-17 and expressed *RORC* mRNA, while the clone isolated from IL-4$^+$ cells produced IL-4 and expressed *GATA3*, but not *RORC* mRNA (Figure 15B,D). These findings demonstrate that intraclonal diversification to exclusive IL-17 and IL-4 production can be imprinted in a naïve T cells by a single round of *in vitro* stimulation.



Sorting	Clone n.	TCR	CDR3
IL-17+	1	Vα	n.d.
		Vβ	CASSPRDYEQYF
	8*	Vα	CAVVIGSARQLTF
		Vβ	CAISPPSGVEKLFF
	12*	Vα	CAVVIGSARQLTF
		Vβ	CAISPPSGVEKLFF
	15*	Vα	CAVVIGSARQLTF
		Vβ	CAISPPSGVEKLFF
	24	Vα	n.d.
		Vβ	CAISPPSGVEKLFF
	28*	Vα	CAVVIGSARQLTF
		Vβ	CAISPPSGVEKLFF
	29*	Vα	CAVVIGSARQLTF
		Vβ	CAISPPSGVEKLFF
	32	Vα	CAAPPVGGATNKLIF
		Vβ	CASRTPTDFTGELFF
IL-4+	2	Vα	CAVTVLNTDKLIF
		Vβ	CAISESRPPGELFF
	6	Vα	n.d.
		Vβ	CASRDQGRETQYF
	10*	Vα	CAVVIGSARQLTF
		Vβ	CAISPPSGVEKLFF
	29	Vα	CAYEGGSNYKLTF
		Vβ	CASSYSLDNEQYF

Figure 15. Generation of multiple fates by *in vitro* priming of naïve T cells. (A) Proliferation of CFSE-labeled naive CD4 T cells primed *in vitro* by *C. albicans* in the presence of autologous monocytes and intracellular cytokine staining on gated CFSE^{lo} cells on day 15. (B) List of clones isolated by cell sorting of IL-17^{+} and IL-4^{+} cells from *C. albicans*-primed cultures. The T cell clones with the same TCR are marked with an asterisk. (C,D) Cytokine production (C), and expression of transcription factors (D) in 3 sister clones isolated from IL-17^{+} and IL-4^{+} cells.

6.2. Additional Results

6.2.1. High throughput approach to unravel the relationship between *C. albicans* antigens and elicited response

The genome of *C. albicans* comprises more than 6,000 ORFs, of which only 1,500 have so far been characterized (Candida Genome Database, 2014). The work described in Section 6.1 of this thesis provided compelling evidences that multiple types of T helper subsets (namely Th1, Th2 and Th17) can be generated in response to this complex microbe. Heterogeneity appears to be represented both at intra-clonal and at population level. The first phenomenon can possibly be explained with well described mechanisms such as asymmetric division or differential environmental cues acting on daughter cells during clonal expansion. However, the vast majority of *C. albicans*-specific clonotypes identified appeared to be unique to one cellular compartment. This argues in favor of driving forces that preferentially skew the polarization of a cell with a given TCR toward a certain fate. Antigen-specific properties, such as post-translational modification, strength of recognition by TCR, produced amount, anatomical localization, are likely factors in determining such fate.

We reasoned that if having the possibility to screen distinct T helper repertoires for the specificity to a wide number of *C. albicans* proteins we would possibly be able to unravel some of the underlying relationships between antigens and their induced response. We therefore selected 80 *C. albicans* proteins from an in depth analysis of the relevant literature (Table 2); the criteria utilized encompassed two main constrains, *i.e.* ultimate characterization of the protein and reported immunogenic activity. Besides, we took care to select proteins with different cellular localization (cytosol, cell wall, secreted proteins) and functions (hydrolysis, adhesion, metabolism, replication, structure maintenance). This would ideally allow us to link a certain class of proteins with shared features to a consistently induced type of response.

We proceeded with a bioinformatic analysis to predict and produce immunoprevalent (dominant within the population) peptides from the selected proteins. This approach presents the great advantage that only a few peptides must be synthesized, thus avoiding yeast-based production of the whole protein, with invaluable spare of time and resources. It must be noted that as *C. albicans* possesses a slightly modified genetic code, production of those proteins in bacteria would not result in correct sequence or structure.

As explained in details in the "Experimental procedures" section, all 15-mer peptides overlapping by 10 residues spanning each sequence were scored for predicted binding to a panel of 24 common different HLA class II DR, DP and DQ molecules. A set of 1,273 peptides was selected as top scoring for binding to 12 or more of the 24 HLA class II molecules probed (in collaboration with Dr. Alessandro Sette, LIAI, CA, USA). These selection criteria are similar to that adopted in other recent works (Lindestam Arlehamn et al., 2013; Oseroff et al., 2012). Each protein was represented by a minimum of 4 to a maximum of 51 peptides (average 16.8). The peptides were synthesized as crude material, individually resuspended in DMSO and pooled accordingly to the protein of origin.

Using the 80 generated peptide pools, we assessed the level of T cell reactivity in healthy donors. Total memory T cells ($CD4^{+}CD45RO^{+}$) were obtained from 5 donors, labeled with CFSE and co-cultured with autologous monocytes pulsed with each of the 80 peptide pools (Figure 16a). Some of the peptide pools induced a high proliferative response in 5/5 or 4/5 donors, as detected at day 12 by CFSE dilution; this is consistent with the notion that some antigens are widely recognized among the human population (immunoprevalent). Some other peptide pools, instead, induced very strong response on a donor-specific basis, which might account for more efficient presentation on specific HLA types. Notably, most peptide pools induced little or undetectable proliferation. Pools representative of metabolic enzymes tended to be poorly immunogenic in our assay, and we speculate that this might depend on the high level of sequence homology for such proteins across the eukaryotic domain (not shown).

We decided to focus our attention on the 15 top-ranking peptide pools; the response of 4 different T helper subsets, as defined on the basis of surface expression of chemokine receptors CCR6, CXCR3, CCR4 (see previous Section 6.1 for details), was analyzed for each peptide pool, and the relative frequency of antigen-specific T cells determined through T cell library assay (Geigeret al., 2009) (Figure 16b). As expected, the magnitude of the response was widely different depending on the analyzed subset. Th17 cells showed high reactivity to most antigens, while Th1 comprised very rare cells specific to the tested pools. Hyr1 and MP65, two already described immunodominant antigens of *C. albicans* cell wall, were confirmed to be among the most recognized proteins from the fungus. S-aspartyl protease (Sap) 4, 5, 6, which constitute a separate group of proteases from Sap 1, 2, 3 in terms of sequence homology, were also identified as novel dominant immunogens inducing exuberant Th17 responses; interestingly, a deeper analysis of the literature revealed that among the Sap family, Sap2 was instead the best characterized immunogen, and is currently under evaluation as a vaccine protein in clinical trials (De Bernardis et al., 2012). The reason for Sap2 being considered a good vaccine candidate relies on the predominant production of this protein from *in vitro* cultured *C. albicans*, and from the wide presence of Sap2 binding antibodies in

human sera (Naglik et al., 2003). However, which member of the family is mostly produced *in vivo* remains unclear, and antibodies have been reported to be broadly cross-reactive among members of this family (Naglik et al., 2003). We propose therefore that Sap2 has erroneously been identified as responsible for an immunogenic effect that has instead to be attributed to Sap4, Sap5, and Sap6 proteins. The frequency of cells recognizing any of these three antigens is extraordinary, with an average of roughly 1 cell out of 700-2,000 Th17 being specific.

Th1* and Th17 showed impressively similar antigenic fingerprints, which would be consistent with high plasticity between these two compartments, as also suggested by the high level of clonotype sharing (see Section 6.1). Several differences in terms of fingerprint could be instead observed among these subsets and the Th2 subset, which seems to preferentially recognize a distinct set of proteins (for instance, it accounts for the highest frequency of cells specific to Als6 and Lip5). Interestingly, in the case of Th1, very little response was induced by any of the tested peptide pools. This finding is in line with the clonotypic analysis we performed on pathogen-specific Th1 population (see Section 6.1), as such population appears to be composed mainly by unique clones that are not shared with any other subset. These results therefore strongly suggest that CCR6^{-} Th1 cells are generated by a distinct set of proteins of *C. albicans* that were not included in our assay. It is also possible that Th1 responding to *C. albicans* are instead cross-reactive T cells that were generated in response other pathogens, an overall scenario that has been suggested by other groups in a different context (Birnbaum et al., 2014). In the attempt to consider the relevance of each analyzed antigen in the generation of a specific type of T cell response, we normalized the obtained frequencies by the total number of cells within the subset responding to any of the screened peptide pools. Not surprisingly, we found that the most dominant antigens (Hyr1, MP65, Sap5) accounted for a very high percentage of the response in the Th1* and Th17 compartments (Figure 16c). Intriguingly though, proteins such as Als6, Lip5 and Plb4 showed a clear skewing toward a Th1 and Th2 phenotype. The reasons for this preferential association are still unclear and are currently object of intense investigation. In conclusion, we have provided evidences for a differential T helper response to antigenic targets within a given microbe. The identification of *C. albicans* prototypic “protective” and “deleterious” antigens (*i.e.* Th17-inducing MP65, Hyr1, Sap 4, 5, 6 and Th2-inducing Lip5, Als6 and Plb4) raises the question whether the antigenic fingerprint may be altered in pathological conditions associated with an overall skewed Th response, such as CMC. We are planning to perform *in vivo* experiments using the characterized antigens to verify whether the *ex vivo* findings would be confirmed; if so, this approach will allow to dissect the antigen borne signals that induce polarization of T cells towards a specific fate.

Pool n.	Protein	Uniprot	N. pept.
1	G3PDH	Q5ADM7	8
2	FBA	Q9URB4	9
3	CCW14	Q5AFN8	4
4	SAP10p	Q5A651	15
5	PLB4	Q59NM2	29
6	SAP3p	P0CY29	16
7	SAP1p	P0CY27	14
8	SAP2p	P0DJ06	17
9	SAP5p	P43094	15
10	SAP4p	Q5A8N2	15
11	SAP6p	Q5AC08	17
12	LIP7	Q9P4E7	28
13	PGA26	Q5AA09	5
14	SOD6	Q5ACV9	10
15	PLB1	Q59W74	26
16	CHT1	Q5AAH2	20
17	ALS5	Q5A8L3	34
18	ALS6	Q5A2Z7	36
19	DCW1	Q5AD78	26
20	TOS1	Q5AJA4	13
21	RBR1	Q5A6M0	5
22	CRH11	Q5AFA2	12
23	LIP2	Q5APG1	15
24	LIP10	Q9P4E5	24
25	LIP3	Q5APA9	21
26	LIP6	Q9P4E8	15
27	LIP1	Q5APE4	24
28	ALS7	Q5A312	51
29	PLB5	Q5A760	23
30	EAP1	G1UBC2	7
31	YWP1	Q59Y31	15
32	HYR1	Q5AL03	21
33	SOD5	Q5AD07	7
34	SOD4	Q5AD05	6
35	SIM1	Q5AKU5	6
36	PIR1	Q59SC4	8
37	RBT5	Q59UT4	6
38	PGA10	Q59UP6	7
39	MP65	Q59XS9	10
40	LIP8	Q59PP4	27

Pool n.	Protein	Uniprot	N. pept.
41	LIP4	Q59RI6	28
42	LIP5	Q9P8W0	24
43	LIP9	Q9P4E6	22
44	PGA4	Q5AJY5	16
45	PHR2	O13318	22
46	ALS4	Q59L09	14
47	ALS2	O74657	16
48	KEX2	Q5APK9	27
49	ALS9	Q5A8K7	15
50	CSA1	G1UB63	27
51	ALS1	Q5A8L0	22
52	ALS3	Q59L12	14
53	CHT2	P40953	14
54	PLB2	Q59W34	26
55	CHT3	P40954	15
56	EXG2	Q5AIA1	13
57	PGA1	Q5ACL7	8
58	PLB4.5	Q5ALY4	13
59	KRE1	P0CY22	9
60	PRA1	P87020	11
61	ADH1	Q5AK23	11
62	SAP7	Q59VH7	20
63	RBT1	Q59TK9	20
64	HWP2	Q59PF9	15
65	SUN41	Q59NP5	5
66	UTR2	Q5AJC0	12
67	SAP9	Q59SU1	27
68	HWP1	P46593	6
69	IHD1	Q5A8I8	4
70	PGA13	Q5A343	11
71	CDC19	P46614	17
72	PGK1	P46273	10
73	ENO1	P30575	15
74	HEX1	Q59NY2	26
75	MET6	P82610	28
76	SAP8	Q5AEM6	17
77	DFG5	Q5ACZ2	21
78	CRH12	Q5AK54	18
79	PHR1	P43076	19
80	RBR3	Q5A5F8	10

Table 2. Selected peptide pools for immunogenicity screening. Eighty *C. albicans* cytosolic, cell-wall associated or secreted proteins were selected (Uniprot accession code is indicated) and predictably immunodominant peptides from each sequence were produced and pooled. Eighty peptide pools were obtained in the end, each composed by a different number of peptides (indicated in last column).

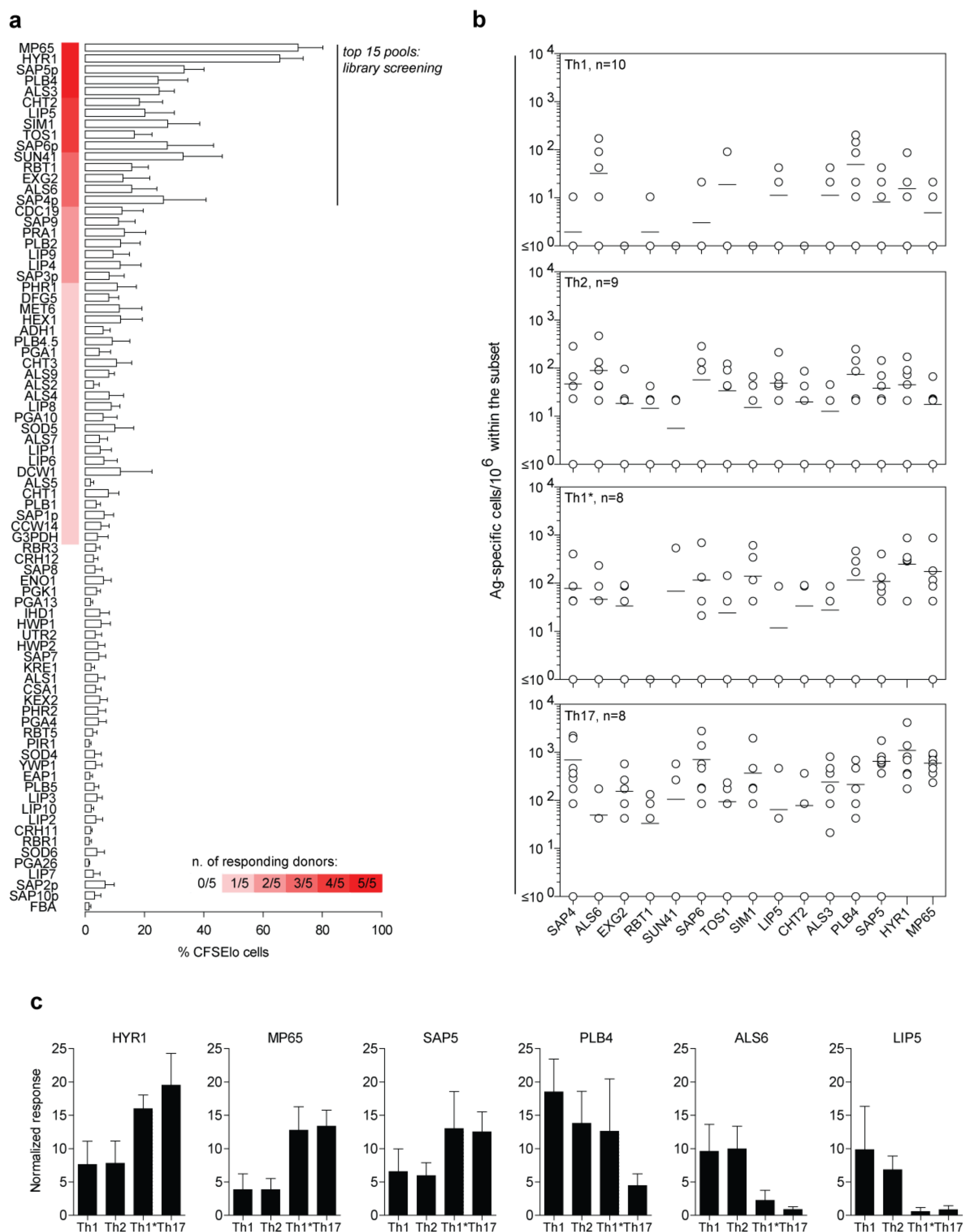


Figure 16. Antigenic fingerprint of *C. albicans*-specific cells in different subsets. (a) T cell memory proliferation in response to 80 *C. albicans*-derived peptide pools. Peptide pools for 80 *C. albicans* proteins were obtained (see text, Table 2); CD4^{+}CD45RO^{+} memory T cells were obtained by negative selection with bead-based enrichment, CFSE-labeled and co-cultured with autologous monocytes pulsed with a peptide pool. After 7 days, 50U/ml IL-2 were added to the culture and CFSE dilution was assessed after additional 5 days. Each pool was tested in duplicate for each donor, 5 donors were tested; bars show average level of proliferating cells in all 5 donors, color scale indicates the number of donors in which a significant response (defined as CFSE^{lo} % > 5 x background proliferation in the absence of stimulation). (b) T cell libraries were run on Th1, Th2, Th1* and Th17 cells sorted from 8-10 donors (each plots represents a donor) using the 15 top ranking peptide pool as assessed in a); average values of frequency are shown as a line. (c) Normalized response is shown for 6 antigens: the values were obtained by dividing the number of peptide pool-specific cells by the total number of T cells responding to any pool in that subset.

6.2.2. Interrogation of the T cell response in patients with chronic mucocutaneous candidiasis

Chronic mucocutaneous candidiasis (CMC) is the collective term for a complex group of disorders characterized by persistent or recurrent infections of the skin, nails and mucosal tissues with *Candida* species, mostly *C. albicans* (Eyerich et al., 2010). CMC is very commonly developed by patients with broad and profound T cell deficiencies and can be provoked by various inborn errors of immunity. Patients with hyper IgE syndrome, caused by dominant-negative mutations of STAT3, are susceptible principally to CMC and staphylococcal diseases of the lungs and skin (Milner et al., 2008). These patients have very low proportions of circulating IL-17A- and IL-22-producing T cells, probably because of impaired responses to IL-6, IL-21, and IL-23. Individuals with mutations in IL-17F or IL-17 receptor genes have also been shown to develop CMC (Puel et al., 2011). APECED, or autoimmune polyendocrine syndrome, is caused by AIRE gene mutation and often associates with CMC; the trigger, in this case, is the production of auto-antibodies against IL-17 and IL-22 (Puel et al., 2010). Overall, it appears clear that all inborn errors leading to CMC underlie impairment of the Th17 response.

A further set of mutations that induce gain of function in the STAT-1 transcription factor (STAT-1 GOF) has been associated with onset of CMC (Liu et al., 2011). Such mutations occur in the coiled-coil domain of STAT-1 protein and impair the nuclear dephosphorylation of the activated form, resulting in stronger cellular responses to STAT-1 inducers IFN-α/β, IFN-γ, and IL-27, and stronger STAT-1 activation in response to the STAT-3-dependent IL-17-inducers, IL-6 and IL-21. Thus, as highlighted previously, the development of T cells producing IL-17A, IL-17F, and IL-22 is impaired also in these patients (Liu et al., 2011). However, the reduced IL-17 production by T cells in these patients was only assessed on the total $CD4^+$ T cell memory compartment following polyclonal stimulation (PMA+Ionomycin or α-CD3/α-CD28). This data do not give therefore any insights on how the memory T cell response to *C. albicans* is shaped in such patients.

We decided to take advantage of the T cell library technique, recently developed and extensively used in our laboratory (Campion et al., 2014; Geiger et al., 2009; Lindestam Arlehamn et al., 2013) to interrogate the repertoire of STAT-1 GOF CMC patients' memory T cells in order to obtain quantitative and qualitative information on its composition.

Blood samples were collected from patients as well as age-matched controls at the Institute Imagine (Paris, France; collaboration with Drs. Anne Puel and Jean Laurent Casanova, Imagine, France and Rockefeller University NY, USA). $CD14^+$ and $CD14^-$ PBMC fractions

were isolated by magnetic bead enrichment after Ficoll-Plaque gradient, frozen and shipped in dry ice to our laboratory. $CD14^-$ PBMCs were thawed and stained for exclusion markers (CD8, CD14, CD16, CD19, CD25, CD56), CD4 memory markers (CD4, CD45RO), and chemokine receptors (CCR6, CXCR3) so to obtain four different memory T cell subsets: $CXCR3^+CCR6^-$ classical Th1, $CXCR3^-CCR6^-$ Th2, $CCR6^+CXCR3^-$ Th17, and $CCR6^+CXCR3^+$ non-classical Th1* (Figure 17a). This sorting strategy slightly differs from the one adopted in the work presented Section 6.1 and aimed to maximize the cellular recovery from the reduced samples in our possess; the main limitation of this strategy would be represented by an over-representation, and possibly "dilution", of the Th2 compartment, which would result enlarged by the inclusion of all $CCR6^-CCR4^-CXCR3^-$ cells generally excluded from our standard sorting strategy. Still, the obtained four populations of memory T cells showed clearly distinct cytokine profiles upon *ex vivo* restimulation, in accordance with the chemokine-receptor based phenotype prediction (not shown and Figure 17f).

Strikingly, most analyzed samples from patients showed a severely impaired expression of CCR6, a chemokine receptor whose expression is considered to be controlled by the Th17-lyneage specifying factor RORγt (Figure 17a,b). Instead, CXCR3 expression, under the regulation of IFN-γ-induced STAT-1, was found significantly increased. As a result, the patients showed a dramatically diminished amount of circulating Th17 cells and increased frequency of classical Th1, as compared to healthy age-matched controls (Figure 17a,b). We next assessed the expression of CCR6 on non-$CD4^+$ non-$CD14^+$ cells (double negative fraction) and surprisingly noticed that this population presented an even higher expression of CCR6 in patients as compared to healthy controls (Figure 17c,d). This finding is extremely important for two different reasons: first, it represents a valuable internal control for the validity of the staining, thus confirming a selective lack of CCR6 expression on memory $CD4^+$ T cells in STAT-1 GOF patients; second, it suggests that CCR6 expression is regulated by different signals in $CD4^+$ T cells and non-$CD4^+$ T cells (possibly in T *vs* B cells).

As $CCR6^+$ cells were very rare in STAT-1 GOF patients, we decided to generate clones by limiting dilution from the two relative subsets (Th1* and Th17) and assess their signature cytokine profile in order to exclude that functional defects accompanied their reduction in number. Figure 17e shows that despite the reduced representation in blood, these cells produced IL-17 and IL-22 in similar amounts when compared to healthy controls. A slight but not significant increase in IL-17 production by Th1* and, most notably, a strong increase in IFN-γ production by Th17 was detected in patients (Figure 17e). This suggests that priming in STAT-1 GOF environment, probably due to contribution of both the APC and naïve T cell, may tend to skew the proliferating progeny toward an IFN-γ producing phenotype (Th1); in this scenario Th1* would reasonably represent ex-Th17 (or "missed" Th17) as suggested

elsewhere (Cosmi et al., 2011), even though clonotypic analysis will be necessary to confirm this hypothesis.

T cell libraries were prepared and expanded as previously described (Geiger et al., 2009), and then screened for reactivity to heat-killed *C. albicans* using autologous monocytes as antigen presenting cells. ^{3}H-thymidine incorporation was used as readout for proliferation after 3 days of co-culture, and frequency of *C. albicans*-specific cells calculated on the basis of the number of non-responding wells, in accordance with Poisson distribution.

Having targeted with our sorting strategy the whole memory T cell compartment, we were able to estimate the total number of circulating memory T cells specific to *C. albicans* just by summing the frequencies obtained in each subset, normalized to the relative proportion of the lineage within the whole population. Unexpectedly, the overall frequency of memory T cells specific to the fungus was unaltered in patients with respect to healthy controls (Figure 17g).

However, the distribution of pathogen-specific cells among subsets differed enormously. Already on a *per subset* basis, an average 4-fold increase ($p<0.05$) was detected in the Th1 compartment of the patients (not shown). When taking in account the relative frequency of each subset within circulating memory T cells, it appeared striking that response in healthy controls was Th17-driven (roughly 55% of *C. albicans*-specific cells belonged to this subset) and very little Th1-polarized (less than 10%), while patients showed a mirroring distribution, with 50% of circulating *C. albicans*-specific cells being Th1 and very few percent Th17 (Figure 17h).

We believe this unbalanced response may be implicated in both susceptibility to *C. albicans* infection and on pathogenesis of CMC. First, the lack of circulating T cells expressing the homing receptor CCR6, considered to be involved in homing to the mucosal sites, might result in very few protective IL-17 producing cells reaching the site of infection. Secondly, it is tempting to speculate that classical CCR6^{-} Th1 may instead reach the site of infection due to expression of CXCR3, homing receptor to inflamed tissues, and worsen the disease by producing not only IFN-γ but also Th2 types of cytokine (it must be noted that IL-4, IL-5 and IL-13 are produced by *C. albicans*-specific classic Th1 in considerable amounts (Becattini et al., *manuscript in preparation*). IL-4, IL-5 and IL-13 have long been associated with non-protective response to *C. albicans*, in both animal models and humans (Romani, 1999), also with respect to CMC (Kobrynski et al., 1996). Such cytokines have been shown to dampen IL-17-mediated neutrophils recruitment *in situ*, induce diseases such as atopic dermatitis and render skin more susceptible to infections due to downregulation of β-defensins (Eyerich et al., 2010). Increased IFN-γ levels at the site of infection could as well play a role in promoting inflammation and worsening overall tissue homeostasis.

Our data provide novel important insights on the understanding of *C. albicans*-specific memory T cell response in patients suffering from CMC, and set the stage for a whole new series of experimental questions. Further studies will be required to assess the relative role of priming *vs in situ* restimulation of memory cells in generating the observed phenotypic skew, the cytokine profile and antigenic specificities of skin-resident *C. albicans*-responding cells as compared to circulating cells, as well as the clonal relationship between cells residing in either of these anatomical compartments.

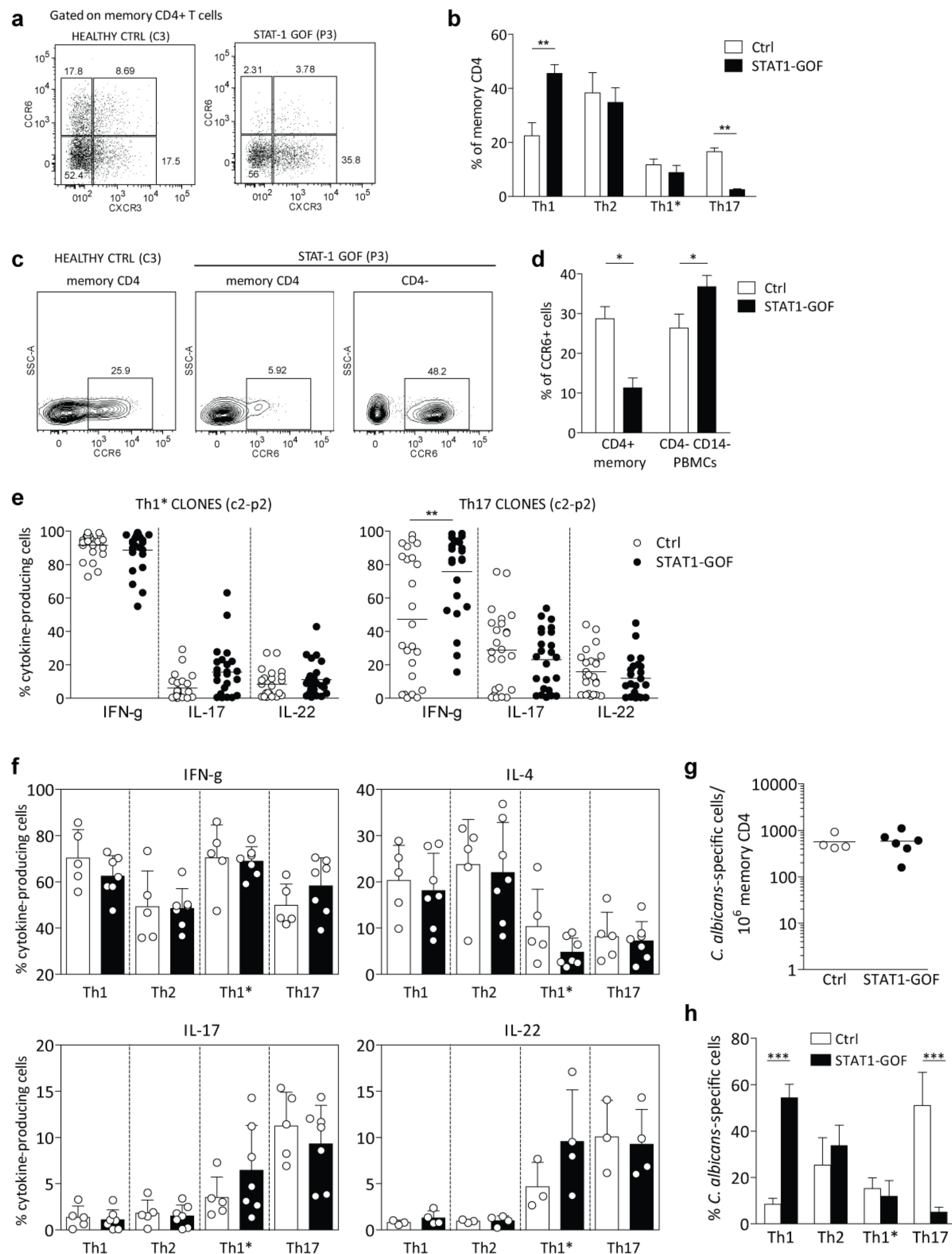


Figure 17. Phenotype and frequency of *C. albicans*-specific memory T cells in STAT1-GOF patients suffering from CMC. (a) Adopted sorting strategy; shown are two representative panels (1 patient, 1 healthy control) for CCR6 and CXCR3 expression in CD4$^+$ T cells (pre-gated as lin$^-$CD4$^+$CD45RO$^+$, not shown). (b) Quantification of cells sorted as in a) from several donors (n=5 controls, n=7 patients). (c) Representative plot for CCR6 expression in CD4$^+$ T cells and CD14$^-$CD4$^-$ cells. (d) Quantification of CCR6 expression as assessed in c). (e) Representative plot showing the percentage of cytokine producing cells within individual T cell clones obtained by limiting dilution from healthy controls or patients. (f) Intracellular cytokine staining of T cell library lines; dots represent average values for each donor. (e) Frequency of circulating *C. albicans*-specific memory T cells calculated through T cell library assay; each dot represents one donor. (f) Distribution of *C. albicans*-specific T cells among different subsets; values were calculated considering the relative expansion of each circulating subset through surface staining as in a). **p<0.01, ***p<0.001

6.2.3. A biochemical approach to identify *C. albicans* dominant Th17 antigens among cell wall mannoproteins

The cell wall of *C. albicans* comprises an outer layer of mannans and mannosylated proteins (mannoproteins) anchored through a GPI molecule to the inner β-glucan layer, that are considered to be highly immunogenic. The immunogenicity of such proteins depends on several synergizing factors: first the conjugation with mannan branches, that are recognized by several innate receptors, such as DC-SIGN, Dectin-2, mannose receptor, Mincle and others (Osorio and Reis e Sousa, 2011), thus facilitating uptake and efficient activation of DCs through innate signaling; secondly, the abundance and availability of such proteins, that are produced in considerable amounts due to their crucial structural or adhesive role, and protrude from the cell wall, becoming easy targets for uptake by innate cells, and increasing the chance of being processed and presented (Chaffin et al., 1998; Gomez et al., 1996).

Only a few immunodominant proteins have been identified among the thousands that are known to occupy this layer; the best studied among those (MP65, Hyr1) are preferentially or uniquely expressed by the hyphal form of the fungus (Gomez et al., 1996; Luo et al., 2010). However, mannoproteins are highly represented on the cell wall of conidia (Chaffin et al., 1998), which are in turn widely recognized by T cells in healthy donors; we therefore reasoned that abundant immunogenic proteins yet unidentified must be present on the yeast surface.

As T cells play a key role in protection against fungal infection, it is believed that a vaccine to protect against *C. albicans* should elicit strong response of the Th17 subset (Bar et al., 2012). The search for T cell immunodominant antigens has therefore a remarkable clinical importance.

We set up a series of biochemical approaches to extract, characterize and identify surface mannoproteins from log phase-growing *C. albicans* conidia. The extraction procedure, described in details in the “Experimental procedures” section, was based on solubilization of the proteins at high temperature in citrate buffer, precipitation through copper tartrate-containing Fehling reagent, purification via acidic treatment and dialysis.

Electrophoretic analysis of the extract revealed an accumulation of high molecular weight proteins at the top of the polyacrylamide gel (Figure 18a, black square). This is consistent with the large size of the mannose branches, that in yeasts have been estimated to be composed on average by over 600 mannose units each (Chaffin et al., 1998). Basically very few proteins were able to enter the gel, either because they had a smaller size or a lower level

of glycosylation (of note the harsh extraction procedure could likely generate breaks in the sugar backbone and therefore give rise to products of reduced dimension).

Treatment of the extract with PNGaseF, an enzyme that catalyzes cleavage of the N-linkage between sugars and Asparagine residues, promoted release of some of the proteins from the high molecular weight aggregate, thus generating a smear at still high molecular weight (Figure 18a, blue square) and a clear band at 15-20 KDa (Figure 18a, red square). Following de-glycosylation we separated the proteic part through acetone based precipitation from the released sugar moieties remaining in the supernatant.

We first assessed the immunogenic activity of the fraction on total memory $CD4^+$ T cells upon stimulation in the presence of autologous monocytes. To address also the relative role of the sugar moiety in this process, as a previous report suggested this would be sufficient to trigger proliferation and polarization of Th17 (van de Veerdonk et al., 2009), we separately stimulated T cells with CWE, deglycosylated CWE and recovered soluble sugars. Heat-killed CA particles were used as positive control. Strikingly, stimulation by CWE induced T cell activation to a similar extent to whole fungal particles, as assessed by CFSE dilution and intracellular staining for IFN-γ and IL-17 production; not surprisingly, the effect was not altered by sugar removal, while mannans alone could not induce substantial T cell proliferation (Figure 18b). This experiment suggested that cell wall mannoproteins comprises the most recognized antigens within CA particles, and their aminoacidic backbone is responsible for recognition by T cells.

We went on trying to determine whether CW mannoproteins would be recognized by different T helper compartments or rather induce a preferential response in supposedly protective subsets ($CCR6^+$). A sorting strategy was used based on chemokine receptors CCR6, CCR4 and CXCR3 that allowed us to sort populations highly enriched for Th1, Th2, Th17 and non classical $CCR6^+$ Th1*. *Ex vivo* parallel stimulation of each subset revealed that $CCR6^+$ cells, Th17 in particular, were preferentially activated by the CWE, as assessed by the level of T cell CFSE dilution in 4 healthy donors (Figure 18c). $CCR6^-$ Th1 and Th2 compartments showed lower response in comparison to that elicited by HK *C. albicans* (Figure 18c); this would imply that antigens recognized by Th1 and Th2 may preferentially be located in the cytosol or be secreted rather than being enclosed in the cell wall. Beside proliferating to a similar extent, Th1* and Th17 cells produced cytokines in comparable amount when restimulated with CWE (Figure 18d).

On the same line, the majority of *C. albicans*-specific T cell clones generated from three different donors responded upon restimulation with CWE (Figure 18e). We therefore decided to determine the frequency of T cells specific to such extract within the Th17 subset; heat-

killed *C. albicans* and two major antigens proposed or under evaluation for vaccine development, MP65 and Sap2 (De Bernardis et al., 2012) were selected as positive control. The extract was recognized by an impressive amount of cells within the Th17 compartment, with a calculated frequency of CWE-specific T cells nearly as high as that obtained for the whole pathogen among several tested healthy donors, and consistently higher with respect to the control immunodominant antigens (Figure 18f, g).

We reasoned that such impressive frequency of CWE-specific cells might be explained either by the presence of a very potent antigen, or by the synergistic effects of different antigens. We calculated the EC50 of CWE-specific clones or polyclonal library lines (whose low initial input, 150 c/w, would reasonably exclude the presence of more than only one responding cell), and found that the functional affinity for the antigens was extremely high, with EC50 ranging from 0.175 to 0.668 µg/ml (one representative clone shown in Figure 18h). Given the likely multi-protein composition of the extract, any recognized antigen must act at a lower concentration with respect to the estimated range, and therefore would represent an interesting target for immunization strategies.

To rule out the possibility that such antigen would be presented through pathways other than the classical MHCII, we restimulated CWE-specific T cell clones in the presence of MHCII-blocking antibodies or using as APCs autologous monocytes fixed with glutharaldeyde prior to pulse, so to exclude the presence of superantigens that would act independently of processing. In neither case we could rescue proliferation which was instead clearly detectable in clones stimulated in standard conditions, thus implying that the CWE-antigen(s) must be processed and presented on MHCII in a canonical manner (Figure 18i). To definitely ascertain that sugars were not directly recognized by the TCR we furthermore stimulated those clones with commercially available protein-free *C. albicans* mannans, but did not detect any proliferation (Figure 18j). Taken together these data suggest that a very potent T cell antigen is comprised in a fraction of cell wall enriched for mannoproteins.

Next, we wondered whether the antigenic potency of CWE would be preserved in the mouse system. To address this question we performed vaginal or oropharingeal *C. albicans* infection of C57BL/6 mice, collected draining lymph nodes after 1 week, isolated cells and restimulated them in *vitro* with either heat-killed fungal particles or CWE. As shown in Figure 19b and c, a very high proportion of Th17 cells were activated by CWE, indicating that most antigens inducing *in vivo* priming of naïve T cells would be contained in our extract. This result has important implications with respect to the debate whether yeast, hyphae or both growth forms of *C. albicans* are involved in infection and elicit immune response, and argues in favor of a strong role for conidia in such contest or, at least, for importance of

antigens present at both fungal stages. We then asked whether this extract might provide any protection if used as a vaccine. C57BL/6 mice were immunized with CWE and then challenged with an intravaginal dose of live *C. albicans* (Figure 19d). Vaginal lavage was performed in the three weeks following infection, and proliferating *C. albicans* particles quantified through plating and CFU count. A significant decrease in vaginal pathogen burden could be observeded at all analyzed time points (Figure 19e), showing induction of a protective response following immunization and suggesting the potential use of the CWE itself (or of its most immunogenic components) to build a T cell vaccine against *C. albicans.*

We finally decided to attempt to identify the immunogens within the cell wall preparation by subjecting CWE to mass spectrometry. First, we tried to narrow down the number of candidates by fractionating the extract via reverse phase HPLC. Using a C8 column, we obtained 6 main fractions, and a seventh fraction at very high acetonitrile concentration containing all remaining (strongly) binding molecules (Figure 20a). When restimulating CWE-specific T cell library lines, however, we were unable to identify a unique fraction retaining the immunogenic activity, most likely due to the elution of any targets over different fractions (as already mentioned, sugar branches can easily break and generate a continuum of "isoforms" with different molecular weights for any given mannoprotein) (Figure 20b). We therefore proceeded to mass spectrometric analysis (MS) of the whole extract. A few candidates were identified with high level of confidence by the run (Figure 20c): Ywp1, in particular, was highly represented, and turned out to be a heavily mannosylated protein whose backbone size was compatible with the lower m.w. band detected in gel upon PNGaseF treatment (Figure 18a, red square). Other potential candidates represented intracellular enzymatic proteins previously reported to be immunogenic (Candida Genome Database, 2014); a deep search in the literature confirmed that these proteins are commonly found on the outer cell wall, even though the mechanism through which they get translocated from the cytosol remains unclear (Chaffin et al., 1998).

The aminoacidic sequences of the identified targets were retrieved from UNIPROT server and produced as pools of overlapping peptides (15mers) spanning the whole sequence; peptide pools were generated accordingly. To our surprise, none of the peptide pools induced significant stimulation neither in libraries from healthy donors (Figure 20d) nor in CWE-specific clones (Figure 20e). Still, this negative result uncovered that several proteins reported in the literature to be highly immunogenic (mainly on the basis of specific antibody detection in blood of immune donors) do not induce a detectable T cell responses.

We reasoned that as the CWE contains proteins, which are highly mannosylated and most likely tightly aggregated due to the extraction procedure, it was possible that enzymatic

digestion preceding mass spectrometric analysis would be extremely inefficient, and the most representative targets remain hidden from our retrieval. We therefore treated the CWE with PNGaseF in the presence of UREA, a condition that we found to maximize the access of the hydrolase to the protein backbone, as shown by the appearance of several novel bands upon silver staining of polyacrylamide gel electrophoresis (Figure 20e). Repetition of MS analysis identified among the top ranking hits a whole new set of heavily glycosylated, high molecular weight proteins belonging to the ALS (agglutinin like sequences) family (Figure 20f). These proteins have been shown to represent important virulence factors in *C. albicans* infection, and a member of this family (ALS3) is currently being tested in clinical trials as a bivalent vaccine against both *C. albicans* and *S. aureus* infection (Bar et al., 2012; Ibrahim et al., 2013).

ALS2 and 4 resulted strongly represented in our sample, ALS1 and 3 possibly also being present in detectable amounts. As expected, very few peptides could be identified (Figure 19g) and only from one N-terminal extremity of these proteins (most likely the portion released from sugars upon enzymatic treatment), while none were retrieved from their core, which resulted to contain an impressive number of potential N- and O-glycosylation sites (not shown), thus supporting our hypothesis on the causes of missed retrieval by the first MS trial. We are currently planning to produce these proteins and test them in cellular assays for immunogenic properties.

In conclusion, we have extracted a mannoprotein-enriched fraction from the cell wall of *C. albicans* conidia that induces a strong T cell response, mainly localized in the $CCR6^+$ Th17 and Th1* compartments, both in humans and mice. The responsible antigen(s) is likely to be a protein and is presented through a canonical MHCII-restricted pathway, acting at very low concentration. The extract as such induced to some extent a protective response in a mouse model of vaginal infection. The identification of antigenic targets is made difficult by the very complex nature and structure of the extract. However, by coupling enzymatic deglycosylation with mass spectrometry, we believe we have identified the potential immunogens responsible for its activity. We have planned to produce those antigens in yeast so to preserve biochemical properties (*i.e.* glycosylation profile) and test them in *ex vivo* as well as *in vivo* assays. If the identity of these immunogens will be confirmed, this work will have provided evidences for a preferential localization of Th17-inducing antigens in the cell wall of *C. albicans* and identified powerful targets suitable for a vaccine against this pathogen.

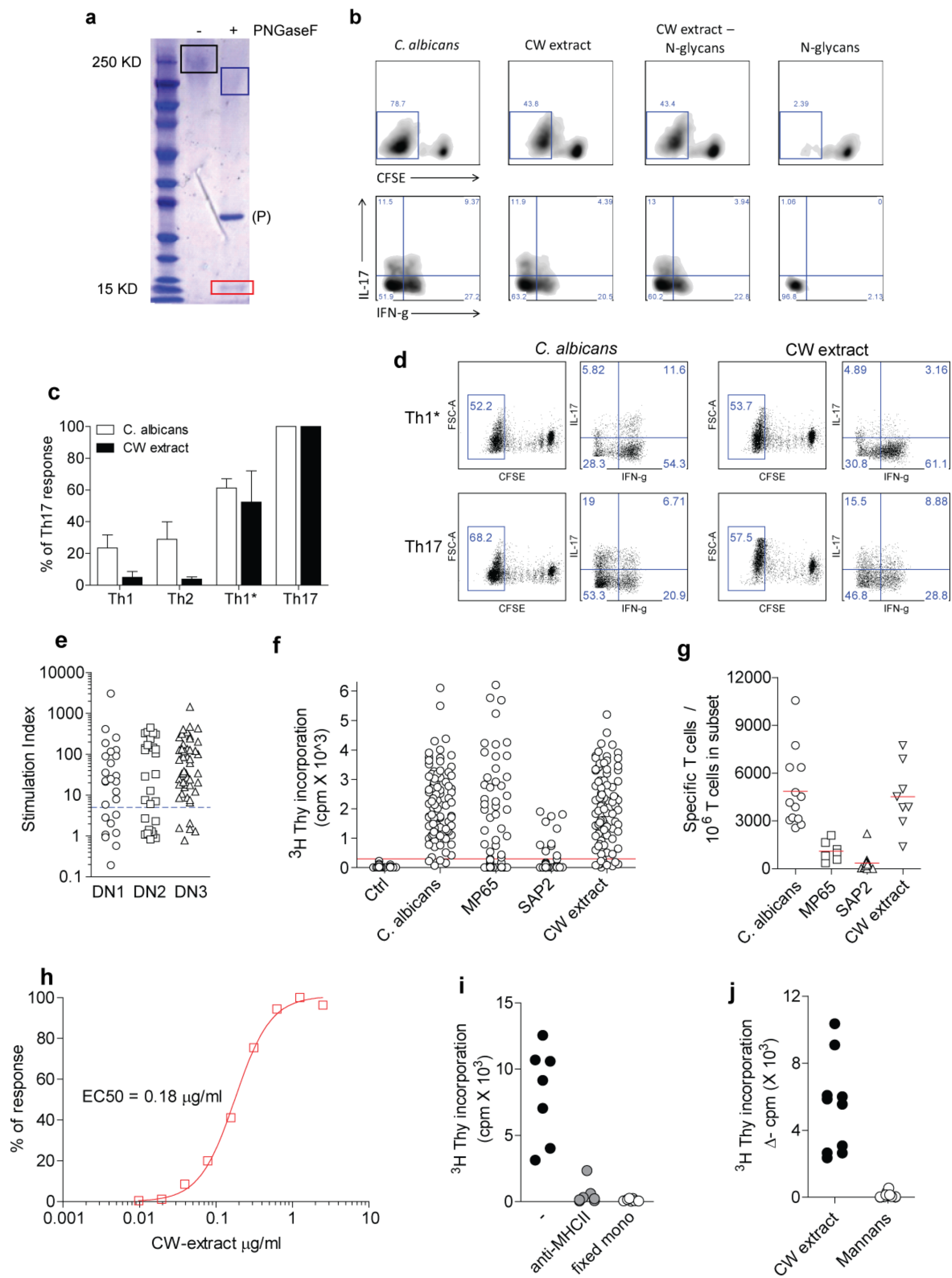


Figure 18. Characterization of immunogenic properties of a mannoprotein-enriched cell wall extract from *C. albicans* conidia. (a) Electrophoresis on polyacrylamide gel of the CWE before and after treatment with PNGaseF glycosidase. Explanation for colored squares are provided in the text. (b) Proliferation and cytokine profile of total $CD4^+$ cells upon stimulation with CWE: cells were labeled with CFSE and co-cultured for 7 days with autologous monocytes pulsed with the indicated antigen. Stimulation with PMA + Iono and BFA was performed prior to staining. (c) Proliferation of different $CD4^+$ T cell compartment in response to CWE. T cells were sorted based on the expression of chemokine receptors and stimulated as in b). CFSE dilution was assessed by flow cytometry and values normalized to the proliferation induced in Th17. (d) Representative intracellular cytokine staining plot of Th1* and Th17 cells stimulated as in b) at day 7. (e) Thymidine incorporation of *C.*

albicans-specific CD4 clones restimulated in the presence of autologous monocytes and CWE or PBS. Stimulation Index represents the ratio between CWE/PBS induced proliferation. (f) T cell library response to indicated antigens as measured by ^{3}H-thymidine incorporation; every dot represent a polyclonal T cell line. Values above 3000 cpm were considered positive. Shown is one representative experiment. (g) Mean frequency values for T cell specificity to the selected antigens. Values were calculated from assays as shown in f), each dot represents a different donor. (h) Antigenic titration for CWE-specific T cell clone. T cell clones or polyclonal lines (see text) were stimulated in the presence of autologous monocytes and decreasing concentrations of CWE; proliferation was measured through ^{3}H-thymidine incorporation assay and EC50 calculated. Shown is one representative clone, the calculated range of EC50 was [0.175-0.668] µg/ml among several donors. (i) Assessment of processing pathway requirements for CWE components. CWE-specific clones were restimulated with CWE in the presence of autologous monocytes with or without MHC II-blocking antibodies; alternatively, autologous monocytes were fixed in 0.02% glutaraldehyde prior to pulse with CWE. (j) CWE-specific clones were restimulated with CWE or protein-free mannans in the presence of autologous monocytes; proliferation was assessed through ^{3}H-thymidine incorporation assay.

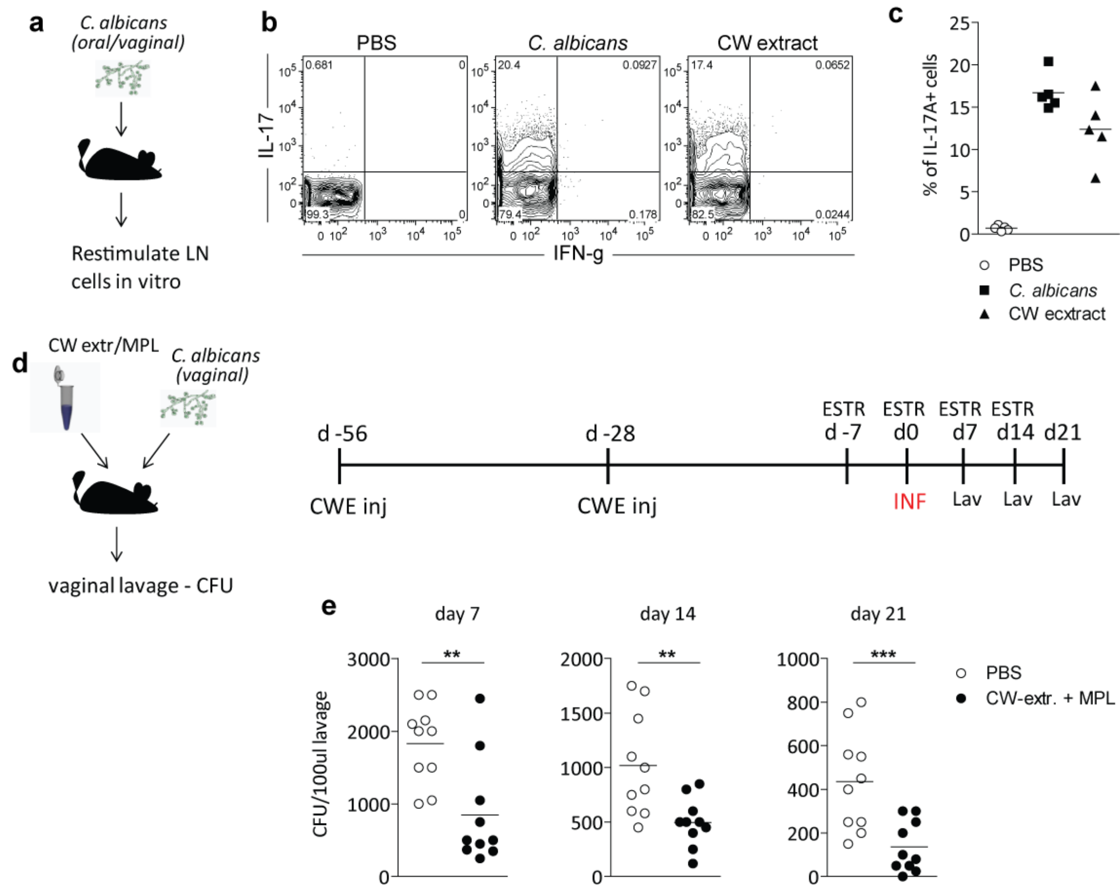
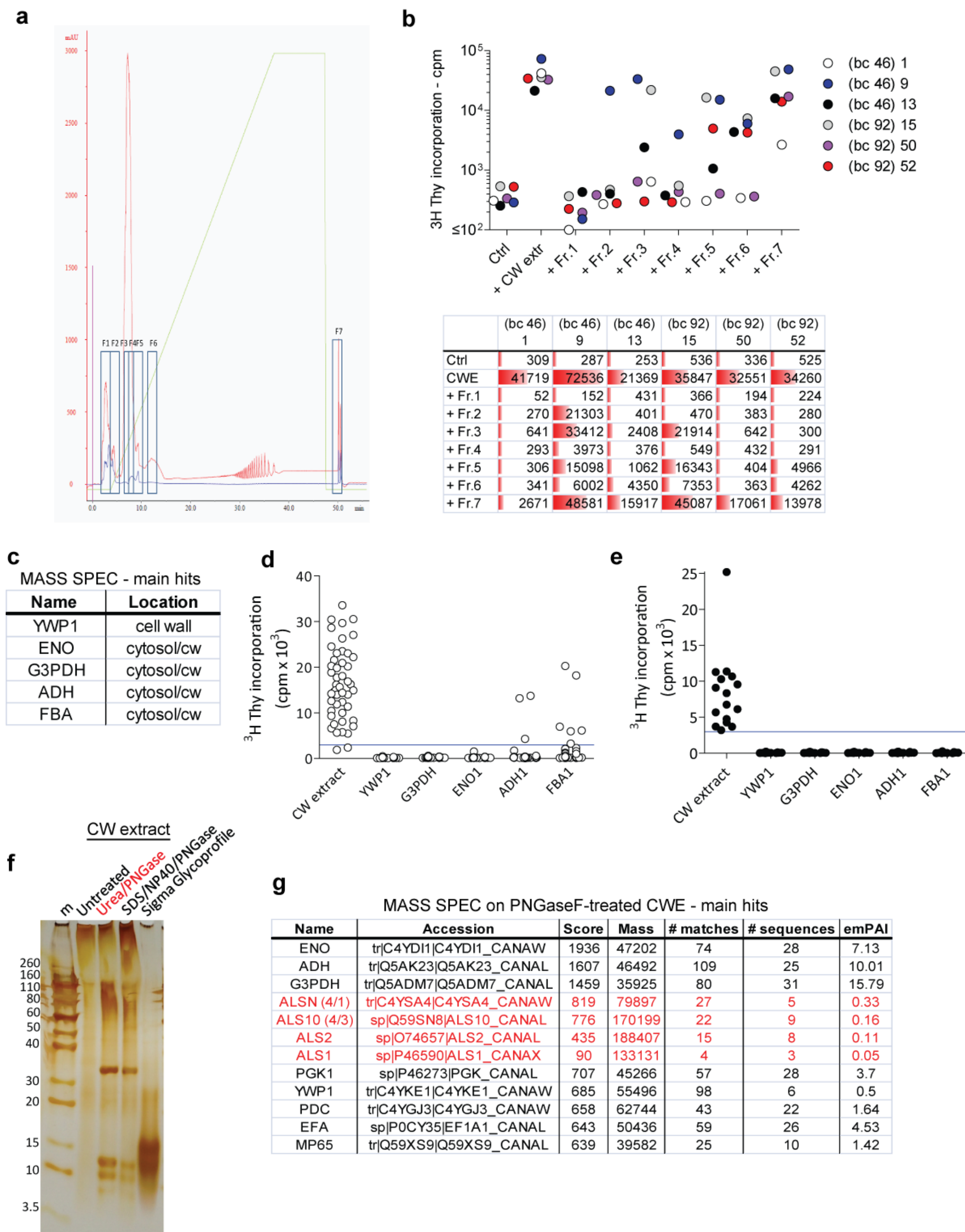


Figure 19. CWE contains antigens that are efficiently recognized in mice and induce protective immune response. (a) Schematic overview of the experiment: oral/vaginal infection of C57BL/6 mice performed upon 17-beta-estradiol treatment, after 1 week draining lymph nodes were extracted, and T cells restimulated *in vitro* in the presence of heat-killed *C. albicans* particles or CWE. After an o.n. incubation with Golgi-stop cells were stained to assess IL-17 and IFN-γ production. (b) Representative plot for an intracellular cytokine staining performed as depicted in (a). Cells were pre-gated on $CD4^{+}CD44^{+}$; the experiments represent oropharingeal infection, similar results were obtained in the model of vaginal infection. (c) Quantification of IL-17A^{+} cells from plots as shown in b). Each dot represents a mouse; representative of at least two independent experiments. (d) Schematic overview of the experimental plan for mouse immunization with CWE. C57BL/6 mice were immunized either with CWE + MPL or with PBS 56 days prior to infection (CWE inj), then received a booster shot at d-28; 17-beta-estradiol was injected every week (ESTR) starting two weeks before infection with 10^{6} *C. albicans* live particles delivered into the vaginal lumen. Vaginal lavage was performed at day 7, 14 and 21 following infection, and plated on Sabouraud plate; CFU were determined after o.n. incubation of the plates. (e) Quantification of fungal particles from vaginal lavage as explained in d). Each dot represents a mouse; data are representative of two independent experiments.



	(bc 46) 1	(bc 46) 9	(bc 46) 13	(bc 92) 15	(bc 92) 50	(bc 92) 52
Ctrl	309	287	253	536	336	525
CWE	41719	72536	21369	35847	32551	34260
+ Fr.1	52	152	431	366	194	224
+ Fr.2	270	21303	401	470	383	280
+ Fr.3	641	33412	2408	21914	642	300
+ Fr.4	293	3973	376	549	432	291
+ Fr.5	306	15098	1062	16343	404	4966
+ Fr.6	341	6002	4350	7353	363	4262
+ Fr.7	2671	48581	15917	45087	17061	13978

MASS SPEC - main hits

Name	Location
YWP1	cell wall
ENO	cytosol/cw
G3PDH	cytosol/cw
ADH	cytosol/cw
FBA	cytosol/cw

MASS SPEC on PNGaseF-treated CWE - main hits

Name	Accession	Score	Mass	# matches	# sequences	emPAI
ENO	tr|C4YDI1|C4YDI1_CANAW	1936	47202	74	28	7.13
ADH	tr|Q5AK23|Q5AK23_CANAL	1607	46492	109	25	10.01
G3PDH	tr|Q5ADM7|Q5ADM7_CANAL	1459	35925	80	31	15.79
ALSN (4/1)	tr|C4YSA4|C4YSA4_CANAW	819	79897	27	5	0.33
ALS10 (4/3)	sp|Q59SN8|ALS10_CANAL	776	170199	22	9	0.16
ALS2	sp|O74657|ALS2_CANAL	435	188407	15	8	0.11
ALS1	sp|P46590|ALS1_CANAX	90	133131	4	3	0.05
PGK1	sp|P46273|PGK_CANAL	707	45266	57	28	3.7
YWP1	tr|C4YKE1|C4YKE1_CANAW	685	55496	98	6	0.5
PDC	tr|C4YGJ3|C4YGJ3_CANAW	658	62744	43	22	1.64
EFA	sp|P0CY35|EF1A1_CANAL	643	50436	59	26	4.53
MP65	tr|Q59XS9|Q59XS9_CANAL	639	39582	25	10	1.42

Figure 20. (a) HPLC fracionation of CWE. CWE was run on a C8 column in acetonitrile gradient and 7 fractions were recovered (highlighted in blue) and separately resuspended. (b) Proliferation of CWE-specific T cell polyclonal lines with autologous monocytes and indicated CWE HPLC fraction; cpm values are reported in the table below. (c) CWE components identified by mass spectrometry on CWE extract and relative location according to the literature. (d) Representative plot of Th17 cell library screened for specificity to CWE or MS-identified targets produced as overlapping peptides spanning the whole sequences. (e) CWE-specific T cell clones from 3 different donors were restimulated in the presence of whole CWE or indicated peptide pools. (f) Electrophoretic run of CWE treated with different deglycosylation protocols; Glycoprofile IV (SIGMA) is an aspecific chemical deglycosylation kit based on trifluoromethansulfonic acid (TFMS). (g) CWE-retrieved proteins upon mass spectrometric analysis of deglycosylated extract. Shown are the 12 top ranking targets, ALS family members are highlighted in red. Note the high score and the low number of identified sequences; explanation is provided in the text.

6.2.4. A biochemical approach to retrieve *C. albicans* antigens inducing Th2 response

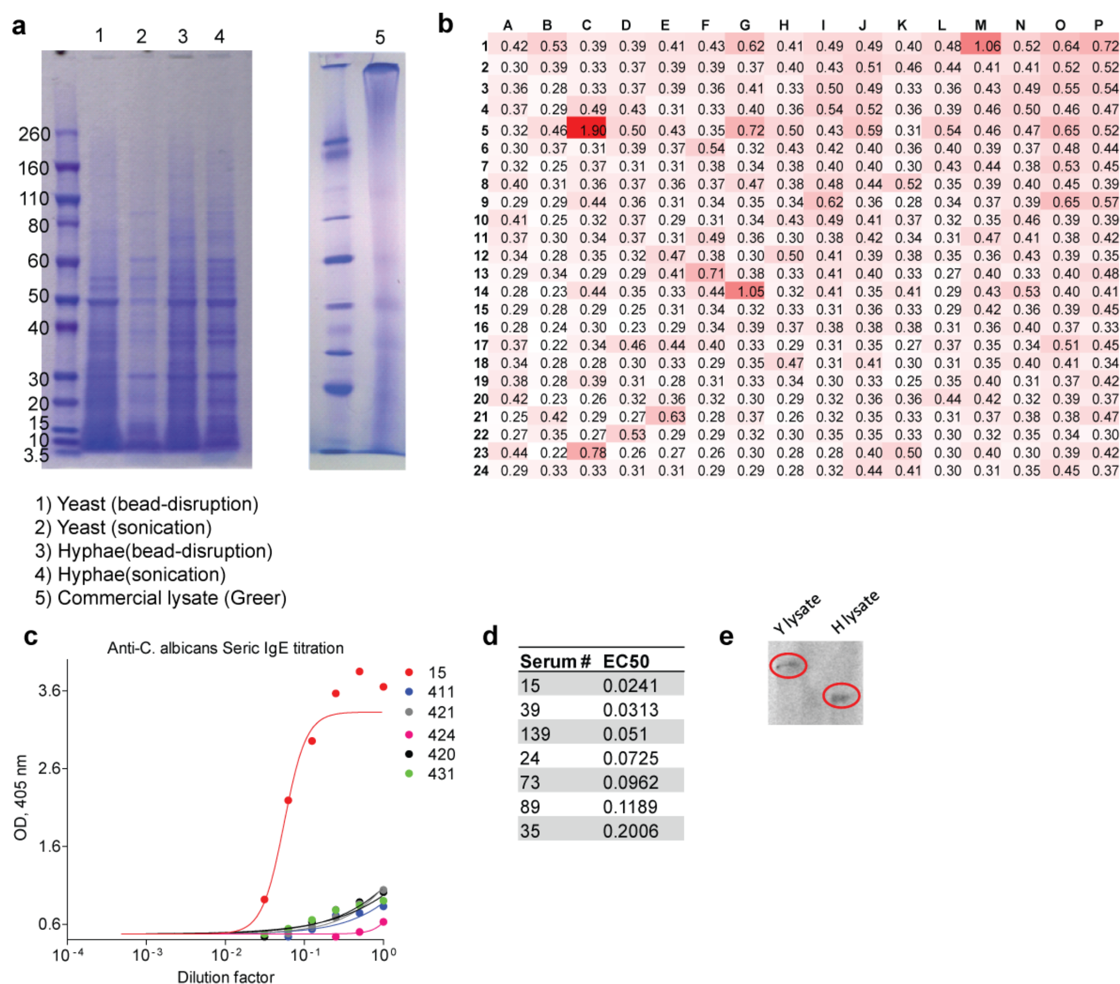
Having identified cell wall mannoproteins as a major source of Th17-inducing antigens, we wondered whether we could retrieve antigens accounting for the Th2 polarization consistently detected in response to *C. albicans* in most healthy donors. The identification of such antigens could have important clinical implications, as a strong correlation between atopy and sensitivity to *Candida* infection is reported, and it has been postulated that an allergic type of inflammation (with *in situ* recruitment of eosinophiils, and production of IL-4, IL-5, and IL-13, as well as IgE) is frequent in patients suffering from RVV (Fan et al., 2008; Romani, 1999; Weissenbacher et al., 2004; Witkin et al., 1989).

We reasoned that Th2 induction could associate to production of specific IgE, as Th2-related cytokines are responsible for the necessary B cell class switch. This is the case for most allergens, and atopic patients have been reported to develop IgE response to fungi (Savolainen et al., 1998). We then set up a series of biochemical assays to retrieve Th2-inducing *C. albicans* antigens using antigen-specific IgE from human sera as probes.

First, a series of *C. albicans* lysates were collected, that were either commercially available or prepared through glass bead-mediated cell disruption or sonication. For the in house preparation, both methods were used on each form of the fungus (yeast, hyphae), and as the composition of the 4 obtained lysates appeared to be slightly different, a pool was created that contained all of them, so to maximize the array of antigens suitable for our search (Figure 21a). The lysates were then used to identify *C. albicans*-specific IgE containing sera in ELISA. Assay plates were coated with lysate pool, sera obtained from a frozen bank of more than 450 samples from healthy donors were used as source of primary antibodies; a secondary anti-human IgE polyclonal antibody and a tertiary AP-conjugated antibody were used for the screening. Results are shown in Figure 21b as heat map, relative OD values are reported for each well. Out of all the screened samples, we could identify only a handful of *C. albicans*-specific IgE containing sera (as defined by OD value > 3 x background). This very low frequency was expected, as the fungus is not reported to induce considerable IgE response in healthy donors; besides, the presence of high titers of *C. albicans*-specific IgG (not shown) most likely overcompeted IgE binding, raising the threshold for detection of the latter. Presence of *C. albicans*-specific IgE in these sera was confirmed in subsequent control assays; titration experiments were performed (Figure 21c), and the relative EC50 was calculated (Figure 21d).

Finally, we set out to use the sera to identify antigens in Western blot. Lysates obtained from yeast or hyphal forms of *C. albicans* were used as a source of antigen, but Western blot failed to retrieve any band. However, when repeating the assay after performing lysate electrophoresis in native (non-denaturing) conditions, in a few cases it was possible to retrieve a band, at slightly different molecular weight in the two preparations, consistent with the notion that proteins undergo different post-translational modifications upon switch to filamentous growth (Chaffin et al., 1998). These results constitute a proof of principle for the use of IgE to identify *C. albicans* antigens. Of note, similar approaches have been successfully used for this purpose elsewhere (Fonseca et al., 2012).

We are now planning to optimize and extensively use this assay to verify whether bands of the same molecular weight can be consistently found in different donors, and then proceed to the identification of the recognized antigens through mass spectrometry. The identified proteins will then be tested with T cell libraries to ascertain the type of response induced *in vivo*. We believe this approach could bring particularly relevant results to those clinical conditions were an increased amount of anti-*Candida* circulating IgE or *in situ* allergic-like reactions have been reported.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	0.42	0.53	0.39	0.39	0.41	0.43	0.62	0.41	0.49	0.49	0.40	0.48	1.06	0.52	0.64	0.72
2	0.30	0.39	0.33	0.37	0.39	0.39	0.37	0.40	0.43	0.51	0.46	0.44	0.41	0.41	0.52	0.52
3	0.36	0.28	0.33	0.37	0.39	0.36	0.41	0.33	0.50	0.49	0.33	0.36	0.43	0.49	0.55	0.54
4	0.37	0.29	0.49	0.43	0.31	0.33	0.40	0.36	0.54	0.52	0.36	0.39	0.46	0.50	0.46	0.47
5	0.32	0.46	1.90	0.50	0.43	0.35	0.72	0.50	0.43	0.59	0.31	0.54	0.46	0.47	0.65	0.52
6	0.30	0.37	0.31	0.39	0.37	0.54	0.32	0.43	0.42	0.40	0.36	0.40	0.39	0.37	0.48	0.44
7	0.32	0.25	0.37	0.31	0.31	0.38	0.34	0.38	0.40	0.40	0.30	0.43	0.44	0.38	0.53	0.45
8	0.40	0.31	0.36	0.37	0.36	0.37	0.47	0.38	0.48	0.44	0.52	0.35	0.39	0.40	0.45	0.39
9	0.29	0.29	0.44	0.36	0.31	0.34	0.35	0.34	0.62	0.36	0.28	0.34	0.37	0.39	0.65	0.57
10	0.41	0.25	0.32	0.37	0.29	0.31	0.34	0.43	0.49	0.41	0.37	0.32	0.35	0.46	0.39	0.39
11	0.37	0.30	0.34	0.37	0.31	0.49	0.36	0.30	0.38	0.42	0.34	0.31	0.47	0.41	0.38	0.42
12	0.34	0.28	0.35	0.32	0.47	0.38	0.30	0.50	0.41	0.39	0.38	0.35	0.36	0.43	0.39	0.35
13	0.29	0.34	0.29	0.29	0.41	0.71	0.38	0.33	0.41	0.40	0.33	0.27	0.40	0.33	0.40	0.48
14	0.28	0.23	0.44	0.35	0.33	0.44	1.05	0.32	0.41	0.35	0.41	0.29	0.43	0.53	0.40	0.41
15	0.29	0.28	0.29	0.25	0.31	0.34	0.32	0.33	0.31	0.36	0.33	0.29	0.42	0.36	0.39	0.45
16	0.28	0.24	0.30	0.23	0.29	0.34	0.39	0.37	0.38	0.38	0.38	0.31	0.36	0.40	0.37	0.33
17	0.37	0.22	0.34	0.46	0.44	0.40	0.33	0.29	0.31	0.35	0.27	0.37	0.35	0.34	0.51	0.45
18	0.34	0.28	0.28	0.30	0.33	0.29	0.35	0.47	0.31	0.41	0.30	0.31	0.35	0.40	0.41	0.34
19	0.38	0.28	0.39	0.31	0.28	0.31	0.33	0.34	0.30	0.33	0.25	0.35	0.40	0.31	0.37	0.42
20	0.42	0.23	0.26	0.32	0.36	0.32	0.30	0.29	0.32	0.36	0.36	0.44	0.42	0.32	0.39	0.37
21	0.25	0.42	0.29	0.27	0.63	0.28	0.37	0.26	0.32	0.35	0.33	0.31	0.37	0.38	0.38	0.47
22	0.27	0.35	0.27	0.53	0.29	0.29	0.32	0.30	0.35	0.35	0.33	0.30	0.32	0.35	0.34	0.30
23	0.44	0.22	0.78	0.26	0.27	0.26	0.30	0.28	0.28	0.40	0.50	0.30	0.40	0.30	0.39	0.42
24	0.29	0.33	0.33	0.31	0.31	0.29	0.29	0.28	0.32	0.44	0.41	0.30	0.31	0.35	0.45	0.37

Serum #	EC50
15	0.0241
39	0.0313
139	0.051
24	0.0725
73	0.0962
89	0.1189
35	0.2006

Figure 21. (a) Gel electrophoresis of *C. albicans* lysates obtained by several procedures. Lysate 5 was obtained from commercial source (Greer) and presents a different pattern of glycosylation. (b) O.D. values from a 384 plate ELISA; coating was performed with lysates shown in a), detection with serum derived IgE. (c) Titration of *C. albicans*-specific IgE contained in a positive serum; for comparison purposes, 5 negative sera were added as control. (d) EC50 value for *C. albicans*-specific sera in 7 donors selected through screening shown in c) and calculated as in d). Each value represents the dilution factor of the serum for which 50% of maximal O.D. is achieved. (e) Native Western blot performed on *C. albicans* lysates and retrieved with one of the 7 sera shown in d).

6.2.5. *In vivo* assessment of the non-redundant role of T cells in long-term protection from *C. albicans* infection

Effective response to different types of infection requires differential involvement of separate arms of the immune system. For instance, it is well established that systemic candidiasis occurs in humans when the granulocyte compartment is defective, while T cell deficiencies lead to chronic or recurrent superficial infections (Hernandez-Santos and Gaffen, 2012).

Th17 have been recognized for several years as the main orchestrator of anti-fungal response, through the production of cytokines such as IL-17, which induces neutrophil recruitment and activation, and IL-22, which promotes antimicrobial peptide production by epithelial cells. However, the recent discovery of ILCs has challenged this view and raised the question whether these cells might be responsible for first-line defense and instruction of the forthcoming T cell response. Indeed, ILCs (together with other cell types like NK and γδ T cells) are characterized by the capacity to become readily activated and react to the presence of the pathogen as well as to tissue-derived signals within hours. For instance, ILCs have been recently shown to be required for protection in a model of oropharingeal candidiasis (Gladiator and LeibundGut-Landmann, 2013). However, IL-17-producing CD4 T cells have also been shown to confer protection in a similar model, and the differential contribution of both cell types at different stages of the infection seems a likely explanation (Conti et al., 2009; Hernandez-Santos et al., 2013).

The vaginal lumen is the most frequent anatomical location for *C. albicans* infection. Vaginal infections occur in more than 75% of women during a lifetime and can become recurrent in 5-10% of cases (Mosci et al., 2013). IL-23-dependent IL-17 but not IL-22 production (attributed to T cell) has been identified as fundamental for containment of *C. albicans* infection in the vagina (Pietrella et al., 2011). However, this work preceded the discovery of ILCs and therefore the relative eventual division of labor between innate and adaptive compartments at this location could not be investigated. Recent work from another group identified in fact ILC-derived IL-22 as the main trigger of a protective anti-*Candida* response in the vagina (De Luca et al., 2013).

We set out to assess the relative contribution of T cells in a model of *C. albicans* vaginal infection. When considering the previous published studies, we made several observations. First, the majority of the experiments were conducted within the first weeks of infection, thus analyzing only the early phases of the host-pathogen interplay, which is generally life-long in humans. Secondly, the commonly adopted treatment with estrogens, which increases mouse susceptibility to the infection, is likely to modify the overall tissue homeostasis and the physiology of the response (Mosci et al., 2013). Third, the use of mouse models that allow

discrimination between activation of different cellular compartments (innate *vs* adaptive, B *vs* T) or cytokine pathways has not been adequately exploited by most works to address this question.

In a first set of experiments (not shown) we could confirm that ILCs are necessary for initial containment of vaginal infection with *C. albicans*, as previously shown elsewhere (De Luca et al., 2013). An early population of $CD3^{-}CD90^{+}CD127^{+}$ ILCs producing the protective cytokines IL-17 and IL-22 was found in WT and *Rag*$^{-/-}$ mice upon infection, both in the vaginal tissue and in the draining lymph nodes. High levels of mortality in the first weeks post infection were displayed by *Rag-gc*$^{-/-}$ mice (that lack B, T, NK, ILCs), by *Rag*$^{-/-}$ mice treated with anti-CD127 antibody (lacking B, T cells and $CD127^{+}$ ILCs but not NK), *Rorc*$^{-/-}$ (lacking ILCs and Th17) and *Il23p19*$^{-/-}$ mice, that were defective in both IL-22 and IL-17 production, but not by *Rag*$^{-/-}$ or WT mice. We went on and found that IL-22 was the fundamental player in protection during this early phase, as *Il22*$^{-/-}$ mice, that produce normal amount of IL-17, displayed overlapping susceptibility to the infection as *Il23p19*$^{-/-}$ mice, thus succumbing to the infection in the first two weeks (data not shown; Gyuelveszi G. et al, manuscript in preparation). It thus appears that IL-23, possibly of DC-origin, is necessary to activate a first wave of response by IL-22 producing ILCs, as reported for other infection models (Kinnebrew et al., 2012).

We next wondered whether ILCs alone would provide long term resistance to the infection, and set up kinetics experiment to study the infection course through all its length. As expected, *Rag-gc*$^{-/-}$ and *Il23p19*$^{-/-}$ died shortly after infection; however, if waiting as long as 70-80 days after infection, we could observe a sudden onset of pathology also in *Rag*$^{-/-}$ mice, that would eventually lead to death in a few days (Figure 22a). On the contrary, WT mice would survive even at these later time points. CFU assessment from collected organs revealed massive *Candida* presence in the vagina, ovary/uterus, gut and stomach (Figure 22b and not shown), implicating failure of containment strategies and multi-organ spread of the pathogen. Notably, pathogen burden was proportionally lower in mice that had resisted longer to the infection, and were virtually absent in WT mice (Figure 22b), possibly reflecting the establishment of different types of equilibrium between the host immune system and the microbe. These data provide evidence that, although not required in the initial phase of the challenge, cells of the adaptive system, most likely T cells, are required for long-term containment of the infection.
All these experiments were performed under constant treatment with 17-β-estradiol, which promotes *Candida* infection by inducing formation of a squamous epithelium, increasing the cellular glycogen load, augmenting the pH and inhibiting the local immune response; however, at later time points this treatment resulted in pathology, provoking liquid

accumulation in the ovary and bladder, weight increase and ultimately death of all animals, including WT (not shown), as previously reported (Mosci et al., 2013).

We therefore decided to assess whether infection would be productive even in the absence of such treatment. To our surprise, despite doubling of the time required to promote *C. albicans* spread, infection was productive in *Rag*$^{-/-}$ animals (Figure 22c). Following animal sacrifice, consistent fungal growth could be detected in the vagina, gut and stomach of *Rag*$^{-/-}$ but not WT mice (Figure 22d).

Having established a more physiological setup for long term infection study, we asked whether T or B cell lack would be responsible for *Rag*$^{-/-}$ mice inability to control *Candida* infection at later time points. The fact that T cell- but not B cell-defects strongly correlate with chronic *Candida* infection at mucosal surfaces in humans argued in favor of the first being fundamental for the response. In fact, when performing vaginal infection in the absence of estrogen treatment, we could clearly observe that, beside *Rag-gc*$^{-/-}$ animals being highly susceptible to the infection, *Cd3e*$^{-/-}$ mice lacking T cells, but not µMT$^{-/-}$ mice lacking B cells, succumbed to the infection, at even earlier time points when compared to *Rag*$^{-/-}$ mice (Figure 22f).

Taken together this data show that IL-22-producing ILCs are important to promote protection during the first phase of vaginal infection with *C. albicans*. However, such early response does not achieve pathogen clearance, and a certain fraction of fungal particles survives, slowly grows and finally takes over the animal in the absence of a productive T cell response. We thus provide evidences that T cell are necessary to control *C. albicans* growth and spreading at later time points during infection, a concept that is compatible with the well established onset of chronic or recurrent candidiasis in patients suffering from T cell deficiencies.

Whether and how ILCs instruct or regulate the forthcoming T cell response, and what type of T cells are required to guarantee protections, are questions that are right now being addressed in our laboratory.

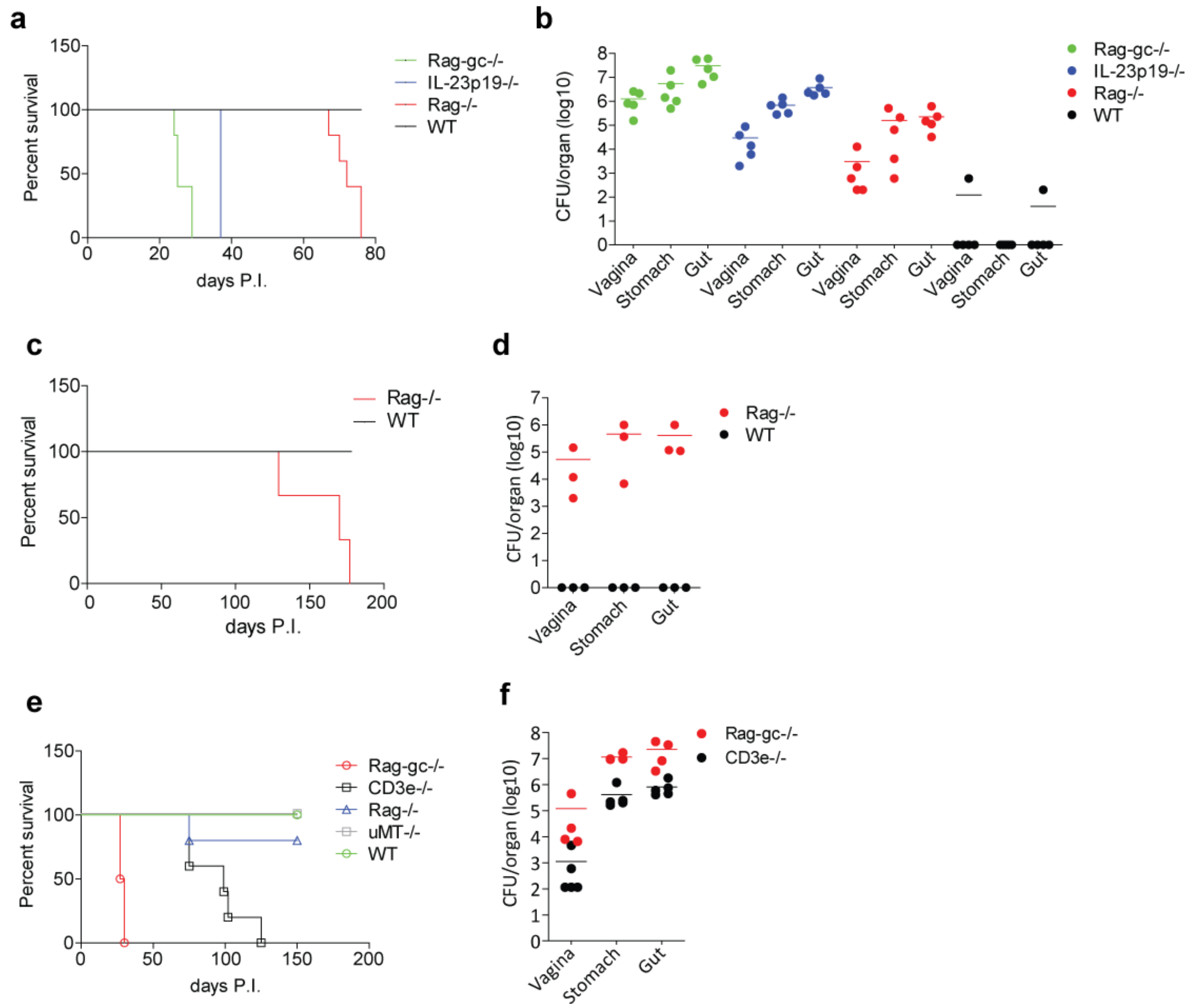


Figure 22. T cells are indispensable to protect the host from *C. albicans* vaginal infection at late time points. (a) Survival profile upon *C. albicans* infection. Mice of the indicated strains were injected with 17-β-estradiol every week starting 7 days before infection and treated for all the duration of the experiment. At d0 and d1 mice were infected with intra-vaginal delivery of $5x10^5$ particles of live *C. albicans* (10^6 conidia in total). Animals were sacrificed according to humane endpoint guidelines. (b) Pathogen burden in organs from animals sacrificed in the experiment shown in a). (c) Survival curve for mice infected in the absence of 17-β-estradiol treatment; other than that, experiment was performed as in a). (d) Pathogen burden of mice from experiment shown in c). (e) Survival curve for mice infected as in c). (f) Pathogen burden of mice from experiment shown in e).

7. Discussion and Outlook

In response to pathogens or vaccines, CD4$^+$ T cells proliferate and differentiate toward distinct fates (namely Th1, Th2, Th17), endowed with peculiar phenotypic and functional properties. Each subset is believed to be optimal for tailoring the response to the eliciting threat, and provide effective memory in case of further attacks. For a long time, the possibility that an heterogeneous response could be generated to any encounterd pathogen was not considered; recent evidences however point towards an overall high plasticity of the immune system, and of the T cell compartment in particular. Preliminary results from our laboratory (Zielinski et al., 2011), showing the incomplete homogeneity of cells sharing gross specificity (*i.e.* responding to the same pathogen or vaccine) prompted us to investigate this issue in the human system.

In the present work, we have interrogated the memory T cell response of healthy donors to *C. albicans*, *M. tuberculosis* and the vaccine antigen TT, with a variety of cellular and molecular high throughput assays. The use of chemokine receptor expression to obtain cells from differently polarized T helper cell subsets (Sallusto and Lanzavecchia, 2009) allowed us to simultaneously dissect the repertoires of pathogen or antigen-specific cells in Th1, Th2, Th17 and non-classical CCR6$^+$ Th1* compartments, each characterized by unique homing properties and effector capacities (cytokine production). We present striking evidences that the pool of T cells that respond to a given microbe or vaccine is indeed extraordinarily heterogeneous, challenging a current dogma in cellular immunology (see *Graphical abstract* below).

In the case of *C. albicans* and *M. tuberculosis*, the response was strongly polarized to one or few subsets (Th17/Th1* and Th1*, respectively), that are known to mediate protection from the eliciting pathogen in healthy donors (Acosta-Rodriguez et al., 2007b; Lindestam Arlehamn et al., 2013). However, other subsets contributed to the response; all the 4 tested compartments, for instance, contained *C. albicans*-specific T cells, and we could estimate that supposedly non-protective Th2 (Romani, 1999) have a only 8-10 times lower frequency than “protective” Th17 in the blood of healthy individuals. Besides, we determined the antigenic fingerprint of each analyzed subset and found that different Th compartments preferentially or uniquely target a peculiar set of *C. albicans*-derived antigens, possibly reflecting the strength of the received TCR stimulation (antigen abundance and physical properties) or the presence of selected antigen-associated PAMPs promoting polarization. Novel Th17-driving immunodominant antigens could be identified, both via high throughput synthesis-based approach and biochemical fractionation of the fungal cell wall, where they appear to be

concentrated. A biochemical approach was also set to retrieve additional Th2-related antigens making use of serum IgE. Expanding previous observations from several groups (Lenarczyk et al., 2000; Livingston et al., 2013; Rivino et al., 2004; Rowe et al., 2000), we also show that response to TT is characterized by the highest level of heterogeneity, with a virtually even distribution of responding cells among all tested subsets.

Through deep sequencing-based analysis of clonotypic composition, we could show that antigen- or pathogen-specific T cell populations contained cells bearing the same Vβ in different subsets. These cells were isolated, and the identity of the TCR was confirmed on both the α- and the β-chain. Assessment of the cytokine profile, chemokine receptor and transcription factor expression, proved the heterogeneity of such clones to hold true. We therefore postulate that heterogeneity relies on distinct clones receiving different signals during priming or antigen re-encounter, as well as on intraclonal differentiation (see *Graphical abstract* below).

These findings are somehow surprising, as they force us to rethink the *in vivo* mechanisms of T cell polarization, as well as the contribution of minor subsets to the generation of an effective response. The large variety of PAMPs displayed by a complex pathogens that activate differential polarizing programs in DCs and the stochastic stimulation and restimulation in different tissues offer likely explanations to our results. Besides, variables such as antigen dose, strength of TCR triggering, type of involved APCs among others could possibly contribute to complicate the outcome of a T cell priming.

It may be the case that minor polarization events are just bystander products of a still remarkably efficient process of fate instruction. However, it is tempting to speculate that types of response that have been considered detrimental in certain infection models, as it is the case for Th2 in candidiasis, may instead be helpful if correctly balanced into a wider context, and that the immune system has positively selected for their occurrence. Broadening the equipment of pathogen specific cells with different migratory capacities would in principle ensure patrolling over an increased body surface; the detrimental effect of a suboptimal type of response would possibly be rapidly overcome by in situ recruitment of the most numerous protective T cells upon inflammation trigger. A further possibility is that cells that have been initially primed toward a certain fate, as it is the case for Th17 in *C. albicans* infection, undergo phenotypic switch upon restimulation under skewing condition; allergic inflammation or vaccination with Th2-polarizing adjuvants (such as the broadly employed alum) could provide such environment. Several reports in the literature furnish proof of concept for this mechanism (Cosmi et al., 2010; Wang et al., 2010b).

The antigenic fingerprint we obtained for *C. albicans* responding cells of different subsets is a valuable source of information on the biology of the infection as well as on the dynamics of T cell response to a microbe. We could indeed identify novel potent proteins that would be suitable as vaccine targets. T cell response is believed to play a non redundant role in mediating protection from fungal attack, and the search of immunodominant antigens to elicit a strong T cell activation started several decades ago (Cassone, 2013). Nonetheless most reports on *C. albicans* antigens were based on induction of a humoral response as a read out for immunogenic properties. Here we determine a broad panel of antigens (at least 15) that are widely and strongly recognized by T cells in a sizable number of healthy donors. Notably, these targets were identified through in parallel-screening of as many as 80 antigens, to our knowledge the broadest analysis of this type ever reported in the literature, thus providing a series of unbiased internal controls to establish immunogenic hierarchy among *C. albicans* proteins. We believe this data will be useful to drive a more rational approach for developing a prophylactic or therapeutic vaccine against *C. albicans.*

On a more fundamental level, this analysis allows us to speculate on the generation of the detected types of response. Antigenic fingerprints of Th17 and Th1* cells appear almost superimposable; this argues in favor of either a similar antigenic requirements for the induction of such phenotypes, or a strong relationship between the two compartments, possibly interconnected by phenotypic switch from one another. This view is supported by other findings in the literature (Cosmi et al., 2008; Hirota et al., 2011) as well as by our clonotypic analysis (see below). Th2 cells show a partially overlapping fingerprint with cells of the $CCR6^+$ compartment, revealing that some antigens preferentially associate with that kind of response, while some would not; again, clonotypic composition was in line with this prediction. Notably, $CCR6^-$ Th1 cells weakly responded to any of the tested antigens, raising the question whether these cells are generated following presentation of a different set of proteins, or may be cross-reactive cells primed in the context of a different microbial infection. Both scenarios appear to be likely in the light of our additional findings on the TCR repertoire, as well as of work from other laboratories in a different context (Birnbaum et al., 2014), as already discussed in the dedicated session (see *Graphical abstract* below).

This set of data has implications for the understanding of the immune response to infectious agents, as it suggests that only few antigens can be efficiently targeted by the immune system, while many others can be exploited as decoys in a previously unseen manner: by inducing a response of ineffective type. Whole pathogen-based vaccines could be built taking advantage of this notion and be generated by inducing over-expression of the protective antigens or forcing down-regulation of the secondary ones.

Isolation of heterogeneous clones provides the first strong evidence in support of a "one cell-multiple fate" model for T cell differentiation in humans. This is in line with many experimental findings in the mouse system, mostly with respect to the CD8$^+$ compartment and the development of heterogeneous population of memory T cells (Gerlach et al., 2010; Reiner et al., 2007; Stemberger et al., 2007). We are aware that homeostatic proliferation generates sizable populations of unexperienced T cells bearing identical TCR; we cannot rule out the possibility that the presence of phenotipically different "sister" memory T cells observed in our system reflects spatially and temporally distinct priming events on "sister" naïve cells. However, the fact that we could retrieve heterogeneous clonotypes even upon *in vitro* priming of a reduced number of naive T cells argues in favor of differentiation happening at a single precursor level.

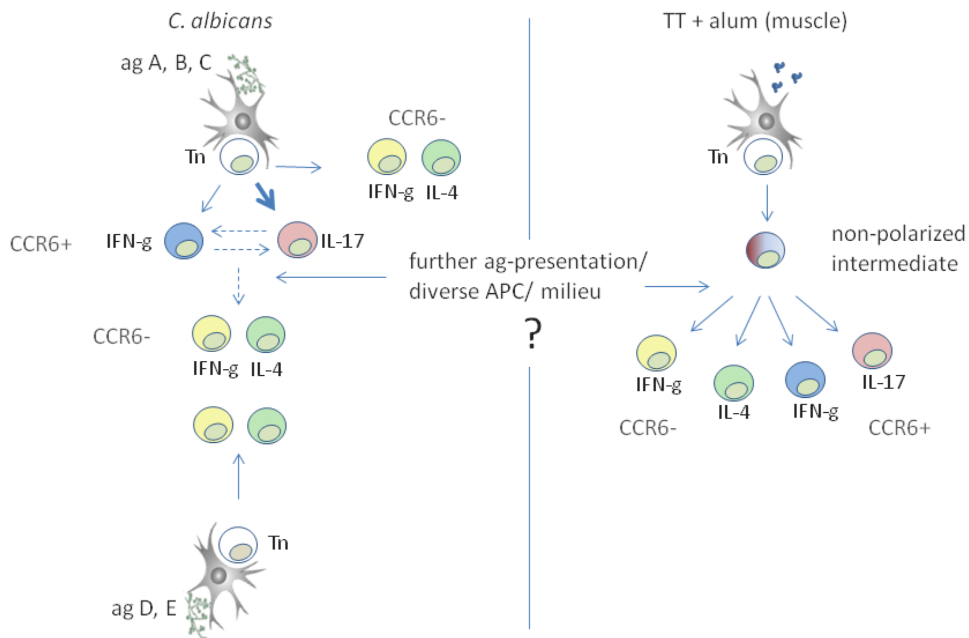
We are planning to confirm this finding in the context of single cell priming: we have estimated that 1 CD4$^+$ T cell out of 5,000 is specific to *C. albicans* in the naïve repertoire, therefore by performing *in vitro* priming of a reduced number of naïve T cells (1,000/well) we could ensure that only one single precursor can be primed in each culture. Single cell PCR or secretion assay could be used to ascertain the fate of the generated progeny in proliferating cultures, and TCR analysis (made possible by our *in house*-developed RT-PCR-sequencing approach) will allow confirmation of clonality. The following step will be to dissect the signals that drive such intraclonal differentiation (asymmetric division, differential APC interaction with daughter cells, role of microenvironment, etc).

We hypothesized that heterogeneity of T cell response must respect some constrains in terms of ratio between different yielded subsets in order to provide effective immunity. Indeed, we found that *C. albicans*-specific circulating T cells in patients suffering from CMC caused by inborn STAT-1-GOF mutations were preserved in number, but severely altered in phenotype. In particular, microbe-specific T cells were mainly found within the IFN-γ/IL-4/IL-5 producing Th1 subset, thus greatly unbalancing the ratio of non-protective *vs* protective (IL-17, IL-22) cytokines in these patients.

We have now identified two main follow up studies to this analysis. On the one hand, we would like to analyze in detail the antigenic fingerprint and fine phenotypic features of *C. albicans*-specific T cells in these patients, taking into account different anatomical locations. Indeed, we wonder whether the information we obtained from the analysis of the circulating compartment is somehow predictive of the peripheral scenario. By utilizing the antigens we have shown to preferentially induce Th1, Th2 or Th17 responses we will address the question whether the antigenic fingerprint remains unaltered in these patients or is rather skewed toward the recognition of secondary or "non-protective" antigens. Secondly, we will try to

characterize the phenotype of T cells in skin biopsies and to compare their TCR repertoire to that of blood borne T cells; a wide variety of assays could suit to this purpose, both on single cells (RT PCR, immunohystochemistry, single cell TCR sequencing) or upon restimulation of tissue-recovered T cells. This should provide insights on the relationship between the circulating and the peripheral T cell compartment and show which type of T cells that reside in tissue are recruited from blood and whether in situ-received signals modify their biology. On the other hand, we are currently expanding our analysis of the T cell memory response to other cohorts of patients that fail to efficiently control *C. albicans* growth at mucosal surfaces. We have obtained samples from women suffering from RVV (in collaboration with Dr. Mihai Netea, Radboud Medical University, The Netherland), and preliminary results from *ex vivo* restimulation and libraries on memory T cells seem to suggest a major involvement of the Th2 subset in *C. albicans* recognition (Becattini S., De Gregorio C., et al., unpublished). Collectively these data point toward a generally unbalanced T cell response as a cause for *C. albicans* overgrowth at mucosal surfaces. Of note, this is in line with our findings on the mouse system, where we could prove T cells to be necessary for *Candida* containment and eventually eradication upon vaginal infection. Transfer experiment of *in vitro* differentiated T cells are ongoing to discriminate the phenotypic requirements for T cell-mediated protection in this experimental set up. Based on data in the literature we can predict that Th17 will prove to be the most effective cells in control of the infection; even in this case, it would be interesting to assess whether the presence of a certain fraction of cells with different homing and effector properties would provide a selective advantage and confer broader resistance or would rather be detrimental.

In conclusion, we believe our work unravels previously unappreciated features of the T cell response to pathogens and vaccines in physiological and pathological conditions, and paves the way for the development of new exciting research areas in the field of T cell immunology.



Graphical abstract. Heterogeneous T cell responses to pathogens and vaccines. Potential mechanisms for generation of heterogeneous T cell responses. Left panel: antigens A, B, C generate primarily $CCR6^+$ Th17, but also minor T helper subsets; Th17 cells tend to switch their phenotype, possibly due to further antigen presentation or exposure to a skewing environment. Other antigens, instead, depicted as D, E, preferentially generate $CCR6^-$ Th1 and Th2 responses. Right panel: in response to vaccination with TT, possibly due to the presence of alum and the site of injection, uncommitted intermediates are generated that subsequently undergo stochastic differentiation toward any Th fate. This would explain why the response appears to be evenly distributed among all different T cell compartments.

8. Experimental Procedures

Cell Enrichment and Sorting

Blood from healthy donors was obtained from the Swiss Blood Donation Center of Basel and Lugano, and used in compliance with the Federal Office of Public Health (authorization no. A000197/2 to F.S). Peripheral Blood Mononuclear Cells (PBMCs) were isolated with Ficoll-Paque Plus (GE Healthcare). Monocytes and total CD4 T cells were isolated by positive selection using CD14 and CD4 magnetic microbeads respectively, or by negative selection using memory CD4 isolation kit (Miltenyi Biotech). Memory T helper cell subsets were sorted to over 97% purity as follows after gating on $CD8^-CD14^-CD16^-CD19^-CD25^-CD56^-$ $CD45RA^-$ cells: $CCR6^-CXCR3^+CCR4^-$ Th1, $CCR6^-CXCR3^+CCR4^-$ Th2, $CCR6^+CXCR3^+CCR4^-$ Th1*, $CCR6^+CXCR3^-CCR4^+$ Th17. Naïve T cells were sorted as $CD8^-CD14^-CD16^-CD19^-CD25^-CD56^-CD45RO^-CD45RA^+CCR7^+CD95^-$. The following α-human surface marker antibodies were used for staining: CD45RA FITC (ALB11), CD45RO PE (UCHL1), CD8 PE-Cy5 (B9.11), CD14 PE-Cy5 (RMO52), CD16 PE-Cy5 (3G8), CD19-PE-Cy5 (J3-119), CD25 PE-Cy5 (B1.49.9), CD56PE-Cy5 (N901) (all from Beckman Coulter), CCR6 PE (11A9), CCR4 PE-Cy7 (1G1) (BD Biosciences), CXCR3 AlexaFluor 647 (G025H7), CCR7 BV421 (G043H7), CD95 APC (DX2) (Biolegend). Cells were stained on ice for 15-20 minutes and sorted with FACSAria III, BD. Viable IFN-γ^+, IL-4^+ and IL-17^+ cells were FACS-sorted using cytokine secretion assay (Miltenyi Biotec).

Ex-Vivo T Cell Stimulation and Intracellular Staining

T cells were cultured in RPMI 1640 supplemented with 2 mM glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin (all from Life Technologies), and 5% human serum (Swiss Red Cross). For some experiments up to 500 IU/ml IL-2 was added to the medium. 50000 sorted T cells were labeled with CFSE and cultured for 6 days (memory) or 12 days (naive) in the presence of 25000 autologous irradiated monocytes pre-pulsed for 3-5h with the antigen of interest. Proliferating cells were then sorted based on CFSE dilution, stained for surface markers or intracellular cytokine production upon stimulation with PMA/Ionomycin for 5h, BFA being added for the last 2.5 h (all reagents from SIGMA-ALDRICH). Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions and then stained with the following anti-cytokine antibodies: IL-17A eFluor660 (64DEC17), IL-22 PerCP-eFluor710 (22URTI)

(eBioscience), IFN-γ APC-Cy7 (4S.B3) (Biolegend), IFN-γ FITC (B27), IL-4 PE (MP425D2) (BD Biosciences). Cytokine concentration in supernatants was assessed using FlowCytomix assay (eBioscience) according to manufacturer's instruction.

Microbes/Antigens

C. albicans strain SC5314 was cultured in YPD medium for 16h at 30°C, extensively washed in PBS and heat-inactivated at 65°C for 30 minutes. Ratio used for stimulation assays was 3 particles per monocyte. Lysate was prepared from mixed cultures of conidia and hyphae. Briefly, *C. albicans* was cultured in YPD medium for 16h at 30°C or in YPD supplemented with 10% human serum for 16h at 37°C. The resulting cultures yielded almost pure populations of conidia and hyphae respectively. Cells from the two cultures were extensively washed, resuspended in PBS with complete protease inhibitor cocktail (Roche), mixed and sonicated on ice for 30 consecutive cycles (45 sec on, 90 sec off, amplitude= 100). The suspension was then centrifuged at max speed for 15 minutes, and supernatant collected and sterile filtered through 0.22 μm pore membranes. Presence of a broad array of proteins was assessed with Imperial Blue staining (Thermo Scientific) of polyacrylamide gel, and quantification was performed using Bradford reagent (Bio-Rad). *C. albicans* lysate was used at a concentration of 2.5 μg/ml in addition to HK conidia, Mycobacterium tuberculosis lysate (strain H37Rv, bei Resources) and Tetanus Toxoid (provided by G. Galli, Novartis Vaccines, Siena, Italy) were used at 5 μg/ml.

T Cell Libraries

The method has already been described in greater details elsewhere (Geiger et al., 2009). Briefly, T cells (500-2,000 cells/well, depending on the subset) were polyclonally expanded in the presence of 1 μg/ml PHA (Remel, Thermo Scientific), irradiated (45Gy) allogeneic feeder cells and IL-2 (500 IU/ml). 24-96 wells per subset were generated for each donor. After 14-21 days, T cells from each well were extensively washed restimulated in the presence of autologous monocytes pre-incubated with the antigens of interest, and proliferation was assessed at day 4 after 16 h incubation with 1μCi/ml [^{3}H] Thymidine (GE Healthcare). Precursor frequencies were calculated based on numbers of wells that scored negative for proliferation according to the Poisson distribution and expressed per million cells (Geiger et al., 2009).

Amplification of TCR-Vβ genes

Individual T cell clone total cDNA was obtained from 1,000-10,000- cells/reaction. Reaction was carried out using Oligo dT(15) primers (Promega) and SuperscriptIII (Life technologies) reverse transcriptase, in a reaction mix containing DTT, NP40, dNTPs, RNAsin (Promega). Reactions were run with the following program: 42°C x 10min, 25°C x 10min, 50°C x 1h, 94°C x 5min. cDNA (3 µl) were added to a PCR mix (final volume 25 µl) containing PfuUltra II Fusion HS DNA Polymerase (Agilent Genomics). Sequences were amplified using one or both the designed TCR Vβ-specific forward primer pools (pool fw1, pool fw2, see extended material) and TBC-rev reverse primer pairing to C1-C2 β chain constant region with the following program: 95°C x 1min; (95°C x 20sec; 50°C x 20sec; 72°C x 30sec) x 45 cycles; 72°C x 3min. Sequence amplification was assessed through agarose gel electrophoresis; successfully amplified fragments were sequenced through Sanger method using TBC-Rev primer.

RNA Extraction and Transcription Factor qRT-PCR:

Total RNA was extracted using TRIzol reagent (Life Technologies) or E.Z.N.A. DNA/RNA Isolation Kit (OMEGA bio-tek) according to manufacturer's instructions. qScript cDNA SuperMix (Quanta Biosciences) was used for cDNA synthesis. Transcripts were quantified by qRT–PCR on an ABI PRISM 7900HT with predesigned TaqMan Gene Expression Assays: *RORC* (Hs01076122_m1), *TBX21* (Hs00203436_m1), *GATA3* Hs00231122_m1 (Life Technologies). Expression of target genes was normalized to 18S ribosomal RNA (Applied Biosystems) and expressed as arbitrary units (A.U.).

TCR Vβ Next-Generation Sequencing

Antigen specific cells were obtained as above. A minimum number of 10^6 cells was obtained for all experiments, and each sample was split in two, half of which was frozen as a backup. In case cells sorted upon stimulation did not reach the required number, they were expanded for 1-5 days in the presence of 50 IU/ml IL-2. Cells to be analyzed through TCR Next Generation Sequences were centrifuged and washed in PBS, and genomic DNA was extracted from the pellet using QUIAamp Micro Kit (QUIAGEN) according to manufacturer's instruction. gDNA quantity and purity were assessed through spectrophotometric analysis. Next generation sequencing (NGS) of CDR3 Vβ regions was performed by Adaptive Biotechnologies Corp. (Seattle, WA) using the ImmunoSEQ assay (www.immunoseq.com).

Briefly, following multiplex PCR reaction designed to target any CDR3 Vβ fragment, amplicons were sequenced using the Illumina HiSeq platform. Raw data consisting of all retrieved sequences of 87 nucleotides or corresponding amminoacidic sequences and containing CDR3 region were exported and further processed using in house-developed software. If not differently stated in the text the assay was performed at Survey level (detection sensitivity: 1 cell in 40,000); for a few samples a Deep level analysis was used (detection sensitivity:1 cell in 200,000).

TCR Vβ Sequence Analysis

Data sets of TCR-β sequences were analyzed using algorithms written in Java. DNA sequences containing frame shift or stop codons were removed prior to analysis. The experimental noise was determined for each batch of experiments with parallel analysis a pool antigen specific memory T cells (obtained as depicted above) that was split in two before DNA extraction and sequencing. Sequences that were present in only split were compared with the shared sequences. Using the BLASTn algorithm (Camacho et al, 2008), each sequence that had one or two mismatches with one of the shared sequences, was considered as a PCR error and removed from the analysis. Finally, all the sequences (shared and not shared) were plotted together. For each control, we selected the non–shared sequence with the highest number of reads and set that value as threshold. Sequences with a value of reads below threshold level were deleted from all experiments. Retrieval of the NGS identified clones was performed through sequence similarity analysis using BLASTn (Camacho et al, 2008).

Control Experiments on TCR Vβ Sequencing

The probability to find the same T cell clone in two different samples is limited by the size of the samples, the frequency and responsiveness of a given cell, and the sensitivity of detection. To take into account these factors in our analysis, we performed two types of controls (Figure 23). In a first set of experiments, we stimulated $1.5x10^6$ Th17 cells with CA, and divided the $CFSE^{lo}$ T cells in two halves that were separately subjected to genomic DNA extraction and sequencing. In the representative experiment shown in Figure 23A (left panel), 905 and 910 clonotypes were found in the two halves. Of these, 727 (representing 99.4% of total reads) were present in both samples. The reason why the remaining 0.6% of the reads were detected only in one of the two halves remains unclear and may be related to the sensitivity of detection or to the unequal split of very low numbers of expanded cells. In a second series of

experiments, we sorted $1.5x10^6$ Th cells from the four subsets *ex vivo* and split the cells in two halves that were stimulated with CA in the presence of autologous monocytes. TCRVB CDR3 analysis was then performed on genomic DNA from $CFSE^{lo}$ cells that had proliferated independently in the two cultures from each subset. In one representative experiments, 97%, 94%, 90% and 90% of total reads were found to be shared between the two samples of Th1, Th2, Th1* and Th17 cells, respectively (Figure 23, right panels). In addition, for most of the clonotypes, there was a remarkable correlation in the number of reads recorded in the two samples. We concluded that, due to the *in vivo* expansion of T cell clones, a sample of $1.5x10^6$ T cells was sufficient to screen more than 90% of the CA-specific repertoire. Based on these findings, we also introduced a cut-off value equal to the number of reads associated with the first clonotypes found only in one of the two halves of a sample included as control. This cut off value excluded clonotypes that accounted for a small percentage of the total response (Figure 23B,C), for both *C. albicans* and TT analysis.

Peptide Bioinformatic Anlalyses-Production

80 *C. albicans* proteins with different cellular location and functions (cytosolic enzymes, cell-wall associated structural and enzymatic proteins, extracellular hydrolases) were selected based on a literature screening for reported immunogenic activity. All 15-mer peptides overlapping by 10 residues spanning each sequence were extracted using bioinformatics tools (8059 15-mers in total). Next, each peptide was scored for predicted binding to a panel of 24 common different HLA class II DR, DP and DQ molecules for which binding assays are available. For 23 of the molecules the peptides were scored utilizing the recommended IEDB consensus algorithm; for one molecule (DRB3*02:02) the IEDB recommended method was NetMHCIIpan. For each molecule we utilized a consensus score ≥ 20 % to define a binder. Finally, a set of 1273 peptides was selected corresponding to those peptides scoring in the top 20% for 12 or more of the 24 class II molecules probed. These selection criteria are similar to what has been recently used in other recent works. (Lindestam Arlehamn et al., 2013; Oseroff et al., 2012). Each protein was represented by a minimum of 4 to a maximum of 51 peptides (average 16.8). The peptides were synthesized by A and A (San Diego, CA) as crude material on a small (1mg) scale, individually resuspended in DMSO and pooled accordingly to the belonging protein. Individual peptides and pools were divided in aliquots and stored at -80°C.

Cell Wall Mannoprotein Extraction

C. albicans strain SC5314 was cultured in YPD or LEE's medium for 16h at 30°C. Harvested conidia were extensively washed in PBS, pellets weighted and resuspended in 0.5ml of 20mM Na citrate buffer per g of yeast wet weight. Samples were autoclaved for 1.5h at 121°C and spun at max speed for 15'. Supernatant, containing highly soluble mannoproteins, was harvested and froze at -20°C. The following day a mix of equal volumes of Fehling solution I (7% hydrate copper(II)sulfate in 100ml H2O) and Fehling solution II (35% potassium tartrate + 10% NaOH in 100 ml H2O) was prepared and added to the thawed supernatant in a ratio 1:1 for 30'. After centrifugation for 15' at max speed a pellet was obtained that derived from precipitation of mannoproteins. Supernatant was removed and pellet dissolved in 70µl of 3N HCl to promote dissociation of Cu^{2+} ions from tartrate complex. Proteins were then precipitated upon addition of a mixture 8:1 MetOH + Acetic acid, incubation for 1h on a rotating wheal at 4°C and centrifugation. This step was repeated at least twice. Finally, two steps of wash/dehydration were performed with MetOH and Ether respectively. Pellets were dried with a vacuum pump and frozen at -80C. Samples were resuspended in water and quantified using Bradford reagent (BIO-RAD) prior to use.

ELISA of Serum Anti-*Candida* IgE Detection

C. albicans lysate (Greer) in Carbonate buffer (pH 9.6) was used at 10ug/ml to coat 96 half area or 384 shallow ELISA plates (Cornig) overnight at room temperature. Plates were blocked by incubation in BSA 1% (Sigma-Aldrich)for 1h. Different dilutions of sera from healthy donors were used as source for primary antibodies (IgE) and incubated for 2h. Secondary antibody polyclonal rabbit anti-human IgE (Dako, 1:1000) was added, and incubated for 1h at room temperature. Goat-anti rabbit IgG-AP (1:500) was finally added and incubated for 1h at room temperature. Used substrate was 4-Nitrophenyl phosphate disodium salt hexahydrate (NPDSH) (Sigma-Aldrich) resuspended in carbonate buffer according to manufacturer's instruction. At least 4 washes in PBS Tween 0.05% were performed in between different incubation steps; all antibodies were diluted in PBS + 1 % BSA. O.D. was determined by consecutive readings at λ=405. All samples were run in duplicate and controls comprising all combinations of coating, primary, secondary and tertiary antibodies included in each assay to determine the level of background.

Non Denaturing Condition Western Blot

C. albicans lysate samples were run on a native pre-cust 3-12% polyacrylamide gel according to manufacturer's instructions (Life Technologies, Native PAGE Novex Bis-Tris Gel System). After electrophoresis run, samples were transferred on a PVDF membrane using fast/dry mode. Blocking was performed in milk 5% for 1h. Blotting was carried out in PBS Tween 0.05%. Membranes were incubated with primary antibody (serum dilutions in PBS + BSA 1%) overnight at 4°C, followed by 3 washes of 5' each. Secondary antibody (anti human IgE-HRP) was added for 30', followed by 3 washes. Blot was developed with ECL substrate (Pierce) for 5 min before reading.

Mouse Infection

Vaginal infection: β-estradiol 17-valerate (SIGMA, 200 µg/mice) was solubilized in 150 µl of ethanol and brought to final volume of 100 µl/mouse in sesam oil. Isofluorane-anesthetized animals were injected on the back every week for the whole duration of the experiment starting from d-7, considering d0 the first day of infection. *C. albicans* strain 3153a, which has been reported to be optimal for induction of vaginal infection, was cultured in YPD medium for 16h at 30°C on rotator. Cultured conidia were confirmed to be in log phase growth by λ 660 nm O.D. measurement, then washed twice with PBS, and delivered into the vagina of asleep animals in a volume of 10ul. $5x10^5$ particles per mouse were delivered for two consecutive days (10^6 particles/mouse in total); particle number was determined through O.D. measurement, considering that O.D.=1 corresponds to 10^7 conidia/ml). Mice were weighted on a weekly up to daily basis depending on the stage of the infection, and euthanized accordingly to humane endpoint criteria. Vaginal lavage was performed in 100µl of PBS on asleep animals, and seeded on Sabouraud plates in duplicates. Colony forming units were assessed upon o.n. culture at 37°C. Organs (vagina, gut, stomach) were removed from sacrificed animals, smashed and seeded on Sabouraud plates, CFU being assessed as for lavage. Oropharingeal infection: C57BL/6 mice were set aspleep with isofluorane and infected by placing a cotton ball saturated with 2×10^{7} CFU *C. albicans* (SC5314) sub-lingually for 75 min, as previously described (Conti et al., 2009). Lymphnodes were collected at day 8 p.i. and processed as described above. For the immunization experiments mice were either infected as described above or challenged with sub-cutaneous injection of CWE (100 µg) + monophosphoryl lipid A (MPL, 10 µg, Sigma). Draining lumbar lymph nodes were collected, smashed, washed with PBS, digested for 75-90 minutes in the presence of Collagenase D and DNAse (both from Roche), stained for flow cytometric analysis. Utilized

antibody clones were: CD4 (RM4-5), CD44 (IM7), IL-17A (TC11-18H10), IFN-γ (XMG1.2). Live-Dead staining (Life Technologies) was always performed on the acquired samples.
Animals were treated in accordance with guidelines of the Swiss Federal Veterinary Office, and experiments were approved by the Dipartimento della Sanità e Socialità of Canton Ticino.

Statistical Analysis

Statistical analysis was performed with the Prism software (GraphPad). Data in columns represent mean ± s.e.m. values, and significance was assessed by non-parametric unpaired (Friedman) or paired (Kruskal-Wallis) tests. Non-parametric Spearman correlation coefficient was determined for shared sequences.

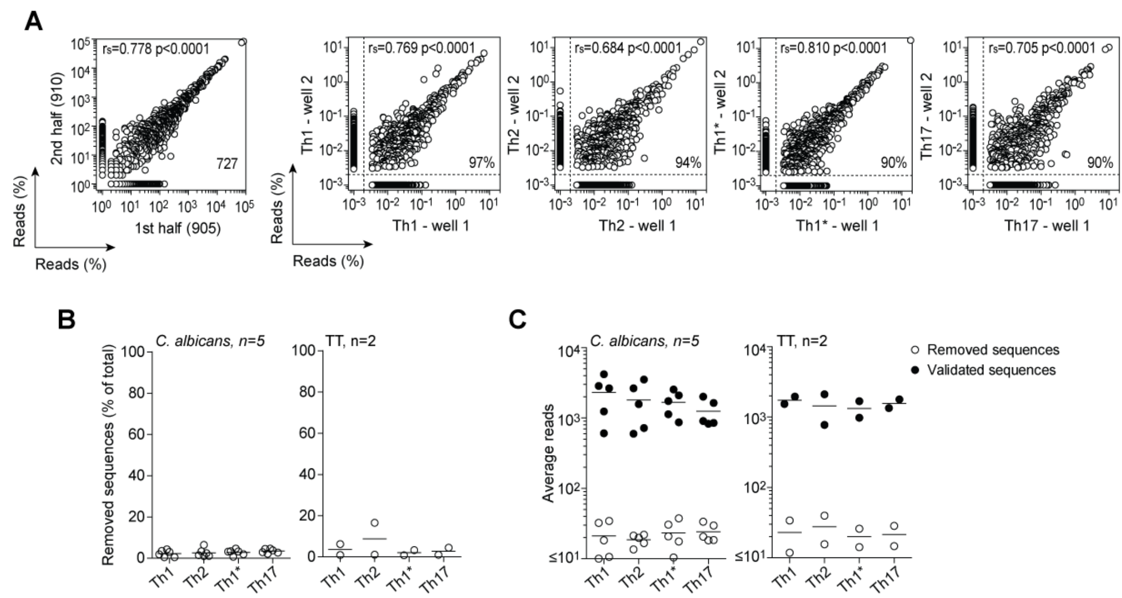


Figure 23. Control experiment on TCR sequencing. (A) Left : Anlaysis of a split sample. *C. albicans*-specific CFSE^{lo} Th17 cells were obtained as described and split in two halves. A pellet was obtained from each half, gDNA was extracted and analyzed through TCR NGS. The plot shows the relative frequency of each identified sequence in the two samples. The value of frequency corresponding to the most expanded clone that was possible to retrieve only in one of the two halves was used as a cutoff. Similar controls were run for each batch of experiments. Right: Parallel stimulation. T cells were sorted as described in the text, each subset was divided in two halves that were stimulated in parallel with the antigen of interest (shown is TT). Values represent Spearman correlation coefficient and statistical significance. (B) Cumulative frequency of sequences deleted after cutoff application. Each dot represents a donor. (C) Average number of reads for deleted sequences (white dots) and validated sequences (black dots); each dot represents a donor.

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10. Curriculum Vitae

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Education

- January 2010 – present: **PhD student** at the Department of Microbiology, ETH Zurich (Switzerland), Experimental work at the Institute for Research in Biomedicine, Bellinzona (Switzerland)
- January 2009: **Board Certification in Biology**, University of Florence (Italy)
- November 2008: **M.Sc. in Medical Biology**, *summa cum laude*, University of Florence (Italy)
- April 2006: **B.Sc. in General Biology**, *summa cum laude*, University of Florence (Italy)
- July 2002: **High School Diploma**, Liceo Scientifico "Leonardo da Vinci", Florence (Italy)

Training experiences

- January 2010 – present: **PhD training**, Institute for Research in Biomedicine, Bellinzona (Switzerland)
  Supervisors: Dr. Federica Sallusto and Prof. Antonio Lanzavecchia
  Research project: "Heterogeneity of the human T cell response to microbes and vaccines"
- June 2009 – December 2009: **Marie Curie Host Fellowships - Early stage research training (EST)**, Karolinska Institute, Stockholm (Sweden)
  Supervisor: Prof. Francesca Chiodi
  Research project: "Role of Fas/FasL in regulation of inflammation in vaginal tissue during HSV-2 infection"
- February 2008 - November 2008: **Master student training**, National AIDS Center, Rome (Italy)
  Supervisor: Dr. Stefano Buttò
  Research project: "Characterization of variable regions (V1-V5) in gp120 of HIV-1 subtype C in Southern Africa: possible implications to antibody-neutralization escape"
- May 2006 - August 2006: Laboratory of Virology, University of Florence (Italy)
  Supervisor: Dr. Krystyna Zakrzewska
  Research project: "Expression of B19V proteins in baculovirus-insect cell systems"
- October 2005-March 2006: **Undergraduate student training**, Laboratory of Virology, University of Florence (Italy)
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Italian: mother tongue **English:** fluent **French:** fair

References:

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11. List of publications, presentations and awards

Publications

- Cenci A., Tavoschi L., D' Avenio G., Narino P., Becattini S., Bernasconi D., Chiappi M., La Torre L., Sukati H., Vardas E., Lo Presti A., Cella E., Ciccozzi M., Picconi O., Monini O., Ensoli B., Buttò S. (2012). "Characterization of variable regions of the gp120 protein from HIV-1 subtype C virus variants obtained from individuals at different disease stages in Sub-Saharan Africa". *J AIDS Clinic Res*, S8:006. doi:10.4172/2155-6113.S8-006
- Cenci A., D'Avenio G., Tavoschi L., Chiappi M., Becattini S., Narino P., Picconi O., Bernasconi D., Fanales Belasio E., Vardas E., Sukati H., A. Lo Presti, Ciccozzi M., Monini P., Ensoli B., Grigioni M. and Buttò S. (2014). "Molecular characterization of HIV-1 subtype C gp-120 regions potentially involved in virus adaptive mechanisms". *PLoS ONE* 9(4): e95183. doi:10.1371/journal.pone.0095183
- Engen S.A., Rukke H.V., Becattini S., Jarrossay D., Blix I.J, Petersen F.C., Sallusto F., Schenck K. (2014). "The oral commensal *Streptococcus mitis* shows a mixed memory Th cell signature that is similar to and cross-reactive with *Streptococcus pneumonia". PLoS ONE* 9(8):e104306. doi: 10.1371/journal.pone.0104306.

Manuscripts submitted and in preparation

- Mele F., Basso C., Leoni C., Becattini S., Aschenbrenner D., Latorre D., Lanzavecchia A., Sallusto F., Monticelli S. "Low threshold of activation of human memory T_H17 cells dependent on miRNA-regulated sustained ERK phosphorylation". *Submitted.*
- Becattini S., Latorre D., Mele F., Perez M., De Gregorio C., Cassotta A., Fernandez B., Kelderman S., Schumacher T.N., Corti D., Lanzavecchia A. and Sallusto F. "Functional heterogeneity of human memory T cell clones primed by pathogens or vaccines". *Submitted.*
- Kabanova A., Lilleri D., Perez L., Marcandalli J., Agatic G., Becattini S., Fuschillo D., Corti D., Gerna G., Sallusto F. and Lanzavecchia A. "Antibody-Driven Vaccine Design: A Human Cytomegalovirus gHgLpUL128L Subunit Vaccine Selectively Eliciting Potent Neutralizing Antibodies". *Submitted.*
- Guylevski G., Becattini S., Basso C., Lanzavecchia A. and Sallusto F. "Innate lymphoid cells and Th17 cells provide early and long-lasting protection against *C. albicans*". *Manuscript in preparation.*
- Teloni R., De Turris V., Chiani P., Becattini S., Sallusto F., Nisini R., Torosantucci A., Gagliardi M.C. "*Candida albicans* targets a lipid raft/Dectin-1 platform to enter human monocytes and induce antigen specific T cell responses". *Manuscript in preparation.*

Active participation to meetings and schools

Oral presentations:

- May 8, 2014: **Zurich Joint Immunology Meeting**, Zurich, Switzerland

  Presentation title: "Heterogeneity of human T cell response to pathogens and vaccines"
- May 27-June 03, 2013: **8th ENII Summer School on Advanced Immunology**, Porto Conte, Italy

  Presentation title: "Deciphering the class and specificity of the T cell response to *C. albicans*"
- April 02-04, 2012: **Wolfsberg Meeting**, Schloss Wolfsberg, Switzerland

  Presentation, title: "Priming of polarized human T cells by microbes"
- March 21-26, 2010: **DC Crest 2010**, Celerina, Switzerland

  Presentation title: "Antibody-driven target discovery: analytic vaccinology"

Poster presentations/others:

- January 12-16, 2014: **Keystone Symposium "Tissue Resident Memory T Cells"**, Snowbird, Utah, USA. Poster title: Diversity of the human T cell repertoire to pathogens and vaccines"
- August 22-27, 2013: **15th International Congress of Immunology,** Milan, Italy. Poster title: "Dissecting the human T cell response to *C. albicans*: subset-specific patterns of antigen recognition and intraclonal heterogeneity"
- May 27-June 03, 2013: **8th ENII Summer School on Advanced Immunology,** Porto Conte, Italy. Poster title: "In search of immunodominant antigens within *C. albicans* cell wall"
- November 08-09, 2012: **SIICA-SSAI Joint workshop "Targeting Dendritic Cells for Immunity and Tolerance"**, Ravello, Italy. Poster title: "Identification of *C. albicans* immunodominant epitopes"
- August 19-21, 2012: **Human Immunity Symposium** (Cell Symposya), Lisbon, Portugal. Poster title: "Human T cell response to *C. albicans*"
- February 12-17, 2011: **Keystone Symposium "Dendritic Cells and the Initiation of Adaptive Immunity"**, Santa Fe, New Mexico, USA. Conference assistant (drafting of a comprehensive summary of all talks)

Honors and awards

- June 2013: Ralph Steinman Memorial Outstanding Poster Award, VIII ENII Summer School on Advanced Immunology, Porto Conte, Sardinia, Italy
- August 2013: Swiss Society for Allergology and Immunology Young Investigator Travel grant to attend the 15th International Congress of Immunology (Milan, Italy)
- May 2013: Institute for Research in Biomedicine 2012-2013 Student Award
- November 2012: Swiss Society for Allergology and Immunology Young Investigator Travel grant to attend SIICA-SSAI Joint workshop (Ravello, Italy)