Universidade de Lisboa

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B cells at the crossroad of immune responses: insights from primary B-cell immunodeficiencies

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Doutoramento em Ciências Biomédicas

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Tese orientada pela Professora Doutora Ana E. Sousa

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“Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.”

Ricardo Reis

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ABBREVIATIONS

ADAM – A Disintegrin and Metalloproteinase

AHA – Autoimmune Haemolytic Anaemia

AID – Activation-induced Cytidine Deaminase

AIDS – Acquired Immunodeficiency Syndrome

APC – Antigen-presenting cell

APRIL – A Proliferation Ligand

BAFF – B-cell Activating Factor of the TNF family

BAFF-R – BAFF Receptor

Bcl-6 – B-cell Lymphoma 6

BCR – B-cell Receptor

BCMA – B-cell Maturation Antigen

Blimp-1 – B Lymphocyte-induced Maturation Protein-1

BLNK – B cell Linker Protein

Btk – Bruton's Tyrosine Kinase

CSR – Class-switch Recombination

CVID – Common Variable Immunodeficiency

DC – Dendritic cell

ELISA – Enzyme Linked Immunosorbent Assay

EndoCAb – Endotoxin Core Antibodies

FcRH4 – Fc Receptor Homologue 4

FDC – Follicular Dendritic cell

FOXP3 – Forkhead Box P3

GC – Germinal Centre

HIGM – Hyper IgM

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

HLA-DR – Human Leukocyte Antigen D-related

HSC – Hematopoietic Stem cell

HSCT – Hematopoietic Stem Cell Transplantation

IBD – Inflammatory Bowel Disease

ICOS – Inducible Co-stimulator

ICOSL – Inducible Co-stimulator Ligand

IFN – Interferon

Ig – Immunoglobulin

IgAD – IgA Deficiency

IL – Interleukin

iNKT – Invariant Natural Killer T cell

IRAK4 – Interleukin-1 Receptor-associated Kinase 4

ITP – Autoimmune Thrombocytopenia Purpura

IVIg – Intravenous Immunoglobulin Therapy

LBP – Lipopolysaccharide-binding Protein

LPS – Lipopolysaccharide

mAb – Monoclonal Antibody

Mcl-1 – Myeloid Cell Leukemia Sequence 1

mDC – Myeloid Dendritic cell

mTORC1 – Mammalian Target of Rapamycin Complex 1

MFI – Mean Fluorescence Intensity

MHC – Major Histocompatibility Complex

mRNA – Messenger RNA

MyD88 – Myeloid Differentiation Factor 88

MZ – Marginal Zone of the spleen

NF-κB – Nuclear Factor κB

NIK – NF-κB-inducing kinase

NK – Natural Killer

PBL – Peripheral Blood Lymphocyte

PBMC – Peripheral Blood Mononuclear Cell

PC – Plasma Cell

pDC – Plasmacytoid Dendritic cell

PD-1 – Programmed Death-1 molecule

PD-L1 – PD-1 Ligand 1

PD-L2 – PD-1 Ligand 2

PID – Primary Immunodeficiency Disease

PI3K – Phosphoinositide 3-kinase

PMA – Phorbol Myristate Acetate

RTE – Recent Thymic Emigrant

SAC – *Staphylococcus aureus* Cowan I

SAP – Signaling Lymphocytic Activation Molecule-associated Protein

sCD14 – Soluble CD14

SCID – Severe Combined Immunodeficiency

SHM – Somatic Hypermutation

sIgAD – Selective IgA Deficiency

SLE – Systemic Lupus Erythematosus

TACI – Transmembrane Activator and Calcium-modulating Cyclophilin Ligand Interactor

TCR – T-cell Receptor

T_{FH} – Follicular Helper CD4 T cell

TGF-β - Tumour Growth Factor β

T_H1 – Helper CD4 T cell Type 1

T_H2 – Helper CD4 T cell Type 2

T_H17 – Helper CD4 T cell Type 17

TLR – Toll-like Receptor

TNF – Tumour Necrosis Factor

TRAF – TNF-receptor associated factor

TREC – T-cell Receptor Excision Circle

T_{reg} – Regulatory T cell

UNG – Uracil-DNA Glycosylase

XLA – X-linked Agammaglobulinemia

SUMMARY

B lymphocytes are important players in adaptive immunity and the main targets of current vaccination strategies, with the interaction between B and T cells being fundamental to generate long-term immunity. B cells also play a key role in linking innate and adaptive immunity by expressing receptors that recognize both specific antigens and microbial patterns.

The general aim of this work was to investigate the interplay between B cells and other components of the immune system through the study of B-cell immunodeficiencies, namely Common Variable Immunodeficiency (CVID), characterized by impaired antibody production due to defective mature B-cell differentiation, and Congenital Agammaglobulinemia, where early B-cell development is abrogated, commonly resulting in the absence of peripheral B cells.

We found BAFF-R expression to be reduced in CVID, particularly in patients with low memory B cells, and associated with high serum levels of BAFF, while TACI expression was significantly increased. BAFF induced BAFF-R down-regulation *in vitro*, both in healthy individuals and CVID patients. However, the degree of modulation in CVID was impaired, suggesting that these dynamics are affected, with a possible impact in B-cell homeostasis.

We also observed that CVID was associated with monocyte activation, irrespective of LPS levels, but in direct association with T-cell activation and B-cell imbalances.

To explore the mechanisms underlying chronic immune activation, we studied the role of IL-17, a major pro-inflammatory cytokine implicated in autoimmunity and inflammatory conditions, frequently found in CVID. However, no increase in T_H17 cells was found and their frequency was inversely correlated with markers of germinal centre impairment. T_H17 cells were severely reduced in Congenital Agammaglobulinemia and directly associated with switched-memory B cells in healthy subjects. Our data support a link between B-cell differentiation and T_H17 homeostasis, with implications for the understanding of the pathogenesis of inflammatory/autoimmune diseases and the physiology of B-cell depleting therapies.

Keywords: Primary B-cell immunodeficiencies, CVID, Congenital Agammaglobulinemia, B cells, BAFF-R, BAFF, Monocyte activation, T-cell activation, T_H17 cells, IL-17.

SUMÁRIO

Linfócitos B têm um papel preponderante na imunidade adaptativa e são os principais alvos das estratégias de vacinação, sendo a interacção entre linfócitos B e T fundamental para desenvolver imunidade a longo prazo. Linfócitos B têm também um papel importante no estabelecimento da ponte entre imunidade inata e adaptativa.

O objectivo principal deste trabalho foi investigar o diálogo entre linfócitos B e outros elementos do sistema imunitário, através do estudo da Imunodeficiência Comum Variável (IDCV), caracterizada por produção deficiente de anticorpos resultante de defeitos na maturação de linfócitos B, e da Agamaglobulinémia Congénita, em que ocorre um bloqueio precoce no desenvolvimento de linfócitos B, resultando frequentemente na sua ausência em circulação.

A expressão de BAFF-R encontrou-se diminuída na IDCV, particularmente em doentes que possuíam uma frequência baixa de linfócitos B de memória, associada a níveis séricos de BAFF elevados. BAFF induziu diminuição de expressão de BAFF-R *in vitro*, no entanto de forma alterada na IDCV, sugerindo que esta dinâmica está afectada, com possível impacto na homeostasia de linfócitos B.

A IDCV associou-se com activação monocitária, não relacionada com níveis de LPS, mas com activaçãode de linfócitos T e alterações de linfócitos B.

Para explorar os mecanismos subjacentes à activação imunitária crónica, estudámos o papel da IL-17, citocina pró-inflamatória envolvida em autoimunidade e manifestações inflamatórias, as quais são frequentemente associadas a IDCV. No entanto, não houve aumento de linfócitos T_H17 e a sua frequência associou-se inversamente com evidências de defeitos de centros germinativos. Linfócitos T_H17 estavam reduzidos na Agamaglobulinémia Congénita, e em indivíduos saudáveis encontravam-se associados com a frequência de linfócitos B de memória com *switch*. Estes resultados mostram que a homeostasia de linfócitos T_H17 se associa à maturação de linfócitos B, com implicações para a compreensão da patogénese de doenças inflamatórias/autoimunes e da fisiologia de terapêuticas depletivas de linfócitos B.

Palavras-Chave: Imunodeficiências de linfócitos B, IDCV, Agamaglobulinémia Congénita, Linfócitos B, BAFF-R, BAFF, Activação monocitária, Activação de linfócitos T, Linfócitos T_H17, IL-17.

CHAPTER 1

INTRODUCTION

This introduction will be divided in two main parts. In the first part will be made an overview of the development of B lymphocytes, as well as of the role they play in the generation of immune responses. In the second part, primary B-cell immunodeficiencies will be addressed, with a brief historical overview followed by a description of the major clinical complications involved, together with a perspective on the aetiology of the different diseases and a short outline of the therapeutic strategies that are currently used in such conditions.

PART I

B CELLS AT THE CROSSROAD OF IMMUNE RESPONSES

a. ONTOGENY, DEVELOPMENT AND DIFFERENTIATION OF B LYMPHOCYTES

B lymphocytes are a population of haematopoietic cells that mainly dedicated to the production of antibodies. Importantly, current vaccination strategies rely on memory B-cell generation and antibody production by B cells. B lymphocytes are thus at the crossroad of immune responses. In evolutionary terms, their origin goes back more than 500 million years to the adaptive immune system of jawed vertebrates[1]. The discovery of B cells was essentially simultaneous to the discovery of T cells. In the mid-1960s, pioneer work by Max Cooper and Robert Good implied that the adaptive immune system was composed of two different populations: one that was accountable for the production of antibodies and derived from the chicken bursa of Fabricius, and another deriving from the thymus that was required for delayed hypersensitivity and graft versus host reactions[2,3]. This functional characterization was also at the basis of the nomenclature used then on: T (*t*hymus-derived) and B (*b*ursa-derived) lymphocytes. In mammals, B lymphocytes are bone marrow derived, also suiting the classification.

Earlier stages of B-cell development occur in primary lymphoid organs, such as the foetal liver and the bone marrow, and then differentiation proceeds in secondary lymphoid organs, such as the spleen and lymph nodes, where B cells achieve functional maturation (**Figure 1**). Haematopoietic stem cells (HSCs) generate lymphoid progenitors that give rise to the earliest cell committed to the B-cell lineage, the progenitor B cell or pro-B cell. Proliferation of pro-B cells in the bone marrow is accompanied by the sequential rearrangement of the immunoglobulin (Ig) heavy chain, originating pre-B cells. The rearranged Ig heavy chain forms, together with the surrogate light chains, $\lambda 5$ and VpreB, the pre-B cell receptor (BCR).

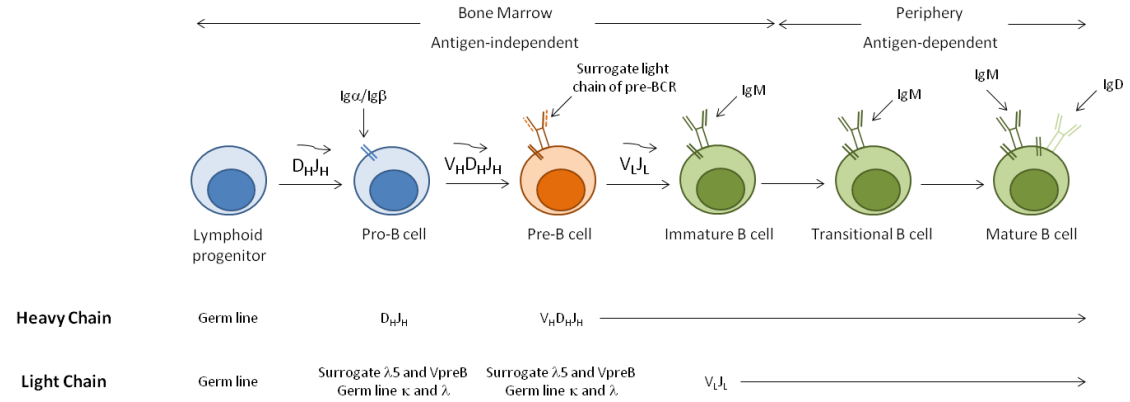

Figure 1. B-cell development. Outline of the events involved, depicting the bone marrow, antigen-independent phase, and the functional maturation in the periphery (adapted from *Immunology*, by Goldsby et al.[4]).

The progeny of pre-B cells will then proceed with the rearrangement of the Ig light chain, developing into immature B cells expressing membrane IgM at their surface, in addition to a functional BCR[5-7]. At this stage, expression of an autoreactive BCR can direct the internalization of the self-antigen-BCR complexes and the activation of a pathway of intracellular signals that arrests differentiation and initiates a secondary rearrangement of the Ig light chain that will replace the original one. This process is called receptor editing and provides the first checkpoint on B-cell development for autoreactivity[8,9]. In addition to receptor editing, B cells with autoreactivities can be eliminated by clonal deletion, undergoing apoptosis if they recognize self-antigens expressed in the bone marrow[10,11]. Even though IL-7 has been shown to be critically involved in murine B-cell development, promoting VDJ rearrangements and inducing proliferation and survival of B-cell precursors[12], such role for IL-7 in human B-cell development remains elusive. Patients with severe combined immunodeficiency (SCID) due to mutations in the IL-7 receptor have normal levels of circulating B cells[13], arguing against a major role for IL-7 in this process.

Following the bone marrow phase of development, immature B cells migrate into secondary lymphoid organs to integrate an antigen-dependent phase of maturation in the periphery[7,14]. Immature B cells, also termed transitional B cells, are fundamentally short-lived and migrate to the spleen through the bloodstream, where they develop into mature long-lived B cells. Upon exiting the bone marrow, transitional B cells acquire expression of markers such as IgD or CD21 (also known as complement receptor 2, CR2)[14]. CD21 is part of the CD19/CD81/CD21/CD225 complex, which is associated with signal transduction from the BCR, thus playing an important role in the bridging of innate and adaptive responses[15]. Transitional B cells have been mainly characterized in the murine system, where they can be subdivided into

T1 and T2 transitional populations. These two populations can be distinguished according to the expression of phenotypic markers, such as CD21 and IgD, with T1 cells being $IgM^{bright}CD21^{-}IgD^{-}$ and T2 cells defined $IgM^{bright}CD21^{bright}IgD^{bright}$. However, some controversy remains regarding whether they are developmentally related or completely independent populations[14]. Transitional B cells have also been described in humans and have been proposed to include two populations expressing differential levels of CD21, which would correspond to the T1 and T2 transitional B-cell populations in the mouse[16,17]. These two populations are thought to represent distinct developmental stages, based on the assessment of B-cell reconstitution following HSC transplantation (HSCT)[17]. A third, more mature, transitional B-cell subset has also been proposed in humans, possessing a more transient nature and rapidly differentiating into mature naïve B cells[18]. Importantly, in addition to BCR signalling, transitional T1 and T2 B-cell populations have differential requirements for B-cell Activating Factor of the TNF family (BAFF)-mediated survival signals, since BAFF-deficient mice present impaired B-cell maturation arrested at the transitional T1 stage[19], while BAFF-transgenic mice have an accumulation of B cells at the transitional T2 stage[20]. It is unclear whether this requirement is also observed for human transitional B cells[16,21]. The transitional B-cell stage is particularly relevant given that it includes a second checkpoint for autoreactivity, in the event of an autoreactive B cell escaping the first checkpoint in the bone marrow. BCR signalling at this stage induces apoptosis and is thus very important to remove autoreactivities[22]. B cells that recognize autoantigens can also be converted to a state where they can no longer respond to BCR engagement, in a process termed clonal anergy or functional unresponsiveness[23,24]. Importantly, human transitional B cells can respond to Toll-like receptor (TLR) 9 stimulation by differentiating into IgM-secreting plasma cells (PCs), arguing that transitional B cells can actually play an important role in building a rapid immune response against bacterial antigens[25,26].

Mature B cells recirculate between the follicles of the spleen and the lymph nodes, playing a crucial role in adaptive immune responses (**Figure 2**). In humans, five major populations are described in peripheral B-cell development: the abovementioned transitional B cells, naïve B cells that have not yet encountered antigen, germinal centre (GC) B cells that are found in peripheral lymphoid organs and are actively involved in ongoing immune responses, memory B cells, expressing the memory marker CD27, that are generated through GC reactions and survive for long periods of time, and PCs that produce antibodies, being the effector arm of the B-cell immune response[7].

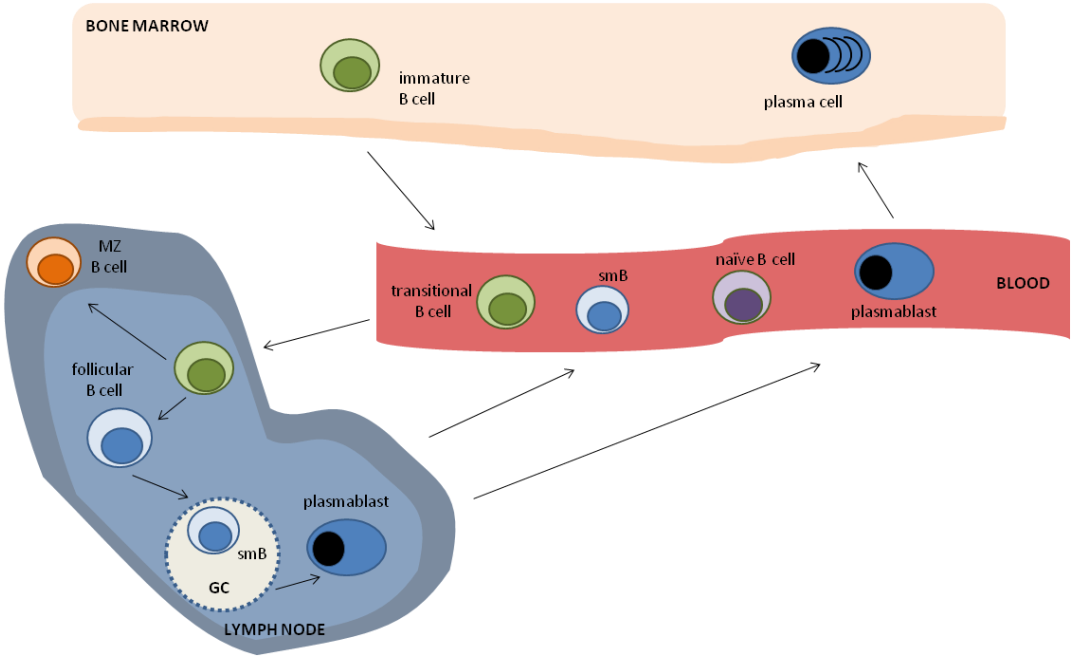

Figure 2. Peripheral B-cell differentiation. Simplified schematic outline, with the main B-cell subsets depicted. MZ: marginal zone; smB: switched-memory B cell.

Naïve B cells constitute the major B-cell subset found in the peripheral blood of healthy adult individuals[27]. Full activation of naïve B cells requires BCR cross-linking by antigen recognition, together with signals coming from CD40 ligation and cytokine stimulation (to be discussed in the next sections) and stimulation by TLR agonists[28] (**Figure 3**). Following activation, naïve B cells can either differentiate into short-lived IgM-producing PCs through extrafollicular reactions or they can enter B-cell follicles and participate in GC reactions, where they differentiate into long-lived memory B cells and PCs[29]. Memory B cells possess less stringent requisites for activation and rapidly produce specific IgG when they re-encounter the antigen, while persisting once the antigen is cleared. Surface expression of CD27 is currently accepted as the best marker for memory, antigen-experienced B cells[27]. PCs directly differentiate from plasmablasts that migrate from the secondary lymphoid organs to the bone marrow[29]. They are normally not found in the peripheral blood of healthy individuals.

The main origin of IgM-expressing human memory B cells is the subject of great controversy. While some argue that they derive from GC reactions[30], others actually propose them to be independent of the GC, since they are present in patients with impaired GC formation and class-switch recombination (CSR)[31-34]. In addition, it has also been suggested that this population includes B cells that are the circulating counterparts of splenic marginal zone (MZ) B cells, which are important in the responses against encapsulated bacteria, and that they would undergo somatic hypermutation (SHM) very early during ontogeny[35,36].

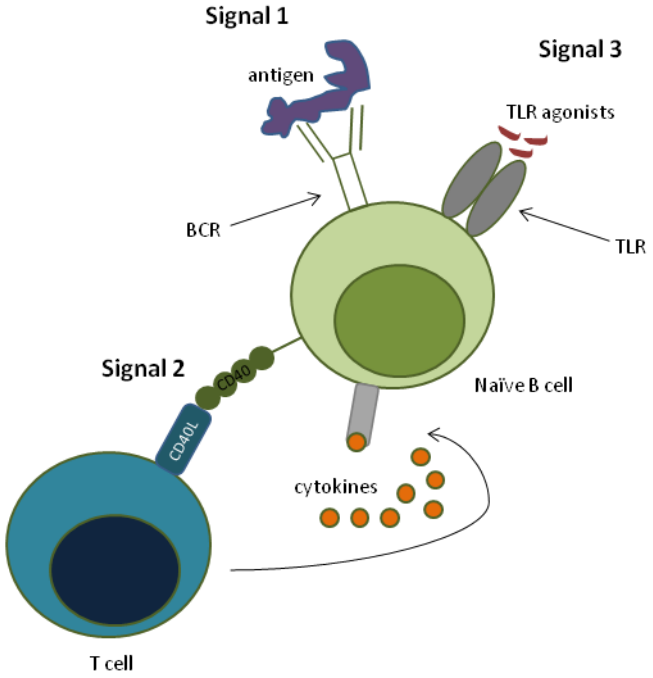

Figure 3. The 3-signal model for naïve B-cell activation. Full activation of naïve B cells requires the integration of BCR stimulation (Signal 1), and help provided by T cells (Signal 2), both in the form of co-stimulation and cytokines. BCR stimulation drives the up-regulation of TLR expression[37], which then provides Signal 3 for B-cell activation.

In the mouse, follicular (also termed B2) B-cell differentiation is essentially similar to that described above for humans. In addition, two separate lineages of B cells have also been well characterized, with features that put them closer to the innate arm of the immune system: B1 B cells and the already mentioned MZ B cells[38]. While MZ B cells share developmental stages with B2 B cells[39], B1 B cells develop from distinct B-cell progenitors in the foetal liver[40]. B1 B cells, originally identified as a population of $CD5^+$ splenic B cells, are a self-renewing population and express germline-encoded antibodies that form the so-called natural antibody repertoire[41]. The terminology derives from the fact that these antibodies are generated in the absence of antigen exposure and hence the proximity to a more innate-like function[41]. B1 B cells are believed to play an important role in the suppression of inflammatory responses[41,42]. Even though human equivalents to mouse B1 B cells have been reported over time[43-45], these cells are not resident in the peritoneal cavity[46], unlike mouse B1 B cells. The recent report that the human equivalent to murine B1 B cells expresses CD27 and IgM, in addition to CD43, argues that at least some B cells that have been previously described as IgM-expressing memory B cells are in fact B1 B cells[44]. MZ B cells are, as the nomenclature indicates, located in the marginal zone of the spleen. They represent a distinct, non-circulating, lineage of murine splenic B cells, critical for the first line of defence against encapsulated bacteria[47]. Contrary to what is seen in the mouse, human MZ B

cells can recirculate and are thus found both in the spleen and the peripheral blood. In addition and as previously mentioned, they express the memory marker CD27 and have been described to undergo SHM in the absence of typical immune responses[36,47,48]. Nevertheless, it has been proposed that, similar to what happens in the murine system, MZ B cells are critical for the fast protective, T-independent response against encapsulated bacteria[47].

Peripheral B-cell survival and differentiation largely depend on two factors belonging to the TNF family, BAFF and A Proliferation Ligand (APRIL), and on their interaction with the receptors BAFF Receptor (BAFF-R), Transmembrane Activator and Calcium-modulating Cyclophilin Ligand Interactor (TACI), and B-cell Maturation Antigen (BCMA)[49]. BAFF signalling through BAFF-R is particularly relevant for the survival and maturation of peripheral B cells, while APRIL acting through BCMA appears to be critical for PC survival in the bone marrow[50-53]. BAFF-R stimulation by BAFF mainly activates the alternative Nuclear Factor-κB (NF-κB) pathway, mediated by the stabilization of the NF-κB-inducing kinase (NIK) induced by TNF-receptor associated factor (TRAF) 3 degradation, thereby resulting in increased B-cell survival[49,54-56]. In addition, BAFF stimulation of BAFF-R also has an impact in the metabolic fitness of B cells by activating the mammalian target of rapamycin complex 1 (mTORC1), in a pathway that sequentially involves Phosphoinositide 3-kinase (PI3K) and Akt[57,58]. The activation of Akt in also critically involved in the up-regulation of the anti-apoptotic molecule myeloid cell leukemia sequence 1 (Mcl-1)[54,58] (**Figure 4**). The integration of the signals delivered through BAFF-R stimulation to these signalling pathways thus mediates the effects of BAFF on B-cell survival and metabolic fitness.

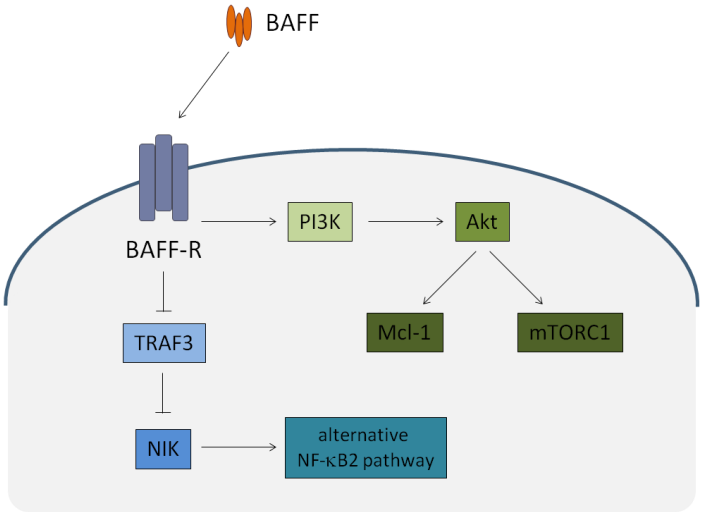

Figure 4. Simplified schematic representation of BAFF-R-mediated signalling events.

b. B CELLS AND THE INNATE IMMUNE SYSTEM

Whenever a pathogen enters the organism, an immune response needs to take place in order to clear it and prevent further damage. Given that failing to control the initial phase of an infection can have serious and even life-threatening outcomes, there is the need for immune defences that are ready-to-go. The innate immune system has the task of initiating a local inflammatory response at the place of pathogen entry, thereby limiting the extent of infection until an adaptive immune response is developed. In addition, the innate immune system also has a crucial role in the priming of the initial adaptive immune response. B cells have an important role in linking cell-intrinsic innate and adaptive immune responses, given the fact that they express both an antigen-specific BCR and TLRs[59]. TLRs are receptors that recognize microbial antigens and activate the innate immune system. The integration of signals coming from these two pathways thus determines the fine-tuning of the overall B-cell response.

As previously mentioned, two subsets of B cells have characteristics that places them closer to the innate immune system: B1 B cells and MZ B cells. These B-cell subsets are crucially involved in T-independent immune responses, where they generate rapid although low-affinity antibody production[60]. Moreover, the BCRs expressed by B1 and MZ B cells are of limited diversity and preferentially recognize microbial antigens and self-peptides, and TLR signalling is important for their responses. In fact, B1 and MZ B cells show higher responsiveness to TLR signals than follicular B cells[61,62]. TLR stimulation induces the up-regulation of Blimp-1 specifically in B1 and MZ B cells, thus driving their differentiation into PCs[61]. In addition, B1 and MZ B cells are highly responsive to BAFF[63,64], which is mainly produced by cells of the innate immune system, such as dendritic cells, monocytes, macrophages, neutrophils and FDCs, and can also be stimulated in response to TLR ligands[49]. Also, naïve and memory B cells respond differently to TLR stimulation, with memory B cells expressing constitutively higher levels of several TLRs[37,65]. This renders memory B cells able to differentiate into PCs through TLR stimulation bypassing the need for T-cell help[37,66].

Such innate-like immune responses of B cells are particularly important in the context of mucosal tissues. Especially in the intestine, B cells mediate immunity against commensal bacteria through the production of IgA by mucosal follicular and extrafollicular reactions[67,68]. At these sites, immune responses are highly dependent on TLR stimulation, as well as on factors such as BAFF, APRIL, TGF-β, and IL-10[69-71].

TLR stimulation of innate cell types can also have an effect in the modulation of B-cell responses. Plasmacytoid dendritic cells (pDCs) were shown to have a better ability to support B-cell differentiation when compared to myeloid dendritic cells (mDCs)[72,73]. Interestingly, TLR-

stimulated mDCs and macrophages may play a suppressive role in controlling the activation of autoreactive B cells, through the secretion of IL-6 and soluble CD40L[74,75]. This suppressive effect was specific for autoreactive B cells, since the activation of naïve B cells was not affected in this system[75]. In line with this observation, patients with defects in TLR signalling (i.e., deficiencies in Myeloid Differentiation Factor 88 (MyD88), Interleukin-1 Receptor-associated Kinase 4 (IRAK4), and UNC93B) have an appearance of autoreactive mature naïve B cells in the peripheral blood[76].

Understanding how B cells translate the different signals around them, coordinating the innate and the adaptive immune systems, is of paramount importance, both for the development of adequate vaccination protocols and treatments for autoimmune diseases[77].

c. THE RELEVANCE OF T-B CELL INTERACTIONS FOR IMMUNE RESPONSES

The interaction between B cells and T cells is fundamental to generate adaptive immune responses. The establishment of long-term humoral immunity, namely in vaccination strategies, relies on B cells receiving appropriate help from CD4 T cells in the context of GC reactions, with the generation of memory B cells and antibody-producing plasma cells[78]. It should also be appreciated that B cells themselves play a crucial role in the development and maintenance of the immune response, in addition to their effector function of antibody production, by acting as antigen-presenting cells (APCs), producing cytokines, recruiting and co-stimulating CD4 T cells[7].

i. ORGANIZATION OF GERMINAL CENTRES

GCs are specific structures within B-cell follicles where antigen-driven SHM and CSR occur[79]. The GC reaction starts with the recruitment and expansion of antigen-experienced B cells to the B-cell follicles of secondary lymphoid organs, which have as main function bringing together APCs and lymphocytes in order to mount immune responses. Whether B cells differentiate into plasmablasts in extrafollicular reactions or seed the GC seems to be related to BCR primary affinity for the antigen. BCRs with high affinity most likely drive B cells into the extrafollicular reaction, whereas moderate or lower affinity will push B cells into the GC[80,81]. As the GC reaction develops, a defined anatomical structure comprising light and dark zones becomes apparent (**Figure 5**). The light zone is mainly occupied by follicular dendritic cell (FDC) processes, while lymphocytes are compacted in the dark zone. The chemokine receptors CXCR4 and CXCR5 are crucial for the positioning of lymphocytes to the dark zone and the light zone. CXCL12, the ligand for CXCR4, is more abundant in the dark zone, while CXCL13, the ligand for CXCR5, is more abundant in the light zone[79,82]. GC B cells in the dark zone are called centroblasts and express high levels of CXCR4, while GC B cells present in the light zone are termed

centrocytes and express high levels of CXCR5. Different events of the GC reaction take place at different zones of the GC; SHM occurs in proliferating centroblasts in the GC dark zone, while it is in the light zone that interactions between T cells, centrocytes and FDCs happen[79,82,83]. During the GC reaction, B cells clonally expand to ultimately generate a high affinity BCR to the given antigen, with the locus of the Ig heavy chain undergoing CSR and its variable regions going through SHM. Both of these processes are mainly mediated by an enzyme termed activation-induced cytidine deaminase (AID)[84].

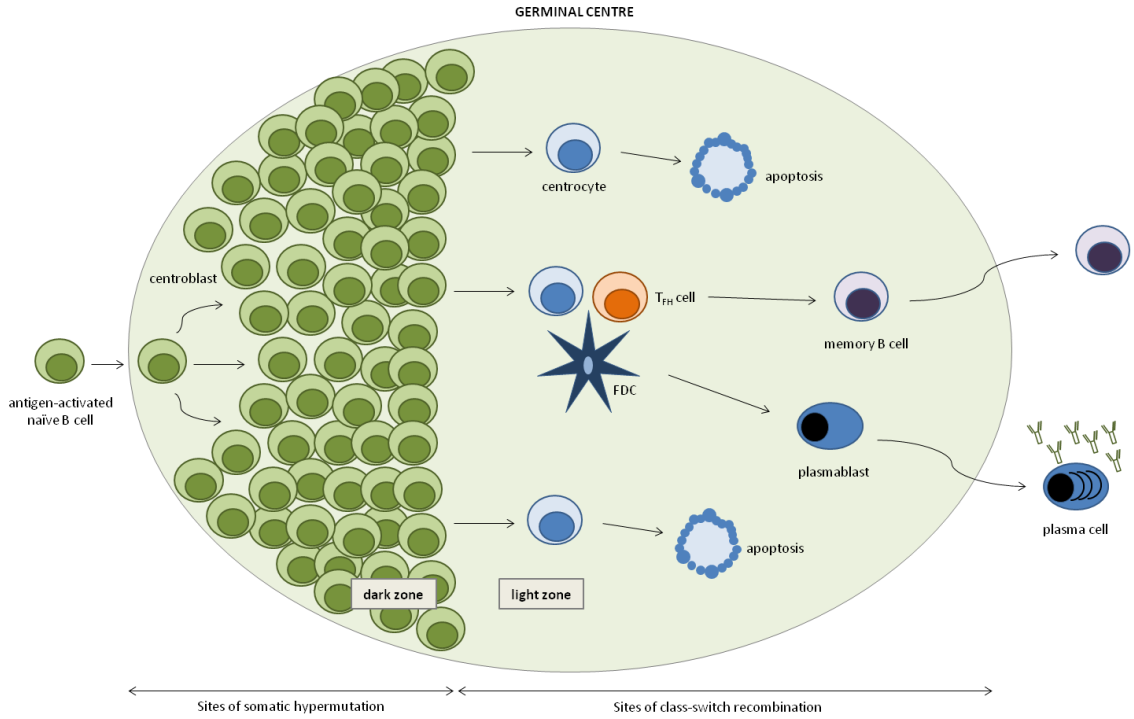

Figure 5. Germinal Centre. Schematic representation of the structure of a germinal centre, with dark and light zones depicted. Somatic hypermutation occurs in the dark zone, while class-switch recombination is limited to the light zone, where B cells interact with follicular helper T cells (T_{FH}) and follicular dendritic cells (FDC) (adapted from Klein and Dalla-Favera[84]).

T-cell help is known to be essential to the induction and subsequent organization of GCs. This help is attributed to a particular subset of T cells, follicular helper T cells (T_{FH})[85,86], which are identified by the expression of the chemokine receptor CXCR5, necessary for their specific homing to B-cell follicles and within GCs[87-89]. In addition to CXCR5 expression, T_{FH} cells are characterized by the expression of the co-stimulatory molecules CD40L and ICOS[85,86], crucial for their function of B-cell help provision (see next section), and the inhibitory receptor Programmed Death-1 molecule (PD-1)[90]. A major function of T_{FH} cells is the production of interleukin (IL)-21[91], a cytokine involved in B-cell differentiation, survival and function (discussed

below). After a long standing debate regarding the functional relationship between other helper T-cell populations and T_{FH} cells, they have been described as a separate lineage of helper T cells[91-93], with its generation being governed by the transcription factor B-cell Lymphoma 6 (Bcl-6)[94-96]. In B cells, Bcl-6 expression is restricted to the GC and it suppresses the differentiation into plasma cells, by inhibiting the expression of the transcription factor B Lymphocyte-induced Maturation Protein-1 (Blimp-1)[97,98]. In addition, Blimp-1 has also been shown to inhibit T_{FH} cell differentiation and function[96]. Thus, Bcl-6 is crucial for GC development and function, both at the B-cell and the T-cell levels[95]. The lack of adequate T-cell help during the priming of B cells results in apoptosis, rather than their differentiation into GC B cells or plasma cells[78,99,100].

Despite the fact that much is known about the attributes of adaptive immune responses, the factors that govern the differentiation of memory B cells and their long-term maintenance remain largely unknown[101].

ii. CO-STIMULATORY MOLECULES

The functional interaction between T cells and B cells within GCs rely on the expression of co-stimulatory molecules, such as CD40-CD40L and the Inducible Co-Stimulator (ICOS) molecule[102].

CD40 is a member of the tumour necrosis factor (TNF) receptor family and a co-stimulatory molecule expressed on B cells that binds CD40L on the surface of activated T cells. This interaction activates B cells and, in combination with soluble cytokines, induces production of different classes of antibodies[103,104]. For instance, CD40 stimulation together with IL-4 induces preferential isotype switch to IgE[105]. Humoral responses are impaired in both mice[106,107] and humans[108-113] with defective CD40L function, underpinning the important role of CD40-CD40L interactions in GC generation, isotype switching and SHM. Also, blocking CD40-CD40L interactions impairs the formation of the GC structure and the proliferation of B cells[114-116]. Importantly, CD40 signals are essential to prevent centrocyte apoptosis[99,100].

ICOS, a member of the CD28 family of T-cell co-stimulatory molecules[117], plays a critical role in the regulation of humoral immunity. ICOS expression is up-regulated on T cells following activation and is expressed at high levels on T_{FH} cells[85,86,117]. ICOS specifically binds to ICOS ligand (ICOSL), constitutively expressed on B cells[118]. ICOS co-stimulation induces IL-4 and, more specifically, IL-10 production by T cells, both of which have an important role in antibody production[117] (discussed in the next section). Of note, ICOS co-stimulation also induces up-regulation of CD40L expression[117], further enhancing the B-cell helper capacity of the T cell. Both ICOS-deficient mice[119-122] and human individuals[31,123] exhibit profoundly defective isotype class-

switch, as well as impaired GC formation. Homozygous deletion of the *ICOS* gene in humans leads to a phenotype of Common Variable Immunodeficiency (CVID), with low numbers of B cells, low serum antibody concentrations and lack of memory B cells[31]. It is noteworthy to mention that patients with defective ICOS or CD40L show a decreased frequency of circulating $CXCR5^+$ CD4 T cells[124], which are suggested to be the counterparts of T_{FH} cells found in secondary lymphoid organs[124,125]. Together with the finding of reduced T_{FH} cells in the spleens and lymph nodes of ICOS- and ICOSL-deficient mice[124,126], this suggests that ICOS is important for the development of T_{FH} cells. In line with these observations, ICOS has recently been shown to supply a vital signal for the early induction of Bcl6 on T cells, which then induces expression of CXCR5, committing cells to the T_{FH} lineage[127].

PD-1 expression by T_{FH} cells also plays a critical role in the GC reaction. GC B cells up-regulate the expression of the PD-1 ligands Programmed Death-1 molecule Ligand 1 (PD-L1) and Programmed Death-1 molecule Ligand 2 (PD-L2). The study of mice deficient for either of the molecules involved in this pathway showed that PD-1 expression on T cells and PD-L2 expression on B cells is in fact regulating T_{FH} cell and PC numbers[128]. PD-1 deficiency also resulted in lower production of IL-4 and IL-21. It has thus been proposed that PD-1 directs the development of long-lived PCs by regulating the survival and selection of B cells in the GC, through its role in the function of T_{FH} cells[128].

In addition to the aforementioned molecules, other factors that affect the interaction between T and B cells also have an impact in GC development and function[101]. One of such cases is the signaling lymphocytic activation molecule-associated protein (SAP), expressed on T cells, whose absence results in defective interaction between T_{FH} and B cells, with a consequent inability of T_{FH} cells to sustain GC reactions[129].

iii. SOLUBLE FACTORS

T-cell derived cytokines present during the events of B-cell activation are crucial in the modulation of the B-cell response, namely by regulating the CSR process and determining the class of antibody that the B cell will produce.

IL-21 is probably the best example of cytokines involved in the functional interaction between T cells and B cells[130-132]. IL-21 was first described as having a critical role in the regulation of antibody production by B cells[131,133,134]. In addition, the GC requirement for IL-21 was also found to reflect an intrinsic requisite for T_{FH} maintenance, acting in an autocrine fashion[132]. Nevertheless, IL-21 is in fact a central player in the B helper function provided by T_{FH} cells. IL-21 produced by T_{FH} cells acts directly in a B-cell intrinsic fashion to induce Bcl-6

expression and thus has a crucial role in the development of GC responses[135,136]. In the absence of IL-21 signalling, GCs are reduced and the production of high-affinity plasma cells is impaired[135,136]. IL-21 production is induced by ICOS stimulation, through the regulation of the transcription factor c-Maf[137]. Paradoxically, IL-21 was also found to promote B-cell apoptosis[138]. This effect is however dependent on the activation signals provided to B cells and may thus represent an important role of IL-21 in regulating B-cell homeostasis[138,139].

IL-4, the master cytokine involved in Th2 responses, was originally identified as a B-cell stimulating factor[140,141]. IL-4 was shown to be critical for isotype switch to IgE and IgG1[105,142], and also to cooperate with IL-21 in regulating isotype switch to different IgG subclasses and IgA[131,143]. Importantly, T_{FH} cells have been shown to produce IL-4 upon immunization with given antigens, in particular with helminths[144,145].

IL-2 was initially described as promoting B-cell growth[146,147]. In addition, it has later been demonstrated to participate in the induction of antibody production[148-152]. However, recent data has challenged this view, with IL-2 administration being described to impair influenza-specific GC formation, long-term IgG responses and limiting the differentiation of T_{FH} cells[153]. However, other recent reports have identified T-cell derived IL-2 to be critically involved in human PC differentiation[154,155]. If these conflicting data represent an essential functional difference between the murine and the human systems is something that needs to be clarified.

IL-6 is another T-cell derived cytokine with a role in the regulation of B-cell responses[156], inducing plasma cell differentiation and antibody production[152,157,158]. Importantly, IL-6 was also shown to promote IL-21 production[159,160] and to drive the differentiation of T cells with the ability to provide B-cell help[160].

IL-10 has a well recognized role in PC differentiation, especially in humans[78,148,157,161,162]. Along with Tumour Growth Factor (TGF)-β, it mediates isotype switch to IgA[163], particularly important at the mucosal sites. IL-10 seems to be especially relevant in stopping B-cell proliferation in the GC and inducing differentiation into PCs[164]. IL-10 production is enhanced by ICOS stimulation[117,165,166], which is particularly relevant in the context of T_{FH} cells. Accordingly, human T_{FH} cells were found to produce IL-10 in an ICOS-dependent manner[167,168], which adds up to their function in providing B-cell help. Recent studies also suggest that IL-10 production by B cells, the so-called regulatory B-cell population, can be important to modulate T-cell responses[169,170].

PART II

PRIMARY B-CELL IMMUNODEFICIENCIES

a. HISTORICAL PERSPECTIVE, CLINICAL MANIFESTATIONS, AETIOLOGY AND TREATMENT OF PRIMARY B-CELL IMMUNODEFICIENCIES

Primary B-cell immunodeficiencies include several disorders that have as a common feature a defect in antibody. Currently, several classes of primary antibody deficiencies are considered - defective early B-cell development in the bone marrow; impairments in CSR, mainly comprising Hyper IgM (HIGM) syndromes; isotype or light chain deficiency, including selective IgA deficiency; specific antibody deficiency; and CVID. Given the fact that this work will be mainly focused on patients affected by defects in early B-cell development and CVID patients, the other classes will only be briefly mentioned.

CSR defects, also referred to as HIGM syndromes, are observed in patients with recurrent infections that have normal or elevated serum levels of IgM, in the presence of reduced IgG, IgA, and IgE. In addition to the CSR defect, SHM can also be affected[171]. This group of genetic defects can be B-cell restricted or it can affect other cell types, such as T cells, macrophages or DCs. More than half of the patients with CSR defects present X-linked mutations in the gene encoding CD40L[108-110,112,113], expressed by activated T cells. In this case, patients usually suffer from opportunistic infections and neutropenia, presenting a clinical picture that is more severe than the one seen in patients with pure humoral defects[171]. CD40 deficiency has also been identified, although in very rare cases, with a clinical phenotype identical to the CD40L deficiency[172]. In terms of CSR defects that are B-cell restricted, autosomal recessive mutations in AID account for the majority of the cases[173,174]. These patients have a later onset of disease, while frequently presenting adenopathies and a high incidence of autoimmunity[175]. Autosomal dominant mutations have also been described in a small number of patients[176]. In addition, autosomal recessive mutations in uracil-DNA glycosylase (UNG), an enzyme responsible for the deglycosylation and removal of uracil residues resulting from the action of AID, have also been described in very few cases of HIGM patients[177]. The clinical phenotype is similar to the one observed in AID-deficient patients.

X-linked Agammaglobulinemia (XLA) was one of the first immunodeficiencies to be described and is often regarded as a prime example. Back in 1952, Ogden C. Bruton reported a case of an 8-year-old male presenting recurrent pneumococcal sepsis with complete absence of

the serum gamma globulin fraction, with otherwise normal serum proteins[178]. These laboratory findings led to the use of tailored therapy, through the subcutaneous administration of human immune serum globulin, while completely explaining the clinical symptoms. In 1953, Charles A. Janeway and colleagues documented the same phenomena (agammaglobulinemia and recurrent infections) in an adult individual, in what was the first report of the nowadays termed CVID[179]. While having a similar outline, it was since early recognized that differences existed between the two observations. Agammaglobulinemia mainly affected males when presenting during childhood, often with an X-linked mode of inheritance, whereas in adults such pattern was not evident, with both females and males being equally affected[179-184]. Later on, it was established that patients with XLA presented virtually no circulating B lymphocytes, at the same time as adult CVID patients typically had close to normal numbers of B cells[185-188]. It was not until more than 40 years after the original description that the molecular defect underlying XLA was unveiled and shown to affect a cytoplasmic tyrosine kinase expressed at all stages of the B-cell lineage and in myeloid cells, later termed Bruton's Tyrosine Kinase, or Btk[189,190]. Mutations in the Btk gene arrest early B-cell development in the bone marrow at the pre-B cell stage[191-194], frequently, but not always[195,196], leading to a complete absence of circulating mature B cells. This block in B-cell development results from defective signal transduction by Btk through the pre-BCR and BCR[197,198].

Regarding clinical manifestations, the most common feature of XLA patients is recurrent infections of the respiratory tract that usually start between 3 and 18 months of age[199-201]. Although some problems can arise from viral infections at early ages, they are well dealt with by XLA patients once T-cell immunity is developed. The most frequent infectious agents are the encapsulated bacteria *Streptococcus pneumoniae* and *Haemophilus influenza*, in terms of respiratory tract infections, and the protozoan parasite *Giardia*, in what concerns gastrointestinal infections[199].

In addition to the X-linked form of agammaglobulinemia, autosomal recessive forms have also been identified, accounting for approximately 15% of the cases of patients suffering from early defects in B-cell development[202] (**Table 1**). As happens with patients with XLA, all of these patients are characterized by presenting an onset of recurrent infections in the first 5 years of life, severe hypogammaglobulinemia, remarkably reduced or absent circulating B cells, and a block in B-cell development in the bone marrow.

Table 1. Genetic defects underlying Congenital Agammaglobulinemia.

GENETIC DEFECT	ASSOCIATED FEATURES	REFERENCES
Btk deficiency	Severe bacterial infections; Absence of circulating B cells; Normal numbers of pro-B cells in the bone marrow	173, 176, 177
μ heavy chain deficiency		198
λ5 deficiency		200
Igα deficiency		201
Igβ deficiency		202, 203
BLNK deficiency		204
PI3K deficiency	Severe bacterial infections; Absence of circulating B cells; Markedly reduced numbers of pro-B cells in the bone marrow	205

Autosomal recessive forms of agammaglobulinemia include mutations in the genes encoding for components of the pre-BCR or the BCR, or in the adaptor protein B cell Linker Protein (BLNK), which also plays a role in signal transduction from the BCR. Mutations in the μ heavy chain, which defines the IgM isotype and is part of the BCR, were identified to underlie a clinical picture similar to XLA[203]. However, the clinical manifestations found in these patients tend to be biased towards a more severe phenotype, with an earlier onset of disease and more clinical complications throughout life[204]. A smaller number of patients have been identified with mutations in the genes encoding λ5[205], Igα[206], and Igβ[207,208], all of which are part of the pre-BCR complex. In addition, mutations in BLNK have also been described to give rise to a phenotype similar to XLA[209]. The vast part of the patients presents null mutations that abrogate protein function. In clinical terms, these patients are identical to XLA patients, even though they normally present an earlier onset of disease. Very recently, it has been described a case of a young female with agammaglobulinemia in the absence of circulating B cells that presented a mutation in PI3K, resulting in the absence of the p85α subunit[210]. Interestingly, the absence of p85α in the patient results in an early and striking defect in B-cell development in the bone marrow, but with a clinical picture restricted to colitis. The block in B-cell development was observed at the earliest stage of commitment to the B-cell lineage, earlier to what is seen for XLA or patients with other forms of autosomal recessive agammaglobulinemia[210].

CVID is the most frequent primary immunodeficiency with clinical relevance, with an estimated prevalence of 1:25,000 – 1:50,000 in the European population[211]. The age of disease onset has a bimodal distribution, with some patients being diagnosed during childhood while the majority presents in early to mid adulthood. CVID is clinically defined by reduced serum levels of IgG and at least another Ig isotype, impaired antibody response to vaccines, and exclusion of defined causes of hypogammaglobulinemia[212]. Nevertheless, CVID patients present highly heterogeneous clinical and immunological profiles. In addition to the high frequency of respiratory and gastrointestinal tract infections, a myriad of non-infectious complications have been reported in more than two-thirds of CVID patients, ranging from chronic lung disease and gastrointestinal inflammatory disease, to autoimmune manifestations, granulomatous disease and neoplasias[211,213,214]. Autoimmunity is being increasingly recognized as a serious medical issue in CVID patients, affecting more than 20% of the patients[215,216]. A recent study has reported the immunological parameters, clinical complications and mortality statistics from 473 CVID patients followed for over 4 decades[214]. In this report, it was found that CVID patients had a significantly shorter survival rate than age- and gender-matched control populations. In addition, this reduced survival was significantly associated with age at diagnosis, lower circulating B cells, and lower baseline serum levels of IgG and higher levels of IgM. Patients affected by non-infectious complications had a significantly higher mortality risk, which was associated with lymphoma, liver or lung disease, and gastrointestinal manifestations, but not with other complications[214]. Moreover, an Italian study has concluded that malignancies are the major cause of death in adult-onset CVID[217]. Another study has analysed and divided 334 CVID patients into distinct clinical phenotypes, but failed to identify useful predictors[213].

Even though the vast majority of CVID cases are sporadic, familial patterns of inheritance are observed in 10 – 20% of the patients, with a family history of autoimmunity or disorders of antibody production[218,219]. In some families, CVID co-exists with IgA deficiency (IgAD). This primary immunodeficiency can be selective for IgA or be associated with deficiency in subclasses of IgG, and is the most common primary immunodeficiency. However, the majority of IgAD patients are asymptomatic and thus the condition is clinically not very relevant[220,221]. Some IgAD patients progress to CVID, suggesting a degree of common genetic aetiology. In this sense, early studies identified certain Human Leukocyte Antigen (HLA) haplotypes to be more common in both conditions[219,222-226].

According to the latest update on the classification of Primary Immunodeficiency Diseases (PIDs)[13], CVID, now standing for Common Variable Immunodeficiency Disorders, groups all patients with a severe reduction of at least two Ig isotypes with normal or low B-cell numbers

of unknown aetiology. In this recent classification, the molecular defects that give rise to a CVID-like phenotype are now in separate subgroups, namely mutations in ICOS, CD19, CD81, CD20, TACI, and BAFF-R[13].

ICOS deficiency was the first genetic defect to be identified in four patients with adult-onset CVID, in 2003[31]. Patients failed to express ICOS at the surface of activated T cells, due to a partial homozygous deletion in the ICOS gene. Five additional patients have been later described to present the same homozygous deletion, indicating that all nine patients probably share a common founder[227]. It is interesting to note that this first description of an autosomal recessive disorder underlying CVID was related to a T-cell intrinsic defect that affects the cross-talk between T and B cells in GCs. In the original report of the first four patients, ICOS-deficient patients were described as lacking major non-infectious complications, such as splenomegaly or autoimmunity[31]. However, the subsequent identification of five additional patients, who included patients with early-onset disease, showed that ICOS deficiency can present with the full clinical spectrum of CVID[123].

Mutations in CD19 were described in 2006, resulting in a phenotype of hypogammaglobulinemia compatible with CVID[228]. There are currently six cases identified, all of which present normal numbers of circulating B cells with minimal or no expression of CD19 at their surface[199,202,228,229]. These include both early- and adult-onset CVID-like phenotypes. Given that CD19 is part of a complex that is associated with signal transduction from the BCR[230,231], mutations in CD19 likely hinder antigenic stimulation and hence result in defective humoral responses. Accordingly, B cells from CD19-deficient patients showed defective calcium fluxes following BCR stimulation. Vaccination responses were also impaired[228]. Patients with CD19 deficiency did not present signs of non-infectious complications, including autoimmunity, lymphoid proliferation or neoplasias[228].

CD81 deficiency was reported in 2010 in one patient with severe nephropathy and hypogammaglobulinemia[232]. B cells from the patient did not express CD19, but had normal *CD19* alleles. Instead, a homozygous mutation in CD81 was found, leading to a complete absence of CD81 expression. Transduction experiments showed that CD81 expression was crucial for CD19 membrane expression. So, CD81 deficiency leads to a functional phenotype that is similar to CD19 deficiency, with impaired responses through BCR stimulation[232].

Also in 2010, CD20 deficiency was described in a young patient with persistent hypogammaglobulinemia[233]. The patient presented normal peripheral B-cell numbers, but a severe decrease in the frequency of memory B cells. SHM was defective and even though the patient mounted adequate antibody responses after vaccination with recall antigens, responses

to pneumococcal polysaccharides (T-independent) were strongly impaired[233], suggesting that CD20 may play an important role in the development of T-independent responses.

TACI mutations were originally reported by two independent studies of two different cohorts of CVID and IgAD patients[234,235]. The pattern of inheritance of these mutations seems to be rather complex, since simple heterozygous, compound heterozygous and homozygous mutations have all been found, involving both autosomal dominant and recessive mechanisms. The most common TACI mutations in CVID are the amino-acid substitutions C104R and A181E[236-238]. These, together with the amino-acid substitution S144X, have been identified both in heterozygosity and homozygosity. The C104R and S144X mutations, when in a homozygous state, abrogate APRIL binding, with loss of TACI function, whereas in heterozygosity APRIL binding is retained[235]. The functional relevance of heterozygous mutations is thus elusive. However, work has shown that the assembly of wild-type and mutant receptors together, as it would be the case when the mutation is present in a heterozygous state, although not having a impact on ligand-binding interferes with the downstream signalling from the receptor[239]. The screening of more than 500 CVID patients from different cohorts has shown that TACI variants are found in around 8-10% of CVID patients[236]. However, the mutations are also found in healthy individuals, although at a lower frequency, questioning the relevance of these mutations in CVID pathogenesis. Nevertheless, presence of a heterozygous mutation was significantly associated with antibody deficiency, particularly relevant in the case of the C104R mutation, indicating that this mutation increases the risk for CVID and affects the clinical presentation[236]. It is worth noting though that the C104R homozygous mutation has not been found in healthy controls and thus seems to be disease-causing. Accordingly, the murine equivalent to the C104R TACI mutation, C76R, significantly disrupts B-cell homeostasis, Ig production, and antigen-stimulated humoral responses[240]. In clinical terms, CVID patients with TACI mutations present a higher prevalence of autoimmune manifestations and splenomegaly[236], in line with the phenotype of TACI-deficient mice[241,242].

Two siblings who present a late-onset CVID-like syndrome have been described to lack BAFF-R protein expression with consequent severe B-cell lymphopenia[243]. The clinical manifestations in these patients were however mild in comparison to other antibody-deficient settings, with no autoimmune nor lymphoproliferative manifestations reported[243]. Even though both patients presented the same homozygous BAFF-R deletion, their clinical presentation differed significantly, demonstrating the variable penetrance of this deficiency[243]. Nevertheless, both patients showed a poor response to T-independent antigens, in correlation with reduced numbers of MZ B cells, while maintaining adequate responses against T-dependent antigens[243].

In addition, polymorphic variants of BAFF-R have been described, affecting both CVID patients and healthy individuals[244]. Such variants do not impact upon BAFF-R expression, neither at the mRNA level nor at the protein level[244], thus questioning the relevance of such alterations.

Finally, CD21 deficiency was very recently added to the list of monogenic defects resulting in a CVID-like phenotype. One adult patient, who presented with a history of recurrent infections, normal B-cell numbers but reduced switched-memory B cells, and hypogammaglobulinemia, was found to have undetectable expression of CD21 on B cells. Sequence analysis revealed a compound heterozygous deleterious mutation in the CD21 gene[245]. Contrary to was is seen in CD19 and CD81 deficiencies, CD21 deficiency did not impair on the expression of other components of the signalling complex, which can explain the fact that CD21-deficient B cells present normal responses to BCR stimulation. Nevertheless, B cells from the patient did not bind or respond adequately to C3d-containing immune complexes. The clinical phenotype of the patient was not severe, since he was able to mount specific antibody responses upon vaccination, although to a lesser degree in the case of polysaccharide antigens[245].

Also quite recently, a genome-wide association and gene copy number variation study was performed in 363 CVID patients[246]. This study confirmed the Major Histocompatibility Complex (MHC) locus as being associated with CVID, in line with previous observations that were done with different technologies[219,222-226]. The study also uncovered a region that was associated with CVID outside of the MHC locus, encompassing several members of the A Disintegrin and Metalloproteinase (ADAM) family[246], which are zinc metalloproteases involved in several biological processes. Importantly, this study confirmed the view of CVID as being a highly heterogeneous disease, even at the genetic level.

The aforementioned genetic defects found to underlie CVID, which together represent no more than around 10% of the cases, elegantly illustrate the complexity of CVID physiopathology. Deficiencies in receptors that are involved in signal transduction through the BCR, molecules expressed by T cells that play an important role in B-cell help, and receptors that have a major impact on B-cell survival and function, have all been shown to give rise to a CVID phenotype, with defective production of antibodies. However, CVID is not merely a deficiency of antibody production. Patients are affected by a myriad of clinical complications that are not solely explained by a loss of B-cell function in terms of antibody production.

Table 2. Known genetic defects underlying Common Variable Immunodeficiency.

GENETIC DEFECT	ASSOCIATED FEATURES	REFERENCES
CD19 deficiency	Mild clinical phenotype; Normal numbers of circulating B cells	223, 224
CD20 deficiency		228
CD21 deficiency		240
CD81 deficiency	Nephropathy; Similar to CD19 deficiency	227
ICOS deficiency	Variable clinical phenotype; Activated T cells fail to express ICOS	32, 118, 222
BAFF-R deficiency	Late-onset, mild clinical phenotype; Severe B-cell lymphopenia	238
TACI deficiency	Complex pattern of inheritance; High prevalence of autoimmunity and splenomegaly	229, 230, 231

The therapeutic management of CVID patients is mainly focused in reducing the morbidity and the mortality that are associated with recurrent infections. IgG replacement therapy is the standard treatment for antibody deficiencies ever since the very first case reported[178], providing patients with the antibodies that they are unable to produce in adequate amounts. Even though IgG replacement therapy is highly efficacious in reducing the frequency of infections, some patients remain susceptible to recurrent infections and the occurrence of non-infectious complications is not resolved by the replacement therapy[213,247-250]. A recent study has analyzed the infection outcomes in patients with CVID and XLA in relation to the replacement therapy for an extended period of time, showing that XLA patients required significantly higher doses to prevent infections[251]. The same study reported that patients with bronchiectasis received higher doses of replacement IgG and that the presence of clinical complications, such as enteropathy, cytopenias or lymphoproliferation, resulted in significantly higher levels of IgG being administered to CVID patients in order to remain infection-free. They have also shown that the infection scores were significantly higher in CVID patients with lymphoproliferation or organ-specific autoimmunity[251]. The adequate doses of replacement IgG vary widely from patient to patient, a consequence of variable baseline IgG levels and rates of IgG catabolism. It is thus necessary to bear in mind that the dose and interval of IgG administration needs to be individually tailored to each patient[251,252]. Interestingly, it has been recently proposed that IgG

replacement therapy can induce B-cell proliferation and Ig production in some CVID patients[253], suggesting that replacement therapy is not a mere passive administration of antibodies but rather has an active role in modulating B-cell function. Nevertheless, longitudinal data on the impact that IgG replacement therapy has on the immune function of CVID patients is still scarce.

Even though IgG replacement therapy is highly efficient in decreasing the frequency of infectious episodes in antibody-deficient patients, antibiotics are still widely used to eliminate on-going infections[248,252,254] and also in a prophylactic manner[211,252]. In addition, IgG replacement therapy is not sufficient to treat non-infectious complications that affect CVID patients, namely autoimmune and granulomatous disease, for which the use of corticosteroids is often required. The treatment of these manifestations remains a challenge, given the potential risk of aggravating the underlying immunodeficiency[211,252]. In this sense, the effectiveness of biological therapies with monoclonal antibodies has been documented in several case reports. Infliximab, a monoclonal antibody against Tumour Necrosis Factor (TNF)-α, has been used in the treatment of Inflammatory Bowel Disease (IBD) associated with CVID[255,256] and also for caseating granulomas, with marked success[257]. Etanercept, another anti-TNF-α monoclonal antibody, has also been successfully used in the treatment of refractory cutaneous granulomas[258,259]. Rituximab, a monoclonal antibody directed against CD20 that depletes circulating B cells, has been shown to be useful in the treatment of medically refractory autoimmune thrombocytopenia purpura[260,261] (ITP) and autoimmune haemolytic anemia[262,263] (AHA). A recent multicentre study has analyzed rituximab treatment for CVID-associated autoimmune cytopenias, showing that this can be an extremely effective and relatively safe therapeutic strategy[262].

The early observation that IL-2 production by T cells from a subgroup of CVID patients was defective and could be corrected by the exogenous provision of IL-2[264,265] led to the *in vivo* administration of recombinant IL-2 to CVID patients, which resulted in enhanced T-cell proliferative responses to mitogens and antigens, and increased responsiveness of B cells to differentiation signals[266-270]. However, the limited evidence for a beneficial effect of IL-2 on *in vivo* antibody production by B cells from CVID patients made it difficult to ascertain which patients would profit from this therapy. It is likely that CVID patients that present with defective T-cell function would benefit from IL-2 treatment, but this line of investigation has not been pursued[252,266].

b. B-CELL DISTURBANCES IN COMMON VARIABLE IMMUNODEFICIENCY

CVID association with intrinsic defects in the B-cell lineage has been recognized early on[185] and extensively documented, including impaired Ig secretion upon several combinations of *in vitro* culture conditions[271-274], decreased class-switched B cells and plasma cells in the gut of CVID patients[275], increased spontaneous B-cell apoptosis[276], reduced expression of the co-stimulatory molecules CD70, CD86 and CD137[277,278], low frequencies of circulating memory $CD27^+$ B cells[279,280], reduced SHM[281], and impaired calcium mobilization following BCR stimulation[282,283].

There has been a long history of attempts to classify CVID patients, a need derived from the acknowledgement of the high heterogeneity of the disease. Back in 1982, two classification schemes were proposed, one regarding the *in vitro* induction of IgM responses to sheep erythrocytes[274], and the other where the distinction was made according to stages of B-cell development affected, as assessed by the ability to respond *in vitro* to *Staphylococcus aureus* Cowan I (SAC) or anti-IgM stimulation[273]. Later on, another attempt to classify CVID patients was made also using a functional assay based on the ability of B cells from CVID patients to produce IgM, IgA, and IgG *in vitro* upon stimulation with SAC plus IL-2 or anti-IgM plus IL-2[271]. Three groups of patients were defined: group A included patients whose lymphocytes fail to produce any Ig isotype in vitro; patients in group B produced IgM but no other isotypes; and group C patients produced normal amounts of all isotypes *in vitro*, similarly to healthy individuals, while failing to produce adequate amounts of antibodies *in vivo*[271]. However, this functional classification of CVID patients revealed to be of low prognostic significance and the methodology was time-consuming and difficult to standardize, raising the need for more reliable and simple procedures. In 2002, the first classification based on flow cytometric phenotypic analysis of peripheral blood B-cell subsets from CVID patients was proposed by Warnatz *et al.*, later known as the Freiburg classification[284]. This was also the first work to report a major reduction of switched-memory B cells in CVID patients, as it had been previously described in patients with X-linked HIGM syndrome[285]. The work also showed that the frequency of switched-memory B cells was directly correlated with the ability of B cells to produce IgG in vitro, both in healthy individuals and CVID patients[284]. They thus proposed a classification based on the frequency of switched-memory B cells in total peripheral blood lymphocytes (PBLs): group I for patients with < 0.4% of switched-memory B cells in PBLs; and group II for patients with > 0.4% (and more similar to healthy individuals). Group I patients were further subdivided according to the expansion (group Ia, > 20% $CD21^-$ B cells) or not (group Ib, < 20% $CD21^-$ B cells) of what they referred to as $CD21^-$ "immature" B cells[284]. Importantly, there was a significant clustering of CVID patients with splenomegaly and autoimmune cytopenias in group Ia, supporting the relevance of the

classification in defining significant clinical associations[284]. In 2003, a second scheme of classification was proposed by Piqueras *et al.*, later to be known as the Paris classification[286]. This classification was based on memory B-cell defects: patients with normal levels of both switched-memory and non-switched memory B cells were termed MB2, whereas CVID patients with reduced switched-memory B cells but normal frequencies of non-switched memory B cells were classified as MB1; patients with defects in both switched and non-switched memory B cells were grouped as MB0[286]. Of note, patients on group MB2 had B- and T-cell phenotypes similar to healthy individuals. Patients with complete memory B-cell deficiency (group MB0) had a significant higher incidence of granulomatous disease, lymphoproliferation and splenomegaly, attesting the usefulness of the proposed classification[286]. Following studies confirmed this association between reduced frequencies of switched-memory B cells and higher incidence of clinical manifestations[287-289]. More recently, a European multicentre trial involving 303 CVID patients developed a consensus between the two previously proposed classification schemes[290]. The EUROclass scheme separates patients with virtually absent B cells (< 1% of PBLs), marked reduction of switched-memory B cells (≤ 2%), and expansion of transitional (≥ 9%) or $CD21^{low}CD38^{low}$ B cells (≥ 10%) (**Figure 6**). Significant clinical correlations were found: patients with a severe reduction of switched-memory B cells had a higher risk for splenomegaly and granulomatous disease; expansion of $CD21^{low}CD38^{low}$ B cells was associated with splenomegaly; and the increase in transitional B cells was connected with adenopathies[290].

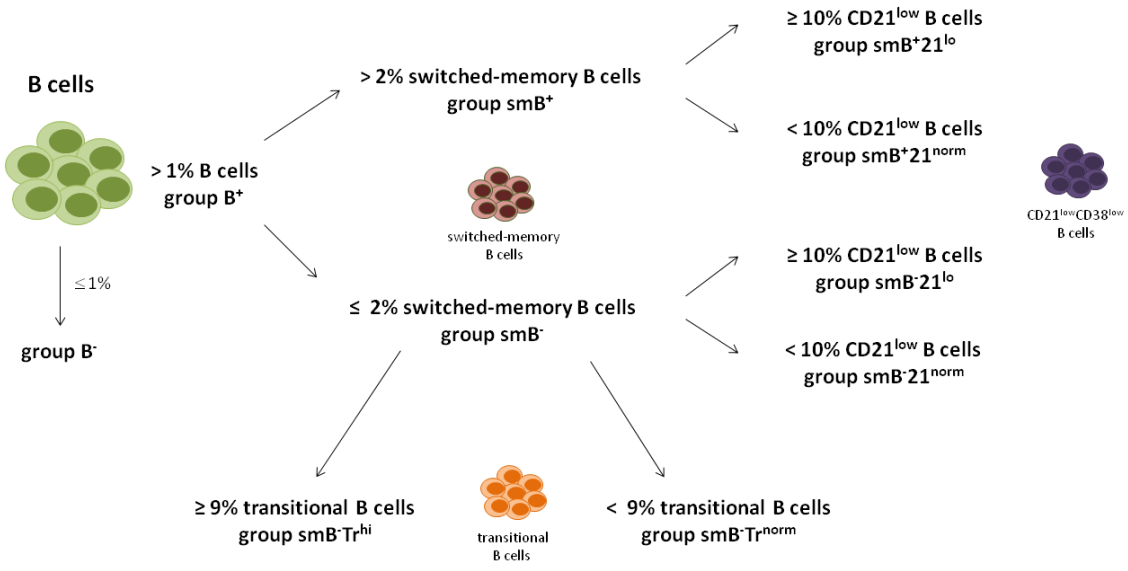

Figure 6. The EUROclass classification scheme for Common Variable Immunodeficiency patients, as proposed by Wehr *et al.*[290].

Even though the expansion of CD21^{low} B cells had been previously described to be a marker for CVID with autoimmune cytopenias and splenomegaly[291], no associations with autoimmune phenomena were found in the EUROclass trial[290]. More recently, distinct pathophysiologic backgrounds were identified in CVID through the combined analysis of B-cell replication history and SHM status[292]. In this work, Driessen *et al.* proposed five different B-cell patterns in CVID patients, in addition to those presenting normal peripheral B-cell development: (1) impairment in B-cell production, (2) early peripheral B-cell maturation or survival defect, (3) defective B-cell activation and proliferation, (4) dysfunctional GC function, and (5) post-GC defect (**Figure 7**)[292]. With this approach, the authors claim that immunologically homogeneous groups of CVID patients can be identified, paving the way for further analysis on the molecular defects involved. Defects in the GC reaction and PC development had been previously documented in lymph nodes from CVID patients[293]. Even though PC differentiation was defective in all three CVID patients analysed in this report, the developmental stage at which this block occurs appears to be variable, once more highlighting the heterogeneity underlying CVID physiopathology.

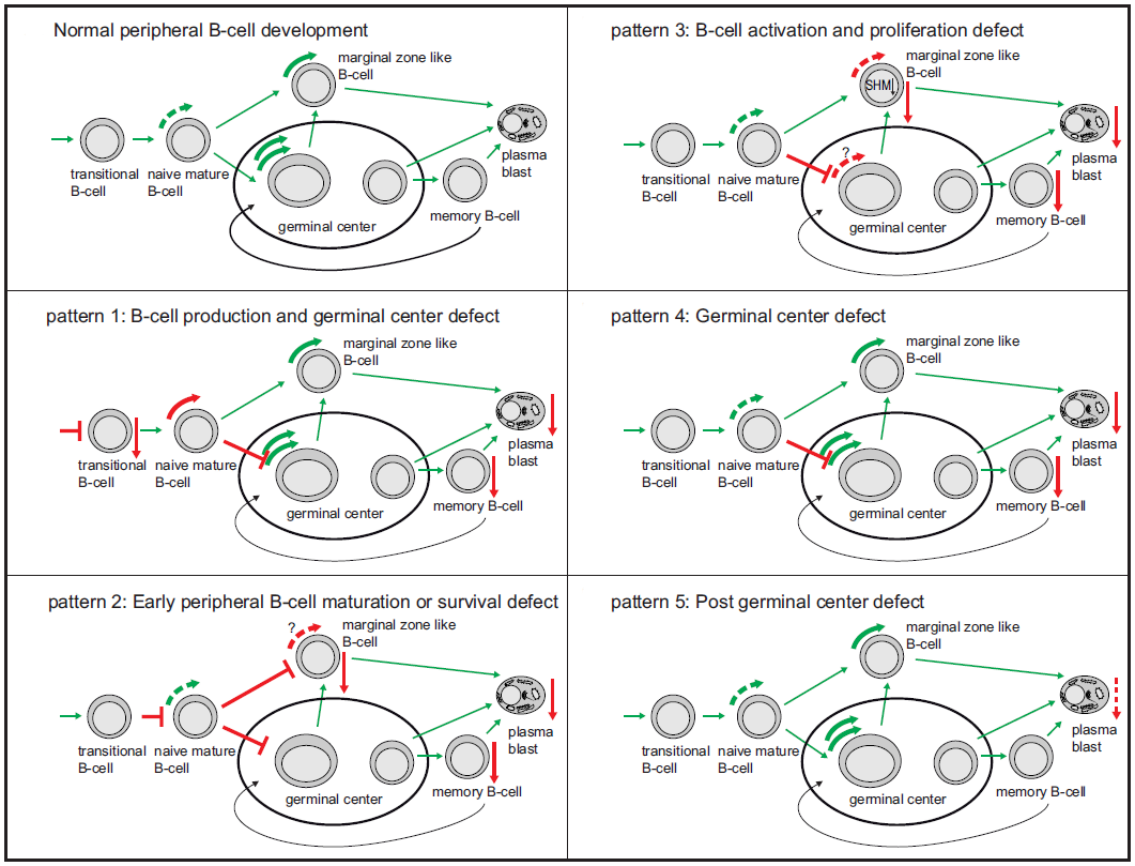

Figure 7. Model of the pathophysiological background of five different B-cell patterns in Common Variable Immunodeficiency patients, based on proliferation history and somatic hypermutation levels. Illustration taken from Driessen *et al.*[292].

Additionally, abnormalities in the bone marrow lymphocyte composition have been recently described in CVID patients, including absence or very low levels of plasma cells, and a partial arrest of early B-cell development in some CVID patients resulting in low peripheral B-cell counts[294], in line with the observations made by Driessen *et al.*[292].

In addition to the effort put into finding more appropriate ways of classifying CVID patients, several other important observations have been made regarding the B-cell compartment of CVID patients. In 2005, Carsetti *et al.* showed that CVID patients with recurrent bacterial pneumonias have very reduced frequencies of IgM^+ memory B cells, while patients who never had pneumonia present normal frequencies of this population and produced IgM antibodies against pneumococcal polysaccharides[295]. This work illustrated the importance of IgM^+ memory B cells in the response against encapsulated bacteria and suggests that the frequency of this population correlates with different susceptibilities to encapsulated bacterial pneumonia in CVID patients. In contrast, the reduction of switched-memory B cells was similar in CVID patients who had or not recurrent pneumonias[295]. IgM^+ memory B cells and the ability to produce IgM antibodies are very important in CVID patients, since the replacement therapy with IgG only provides IgG antibodies and cannot substitute for the function of IgM antibodies. Quite recently, it has been shown that the frequency of the population of B cells described as being equivalent to the mouse B1 cells, important producers of natural antibodies, is not significantly different in CVID patients and healthy individuals, when taking into account the reduction in the total memory B-cell pool[296]. This suggests that CVID patients may be able to maintain, at some level, the production of natural antibodies.

Interestingly, a recent report has shown that the combination of IL-21, IL-4 and anti-CD40 stimulation *in vitro* was able to induce CSR in B cells from CVID patients and differentiation into PCs. In addition, this combination of stimuli prevented spontaneous B-cell apoptosis, suggesting that some defect in IL-21 may be involved in CVID. However, genetic analysis did not reveal any mutations in the *IL-21* gene of CVID patients, and the expression of both IL-21 and the IL-21R was also not impaired[297].

The observation of expanded populations of B cells expressing low levels of CD21 in CVID patients[284,290,291] has led to intense research regarding the origin and functionality of these cells. Rakhmanov *et al.* made a comprehensive phenotypic and functional analysis of circulating $CD21^{low}$ B cells from CVID patients and suggested that these cells show a phenotype that is mostly compatible with that of naïve B cells, but they are actually a distinct population of pre-activated, polyclonal, partially autoreactive cells that are functionally less responsive to BCR stimulation and have a preferential enrichment in peripheral tissues[298]. The fact that $CD21^{low}$ B

cells are less responsive to BCR stimulation can be simply explained by the low levels of CD21 expression, which result in impaired signal transduction from the BCR. The authors acknowledged a great similarity of $CD21^{low}$ B cells in CVID and a previously described population of tonsillar memory B cells expressing the immunoregulatory molecule FcRH4[299], but argued that $CD21^{low}$ B cell in CVID are not memory cells in the sense that they lack CSR, SHM, and oligoclonality[298]. Contrary to what was described by Rakhmanov *et al.*, a following study reported the existence of two different populations within $CD21^{low}$ B cells according to the expression of CD24[300]. However, the functional relevance of those two different populations has not been assessed. Isnardi *et al.* expanded the knowledge on this B-cell population by performing a transcriptome analysis, showing that $CD21^{low}$ B cells up-regulate genes encoding molecules likely to inhibit B-cell activation, proliferation, and survival, at the same time that they down-regulate genes involved in B-cell activation, supporting the view that these B cells are indeed refractory to antigenic stimulation[301]. Even though CVID patients who present expanded populations of $CD21^{low}$ B cells have a higher incidence of autoimmune manifestations, the role of these B cells in the development of autoimmunity is still not clear.

CVID patients with known genetic defects sometimes feature distinct impairments on B-cell phenotype and function. For instance, adult patients with ICOS deficiency present reduced frequencies of peripheral B cells, while affected young children do not[123]. The memory B-cell compartment is decreased in all patients, with a severe reduction of switched-memory B cells, likely resulting from poor GC formation[31,123]. Memory IgM^+, or MZ, B cells were also found to be reduced in the majority of ICOS-deficient patients. However, memory B cells isolated from ICOS-deficient patients were able to produce all Ig isotypes upon *in vitro* culture with autologous T cells in the presence of SAC and IL-2, demonstrating that these patients do not have a B-cell intrinsic defect and that the lack of ICOS:ICOSL interaction can be functionally overcome[31]. These B-cell defects are not exclusive of CVID patients with defective ICOS, but rather typify the common CVID phenotype. In the cases where SHM could be analyzed, it was found to be normal or slightly reduced[123]. CD19-deficient patients present normal numbers of circulating B cells, with an apparently normal precursor B-cell compartment in the bone marrow[228]. In addition to the lack of CD19 expression, CD21 expression is also reduced. Nevertheless, CD81 and CD225, the other members of the signalling complex, were normally expressed at the surface of B cells[228]. The frequencies of both total memory and switched-memory B cells were markedly reduced, even though GC formation was normal. In addition, SHM was also comparable to normal individuals[228]. CVID patients with TACI mutations have increased B-cell numbers but

decreased immunoglobulin production, contrary to what is observed in the mouse[234,235]. In addition, switched-memory B cells were reduced in these patients[236], comparable to other CVID phenotypes without TACI mutations. On the contrary, lack of BAFF-R expression in CVID patients leads to severe B-cell lymphopenia, with an increase in transitional B cells paralleled by a decrease in all mature B-cell populations[243]. While both IgG and IgM serum levels were severely reduced, IgA production was intact *in vivo* and *in vitro*, suggesting that BAFF-R signals are dispensable for human class-switch to IgA[243]. All in all, while presenting distinctive alterations at the level of the B-cell compartment, CVID patients with known genetic alteration basically show the main characteristic defects that are common to all CVID patients.

c. How are T cells affected by B-cell immunodeficiencies

Given that impaired B-cell function can arise both from B-cell intrinsic defects and lack of appropriate B-cell help by T cells, the T-cell compartment has also been extensively studied in CVID patients. Early studies reported defects in T-cell activation and proliferation[302-306], T-cell lymphopenia, particularly of the naïve CD4 T-cell subset[307-309], abnormal cytokine production[265,310-315], impaired expression of CD40L[316], of the C-type lectin attractin[317], and of the adhesion molecule CD62L[318], defective TCR signaling[315,319-322], perturbed generation of antigen-specific memory T cells[323-325], increased T-cell apoptosis[309,326], and oligoclonal expansions of CD8 T cells[327], among others. More recently, it was also described that invariant natural killer T (iNKT) cells, a subset of thymically-derived T lymphocytes that have the ability to rapidly produce a wide range of cytokines, are markedly decreased in CVID patients, both in terms of frequency and absolute numbers in circulation[328]. In addition, this lost of iNKT cells was more pronounced in patients with low switched-memory B cells and no association was found with clinical features, arguing that low frequencies of iNKT cells might be contributing to the failure of memory B-cell generation[328]. A different study has however reported that the frequency of circulating iNKT cells in CVID patients was similar to healthy subjects, even though iNKT cell subsets were skewed and presented an activated phenotype in CVID patients[329]. Analysis of bone marrow biopsies from CVID patients revealed the existence of significantly more diffuse and nodular T-cell infiltrates than in healthy subjects, which were correlated with the presence of autoimmune cytopenias in CVID patients, but not other clinical manifestations. Nodular T-cell infiltrates in the bone marrow were directly correlated with the frequency of circulating memory $CD45RO^+$ CD4 T cells[294].

Naïve CD4 T-cell lymphopenia has been linked to defects in thymic function through the finding of decreased levels of TCR excision circles (TRECs) on peripheral T cells[330,331], an estimate

of recent thymic output. In addition, recent thymic emigrants (RTEs), as defined by the expression of CD31 on naïve CD4 T cells, were also found to be significantly reduced in CVID patients[332], in line with the idea of a defective thymic function in these patients. Increased levels of circulating IL-7 have been reported in CVID patients[333], but these were not related to CD4 T-cell lymphopenia, contrary to what is found in several clinical settings[334-337]. Instead, CVID patients with high IL-7 levels present increased numbers of CD8 T cells, increased proportion of terminally-differentiated T cells and reduced levels of apoptosis[333], suggestive of an impairment in IL-7-mediated T-cell homeostasis. In 2007, Giovanetti *et al.* reported an extensive analysis on T-cell abnormalities in CVID patients, proposing a new classification scheme based on the frequency of naïve CD4 T cells that resulted in a significant association with clinical features, such as splenomegaly[338]. This study showed that naïve CD4 T-cell lymphopenia was accompanied by reduced thymic output and contracted CD4 and CD8 TCR repertoires in CVID patients, while paralleled by strong T-cell activation, increased T-cell turnover and apoptosis, pointing to a perturbed homeostasis of the peripheral T-cell compartment[338]. Given that the concordance between this T-cell based classification scheme and the one based in the frequency of switched-memory B cells[284] was only partial, the authors propose that the function of T cells should also be taken into consideration in order to attain a proper classification of CVID patients[338]. In this sense, the DEFI Study Group has made an attempt to develop a classification system based on the frequencies of total B cells, switched-memory B cells and naïve CD4 T cells[339]. With this approach, the authors observed that CVID patients with clinical complications such as lymphoproliferation and autoimmune cytopenias more frequently present a phenotype with major B- and T-cell alterations, while patients presenting with only infections have no significant alterations of the B- and T-cell compartments[339]. Another study has reported that CVID patients with expanded $CD21^{low}$ B cells simultaneously present a severe reduction of naïve CD4 T cells associated with decreased levels of TRECs, showing a combined defect in B- and T-cell subsets[340].

It had been early on suggested that T cells from CVID patients could exert a suppressive effect on B-cell differentiation and antibody production[341,342]. This idea is however far from the current view on regulatory CD4 T cells (T_{reg}). T_{reg} cells are defined by the expression of the forkhead box P3 (FOXP3), a transcription factor associated with T_{reg} differentiation[343-345], which is considered the best available T_{reg} marker, in addition to high levels of CD25. T_{reg} cells have a crucial role in maintaining tolerance to self-antigens and preventing autoimmunity, as demonstrated both by the mouse and human models of FOXP3 deficiency[346-349], in which individuals develop fatal autoimmunity. In recent years, T_{reg} cells have been described to be reduced in CVID patients[350,351] and this reduction has been proposed to be linked to autoimmune

manifestations in the patients[350,352,353]. In addition, the frequency of T_{reg} cells has been shown to negatively associate with the expansion of $CD21^{low}$ B cells[352,353], further supporting a link with the development of autoimmunity. It has also been reported that T_{reg} cells from CVID patients with autoimmune manifestations have a lower suppressive capacity, together with lower expression of molecules normally related to T_{reg} function[354].

In the first four patients described with ICOS deficiency, apart from the lack of ICOS expression, T cells were found to be normal with regard to activation, proliferation, cytokine production and subset distribution[31]. However, subsequent studies have reported impaired IL-10 and IL-17 production by ICOS-deficient T cells[123]. Furthermore, it has been shown that circulating $CXCR5^{+}$ CD4 T cells are severely reduced in these patients, reflecting the disturbed generation of GCs[124]. CVID patients with TACI mutations present a normal T-cell compartment, with normal T-cell subsets and maintained proliferative responses[235]. This is also the case in BAFF-R-deficient patients, who have a normal distribution of T-cell populations[234].

Contrary to what is found in CVID patients, little abnormalities have been described at the level of the T-cell compartment of patients with Congenital Agammaglobulinemia. Normal mitogenic responses were early on described[355,356], together with normal T-cell numbers and subsets[357]. Additionally, XLA patients were shown to possess intact T-cell responses[305] as well as normal CD4 T-cell helper function[358]. However, other studies have reported a poor helper function in XLA[359]. It had actually been proposed that T cells from XLA patients were phenotypically similar to those found in healthy newborns, suggesting immaturity of the T-cell compartment[360,361], but this is not likely the case since memory T cells are present in XLA in normal proportions, contrary to previous observations[362], while adequately responding to recall antigens, such as tetanus toxoid[323,355]. In addition, the development of memory T-cell responses to viral infections, such as influenza, has been shown to be normal and to not depend on the presence of B cells[363]. On the other hand, it has been recently reported that naturally acquired T-cell memory responses to meningococcal antigens are reduced in XLA patients as compared to healthy individuals, implicating B cells in the induction and maintenance of T-cell memory to given pathogens[364]. Antigenic and mitogenic responses in XLA patients have been shown to present a predominant T_H1 phenotype, contrary to what is found in healthy individuals[365]. In contrast, a different study has shown that both T_H1 and T_H2 memory-effector and resting T cells develop in XLA patients vaccinated against Hepatitis B Virus, suggesting that B cells are not absolutely necessary for antigen-specific T-cell memory development, nor do they appear to affect the T_H1/T_H2 balance[366]. Quite recently, the importance of B cells in CD4 T-cell differentiation has been given further support by the finding that, similar to what is observed in

ICOS-deficient patients, XLA patients show decreased frequencies of circulating $CXCR5^+$ CD4 T cells, together with a reduction in the total memory T-cell pool, suggesting that B cells are crucial for the generation of GCs and the development of T_{FH} cells[367], of which circulating $CXCR5^+$ CD4 T cells are believed to be counterparts[125].

Along with the observation that IgG replacement therapy can induce B-cell proliferation and Ig production in some CVID patients[253], several other reports have suggested that it can also have an immunomodulatory role on T-cell function. Long-term administration of IgG replacement therapy in CVID patients was shown to significantly reduce the numbers of circulating T cells[368]. Also, administration of intravenous IgG (IVIg) leads to an instant increase in the production of IL-2 and TNF-α by T cells from CVID patients, but not from individuals with XLA[369], suggesting a disease-specific pattern of action. In addition, IgG replacement therapy appears to have an important anti-proliferative effect on T cells from both CVID and Congenital Agammaglobulinemia patients[370]. It has also been shown to significantly increase the *in vivo* expression of both CD25 and Fas within T cells, with a possible impact on T-cell function[371]. Of note, IVIg has also been shown to inhibit the differentiation of T_H17 cells from naïve CD4 T cells *in vitro*, but also their amplification and effector function, suggesting that this can be an important mechanism in the beneficial therapeutic effect of IVIg in patients with inflammatory or autoimmune diseases[372].

d. OTHER IMMUNE COMPARTMENTS AFFECTED IN B-CELL IMMUNODEFICIENCIES

Apart from the impairments found to affect both B- and T-cell compartments in patients with antibody deficiencies, other cellular compartments have been shown to be similarly compromised. It has long been recognized that macrophages from CVID patients have a lower antigen-presenting capacity[373,374], together with a defective interaction between macrophages and T cells[375]. Also, DCs from CVID patients were shown to induce abnormal Ig production by healthy B cells, suggesting that an impaired accessory function may play an important role in B-cell differentiation in CVID[376]. However, normal antigen presentation has also been reported in CVID[377,378]. Monocyte chronic activation has been proposed, relating to raised levels of serum neopterin in both CVID and XLA patients[379,380], as well as increased production of reactive oxygen species, which was only observed in CVID and not in XLA patients[381]. It has also been reported that monocyte production of IL-6 and TNF-α in response to Pneumovax-23 (Pneumococcal Vaccine Polyvalent) was impaired in CVID patients, possibly underlying the increased susceptibility of these patients to *Streptococcus pneumoniae* infection[382].

CVID has been associated with defective *in vitro* maturation of DCs from monocytes, at least in a subset of patients[383,384], and with disturbances in the monocyte responses upon lypopolysaccharide (LPS) stimulation *in vitro*[385]. Additionally, CVID patients were shown to have reduced numbers of circulating DCs[386,387], with perturbed differentiation and function, namely reduced expression levels of the co-stimulatory molecules CD80 and CD86, an impaired ability to produce IL-12 upon stimulation[388-390], as well as lower antigen-presenting capacity in mixed lymphocyte reactions[389,390]. It has also been recently shown that immediately after administration of IVIg there is a reduction of pro-inflammatory $CD14^+CD16^+$ monocytes in CVID patients, and that TNF-α production in response to *ex vivo* LPS stimulation is also reduced, implying that IVIg at replacement doses may in this way play an important anti-inflammatory role[391]. It should be noted however that this effect is transient and numbers of $CD14^+CD16^+$ monocytes return to baseline 20h after IVIg has been administered[391].

Monocyte-related alterations, namely an increased frequency of CD16-positive monocytes[392] and decreased numbers of mDCs[393] have also been reported in XLA patients. A previous study reported a lack of major alterations in monocyte function as assessed *in vitro* in patients with XLA[385]. The same study showed an up-regulation of Human Leukocyte Antigen D-related (HLA-DR) within the whole monocyte population in CVID patients[385]. CD86, a marker of monocyte differentiation and acquisition of antigen-presentation properties, has been shown to be expressed at reduced levels in monocyte-derived DCs generated *in vitro* from CVID patients, supporting an impaired function as APCs in CVID[383,394].

It has also been shown that TLR7 and TLR9 function is defective in CVID patients[395,396]. pDCs from CVID patients produced markedly less IFN-α upon TLR9 stimulation, even though TLR9 expression was normal[395]. Stimulation through these endosomal TLRs resulted in defective B-cell proliferation, lack of IL-6 and IL-10 production, defective up-regulation of AID mRNA, low isotype switch, and impaired IgG and IgA production in both $CD27^-$ naïve and $CD27^+$ memory CVID B cells.

Given that Btk is not only crucial for B-cell development, but is also expressed in cells of the myeloid lineage[202], including DCs and monocytes, it could be anticipated that these cellular compartments would be affected in XLA patients. Btk has been found to interact with TLR8, and to TLRs 4, 6 and 9 to a lesser degree. In addition, Btk also interacts with proteins of the TLR signalling pathway, suggesting that Btk may be part of the protein complex recruited upon signaling[397]. However, it has been shown that DCs from XLA patients can be fully differentiated from monocyte precursors, as well as matured in response to LPS[398]. In addition, these DCs were entirely capable of priming naïve CD4 T cells, arguing against a role for Btk in DC differentiation,

maturation and function[398]. Another study has nevertheless reported that differentiation and maturation of DCs from monocytes of XLA patients in the presence of autologous plasma is impaired, but it could be restored upon stimulation with CD40L or supplementation with IVIg[399]. It has also been reported that peripheral blood mononuclear cells (PBMCs) from XLA patients produce reduced amounts of TNF-α when stimulated with LPS *in vitro*[400]. A subsequent study has contradicted this observation by showing that the early events of LPS signalling in monocytes from XLA patients, including TNF-α and IL-6 production, are similar to those in healthy subjects[401]. It was later shown that both mDCs and pDCs are present in normal numbers in the peripheral blood of XLA patients[402]. Nevertheless, the same study reported impaired TLR8-mediated IL-6 and TNF-α production in DCs from XLA patients, while phenotypic maturation, APC function and responses to TLRs 1/2, 2/6, 3, 4, and 5 were normal, suggesting that defective TLR8 signalling can underlie the increased susceptibility of XLA patients to enteroviral infections[402]. Another study has shown that PBMCs from XLA patients produce significantly less TNF-α and IL-1β than healthy controls when stimulated through TLR2 or TLR4, while IL-6 and IL-10 production were unaffected[403]. This was also observed in another report, where it was shown that the expression of TLRs is not defective in monocytes and DCs from XLA patients, but stimulation through TLRs 2, 4, or 8 elicited significantly less maturation of DCs[404]. Importantly, DC responses to influenza virus were found to be normal in XLA patients, with normal ability to produce IFN-α[363].

Neutropenia has been reported as a frequent finding at diagnosis in XLA patients, although with an unclear etiology[405,406]. Episodes of neutropenia are usually resolved upon starting IgG replacement therapy[406]. Nevertheless, it has been recently shown that Btk is necessary for neutrophil development and function in a mouse model of XLA[407]. In this work, the authors have shown that, despite an increased granulopoiesis, Btk-deficient neutrophils have defective maturation and function in an acute inflammatory response[407]. However, it has been shown that Btk-deficiency in XLA patients does not impair essential functional responses of neutrophils to TLR stimulation[408], suggesting a fundamental difference between the human and the mouse model of Btk-deficiency in what regards the myeloid compartment.

Natural Killer (NK) cells, an important arm of the innate immune system, have been reported to be severely reduced in both CVID and XLA patients, although with no clear clinical relevance[409,410]. Although decreased, NK cells seem to be fully functional and may thus functionally compensate for the low numbers present[410]. It has been very recently described that Btk expression is up-regulated during maturation of murine NK cells, while being involved in

TLR3-mediated NK activation[411]. In this study, XLA patients were reported to have normal NK cell numbers, while TLR3-mediated NK activation was decreased, with reduced expression of IFN-γ and killing capacity, implying that Btk is important for NK function[411].

This overview illustrates how primary B-cell defects can impact in all compartments of the immune system. It seems unlikely that these impairments can be solely attributed to a lack of B cells, as in the case of XLA patients. Immunological alterations most likely result from the interplay of defective B cells and other cells of the immune system, an area of research that remains largely unexplored.

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CHAPTER 2

AIM AND WORKPLAN

B lymphocytes are central players of the immune system, mainly recognized by the production of antibodies. Moreover, the interaction between B and T cells has a crucial role in the generation of adaptive immune responses. B cells are also important in linking innate and adaptive immune responses, given the fact that they express receptors that recognize both specific antigens and microbial patterns. The integration of signals coming from these pathways thus determines the fine-tuning of the overall B-cell response.

The general aim of this work was to study the interplay between B cells and other elements of the immune system, for which we have taken advantage of two clinical models of primary B-cell immunodeficiencies, CVID and Congenital Agammaglobulinemia. We reason that studying these human clinical models will not only extend our knowledge on the function of the immune system, but will also provide new tools regarding the treatment and follow-up of these patients.

CVID is a primary immunodeficiency defined by impaired antibody production. CVID patients have heterogeneous immunological and clinical profiles, and although some cases have been associated with monogenic defects affecting CD19, ICOS, and the B-cell-associated receptors TACI and BAFF-R, the immunodeficiency is fundamentally considered to be of polygenic aetiology. The defects in mature B-cell development that characterize CVID are thought to mainly result from impairments in GC organization. Although being a B-cell immunodeficiency, CVID is associated with perturbations of the T-cell compartment, namely naïve/memory disturbances and increased levels of cellular activation, among others, which are reported even in patients under replacement therapy with IgG. Indeed, non-infectious complications, such as lymphoproliferation, granulomatous disease and autoimmune manifestations, are currently main causes of morbidity and mortality in CVID. The mechanisms underlying the chronic immune activation associated with CVID remain largely unclear.

Congenital Agammaglobulinemia is associated with a disruption of early B-cell development in the bone marrow that results, in the majority of cases, from mutations in the Btk gene, frequently leading to a complete lack of circulating B cells. As expected, these patients have a high frequency of respiratory infections. However, they do not have major non-infectious complications. We found that despite the absence of B cells, patients with Congenital

Agammaglobulinemia do not normally present with major T-cell imbalances, thus providing us with an excellent model to study the interaction between the different compartments of the immune system, in contrast to CVID patients.

The specific aims of this work were the following: 1) to study the modulation of BAFF-R and TACI expression in CVID, in what regards its role in B-cell homeostasis; 2) to investigate a putative relationship between B- and T-cell imbalances in CVID and Congenital Agammaglobulinemia, namely regarding the autoimmunity-associated CD4 T-cell population that produces IL-17, T_H17, and the differentiation of mature B cells; and 3) to assess monocyte imbalances and their possible relationship with increased microbial translocation in CVID patients and with chronic immune activation. Additionally, the CVID patients enrolled in this study, which are followed at the Serviço de Imunoalergologia from the Hospital de Santa Maria, Lisbon, were characterized and classified according to their B-cell phenotypes, in line with the most recent classification of CVID, as proposed by Wehr *et al.*.

I. Modulation of BAFF-R and TACI expression in CVID

Peripheral B-cell survival and differentiation mainly rely on two major factors, BAFF and APRIL, and on their interaction with the receptors BAFF-R, TACI, and BCMA. BAFF serum levels have been shown to be elevated in numerous clinical settings, such as in autoimmune diseases or lymphoid cancers. Both BAFF and APRIL circulating levels have also been shown to be highly increased in CVID patients. However, no relationship has been found between high BAFF and APRIL levels and clinical manifestations in CVID patients, such as autoimmunity or splenomegaly. In addition, mutations in *TNFRSF13B* and *TNFRSF13C*, encoding TACI and BAFF-R, respectively, have been associated with CVID.

The first aim of this work was to investigate BAFF-R and TACI expression in CVID. For this purpose, the levels of protein expression of BAFF-R and TACI at the surface of B cells were compared in cohorts of CVID patients and healthy controls. The *in vitro* modulation of BAFF-R expression by recombinant BAFF, recombinant APRIL, and autologous serum was also assessed. Additionally, the levels of mRNA transcripts of *TNFRSF13C*, encoding BAFF-R, were quantified in these conditions, in order to evaluate a possible transcriptional regulation. Finally, longitudinal data on BAFF-R expression and serum levels of BAFF were collected, with the purpose of determining the impact of the introduction of IgG replacement therapy on these parameters.

The results obtained are shown in the chapter 1 of the Results.

II. Relationship between B- and T-cell imbalances in primary B-cell immunodeficiencies

The interaction between T cells and B cells is fundamental to generate adaptive immune responses. The establishment of long-term humoral immunity implies that B cells receive appropriate help from CD4 T cells in the context of a GC reaction, with the generation of memory B cells and antibody-producing plasma cells. T-cell help is known to be essential to the induction and subsequent organization of GCs. This help has mainly been attributed to a particular subset of T cells termed follicular helper T cells, T_{FH}, which are identified by the expression of the chemokine receptor CXCR5, necessary for their specific homing within GCs. The lack of adequate T-cell help during the priming of B cells results in apoptosis, rather than their differentiation into GC B cells or plasma cells. The functional interaction between T cells and B cells rely both on the expression of co-stimulatory molecules, such as CD40-CD40L and ICOS molecule, as well as in cytokine production, with the best example being IL-21. These pathways have also been implicated in the induction and/or survival of T_H17 cells.

IL-17 is a pro-inflammatory cytokine implicated in autoimmune and inflammatory conditions. The development/survival of T_H17 cells share critical cues with B-cell differentiation and the circulating T_{FH} subset has been shown to be enriched in T_H17 cells able to help B-cell differentiation, so potentially the homeostasis of the circulating T_H17 compartment may be related to B-cell differentiation. CVID is frequently associated with autoimmune and inflammatory manifestations, and thus it is plausible that IL-17 may play a role in these processes.

As a strategy to understand the contribution of T_H17 cells to the mechanisms underlying inflammatory manifestations, the frequency of T_H17 cells was compared in CVID patients, Congenital Agammaglobulinemia patients and healthy individuals. The relationship between T_H17 cells and parameters of naïve T-cell depletion and T-cell activation was determined, in comparison to the populations of CD4 T cells producing other pro-inflammatory cytokines, such as IFN-γ and TNF-α. In addition, it was also studied the association of T_H17 cells with populations of B cells that are commonly impaired in CVID. In Congenital Agammaglobulinemia patients, who are expected to have severely impaired GC formation, circulating T_{FH} cells were additionally studied. With the aim of better understanding the relationship between cues involved in B-cell differentiation and the homeostasis of T-cell populations, serum levels of BAFF, a crucial cytokine for B-cell survival and maturation, were quantified in CVID and Congenital

Agammaglobulinemia, and their relationship with T_H17 cells was investigated. In addition, in order to understand if the relationship between T_H17 cells and B cells relies on cues involved in class-switch to IgA, T_H17 cells were measured in patients with selective IgA deficiency, a primary B-cell immunodeficiency that is characterized by a strict deficiency in IgA production, with all other Ig subclasses normal. To test the possibility of an intrinsic impairment in the ability of CD4 T cells from Congenital Agammaglobulinemia patients to differentiate into T_H17 cells, *in vitro* T_H17 differentiation assays were performed and compared to healthy individuals.

T_{reg} and T_H17 cells are believed to possess reciprocal pathways of differentiation. In order to determine the relationship between T_{reg} cells and T_H17 cells in primary B-cell immunodeficiencies, T_{reg} cells were quantified in terms of FOXP3 and CD25 expression in CVID patients, and their association with T_H17 cells and the abovementioned B- and T-cell subsets was analysed.

The results generated are presented in the chapter 2 of the Results.

III. Monocyte imbalances in primary B-cell immunodeficiencies and their possible relationship with increased microbial translocation and chronic immune activation in CVID patients.

T-cell activation and granulomatous manifestations represent main causes of CVID morbidity, even in patients under IgG replacement therapy. The mechanisms underlying the chronic immune activation associated with CVID remain largely unclear. Another main cause of CVID morbidity is gastrointestinal pathology, with or without mal-absorption. The impairment in IgA production and other CVID-associated mucosal alterations have been shown to be often associated with increased intestinal permeability. Thus, it is plausible that increased levels of microbial translocation, particularly of bacterial products such as LPS, with consequent monocyte stimulation, may contribute to the chronic immune activation observed in CVID patients, as reported for HIV-1 infected individuals.

To assess this premise, the monocyte compartment was characterized in patients with CVID and Congenital Agammaglobulinemia, and compared to healthy controls. The possible relationship with increased microbial translocation in CVID patients was evaluated by the quantification of plasma LPS, and serum levels of soluble CD14 (sCD14), LPS-binding protein (LBP), and anti-LPS antibodies in samples from CVID patients. In addition, to assess the impact of IgG replacement therapy on these parameters, monocyte and T-cell subsets were analyzed longitudinally in a group of CVID patients, before and after starting IgG replacement therapy.

These results are presented in the chapter 3 of the Results.

In agreement with the Decreto-Lei 388/70, art. 8o, parágrafo 2, the results presented here were published or are currently being prepared for publication in the following scientific journals:

Rita R. Barbosa, Susana L. Silva, Sara P. Silva, Alcinda Campos Melo, M. Conceição Pereira-Santos, João T. Barata, Ana E. Sousa. Decreased BAFF-R and increased TACI expression on B cells are features of Common Variable Immunodeficiency Disorders.
Manuscript in preparation.

Rita R. Barbosa, Sara P. Silva, Susana L. Silva, Alcinda Campos Melo, Elisa Pedro, Manuel P. Barbosa, M. Conceição Pereira-Santos, Rui M. M. Victorino, Ana E. Sousa. Primary B-cell deficiencies reveal a link between human IL-17-producing CD4 T-cell homeostasis and B-cell differentiation. *PLoS ONE*, 2011;6(8):e22848.

Rita R. Barbosa, Sara P. Silva, Susana L. Silva, Rita Tendeiro, Alcinda Campos Melo, Elisa Pedro, Manuel P. Barbosa, M. Conceição Pereira Santos, Rui M. M. Victorino, Ana E. Sousa. Monocyte activation is a feature of Common Variable Immunodeficiency irrespective of plasma lipopolysaccharide levels. *Clinical and Experimental Immunology*, 2012 Sep;169(3):263-72.

CHAPTER 3

RESULTS

3.1 – MODULATION OF BAFF-R AND TACI EXPRESSION IN COMMON VARIABLE IMMUNODEFICIENCY

including

Decreased BAFF-R and increased TACI expression on B cells are features of Common Variable Immunodeficiency Disorders

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Manuscript in preparation

Abstract

Peripheral B-cell survival and differentiation critically depend on the interaction of B-cell activating factor receptor (BAFF-R) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) with their ligands, BAFF and a proliferation-inducing ligand (APRIL). We hypothesize that disruption of these pathways plays a role in Common Variable Immunodeficiency Disorders (CVID), which are defined by defects in mature B-cell function with impaired antibody production. We found that BAFF-R expression was significantly reduced in all B-cell subsets in CVID, both at the protein and the mRNA levels, particularly in patients with very low memory B-cell frequencies. Conversely, TACI expression was increased in CVID patients, especially in the memory B-cell compartment. Relevant polymorphisms in the genes encoding these receptors were excluded in the studied CVID patients, given that monogenic defects have been described in rare cases. Reduced BAFF-R and increased TACI expression were both directly associated with high BAFF serum levels, but not APRIL, even though being a ligand for TACI. Using an *in vitro* culture approach, we observed that recombinant BAFF, as well as autologous serum, induced down-modulation of BAFF-R protein expression, both in healthy individuals and CVID patients. However, the degree of modulation seemed to be impaired in CVID patients, suggesting that these dynamics are affected. In addition, BAFF did not seem to regulate BAFF-R expression at the transcriptional level in this *in vitro* model. Understanding the mechanisms involved in the modulation of BAFF-R expression by its ligand BAFF will be crucial to clarify the role of BAFF-R dysregulation in CVID pathogenesis, in particular, and in B-cell biology, in general.

Introduction

Peripheral B-cell survival and differentiation mainly rely on two major factors, BAFF (B-cell activating factor of the TNF family; BLyS/TALL-1/THANK/zTNF4) and APRIL (a proliferation ligand), and on their interaction with the receptors BAFF-R (BAFF receptor; BR3), TACI (Transmembrane Activator and CAML Interactor), and BCMA (B-cell maturation antigen)[1]. BAFF-R is expressed by all peripheral B cells and is critical for their survival and maturation. TACI is mainly expressed on memory peripheral B cells and it is suggested to act as a negative regulator of B-cell development. BCMA is expressed on memory B cells in secondary lymphoid organs but its highest levels are found in plasma cells[1]. BAFF-R only binds BAFF, whereas TACI and BCMA can bind both BAFF and APRIL, and BCMA binds APRIL with a 10-fold higher affinity than BAFF[1].

BAFF and APRIL are primarily produced by neutrophils, monocytes, macrophages and dendritic cells. They are also produced by non-hematopoietic cells at local niches to promote survival and function of B cells and plasma cells[1]. T cells may also produce BAFF[2,3]. In addition, BAFF and APRIL can be produced by lymphoid tumour cells to promote survival in an autocrine fashion[1,4-6]. It has been shown in the mouse that two distinct pools of BAFF actually exist: a constitutive pool that is produced by radiation-resistant cells and controls the size of the peripheral B cell pool; and a cytokine-induced pool that acts at sites of inflammation[7,8].

BAFF transgenic mice present B-cell hyperplasia and autoimmune manifestations that resemble systemic lupus erythematosus (SLE) and Sjögren's syndrome[9-11], pointing out the immunoregulatory potential of the BAFF system on B cells. BAFF serum levels have been shown to be elevated in numerous clinical settings, such as in autoimmune diseases, or lymphoid cancers. BAFF can also be increased during inflammation and infection, possibly modulated by type I interferons[1]. BAFF-deficient animals have a similar phenotype to A/WySnJ mice, which have a mutant BAFF-R gene, and to BAFF-R-null mice, all of which present severe mature B-cell lymphopenia[12-15]. In agreement, both BAFF-deficient and BAFF-R-deficient mice show abnormal germinal centre (GC) development and progression[16]. TACI-deficient mice, on the contrary, show a marked expansion of B cells that includes almost all mature peripheral B cell populations, splenomegaly and over-production of immunoglobulins[17,18], suggesting a negative regulatory role for TACI in B-cell homeostasis. Nevertheless, TACI-deficient mice also present defective T-independent type 2 responses *in vivo*, indicating a positive outcome of TACI stimulation[18,19]. APRIL-deficient mice have normal B and T-cell development, but impaired class-switching to IgA[20]. APRIL-transgenic mice, on the other hand, develop B-cell neoplasias as they age[21] and present increased T-cell survival[22].

Common Variable Immunodeficiency (CVID) is the most prevalent symptomatic primary immunodeficiency. Patients present a heterogeneous immunological and clinical profile, and although some cases have been associated with monogenic defects, the disease is largely considered to be of polygenic etiology[23]. Importantly, some of the referred monogenic defects include polymorphisms in the coding genes for BAFF-R and TACI[24-27]. Humans lacking TACI expression have a CVID clinical phenotype, with increased B-cell numbers but decreased immunoglobulin production, contrary to what is observed in the mouse[24,27]. Nevertheless, the murine equivalent to the C104R TACI mutation, C76R, significantly disrupts B-cell homeostasis, immunoglobulin production, and antigen-stimulated humoral responses[28]. Two siblings who present an adult-onset CVID-like syndrome have been described to lack BAFF-R protein expression with consequent severe B-cell lymphopenia[26]. The clinical manifestations in these patients were however mild in comparison to other antibody-deficient settings[26]. In addition, polymorphic variants of BAFF-R have been described, affecting both CVID patients and healthy individuals. Such variants do not impact in BAFF-R expression, neither at the mRNA level nor at the protein level[25]. However, ligand-binding capacity has not been evaluated in these subjects[25].

Little is known about the expression of these receptors in CVID patients and how it is related to disease pathogenesis. Both BAFF and APRIL circulating levels have been shown to be highly increased in CVID patients[29-32]. However, no relationship has been found between high BAFF and APRIL levels and clinical manifestations in CVID patients, such as autoimmunity or splenomegaly[30,31].

The aim of this work was to study BAFF-R and TACI expression in CVID patients, and its relationship to BAFF and APRIL serum levels. In this study, we show that BAFF-R and TACI expression is altered in CVID patients, likely contributing to disease pathogenesis.

Materials and Methods

Cohorts

The study involved 33 patients with CVID and 19 healthy individuals. All subjects gave written informed consent for blood sampling and processing. The study was approved by the Ethical Board of the Faculty of Medicine of Lisbon. CVID patients were diagnosed according to the European Society for Immunodeficiency criteria (www.esid.org), namely IgG levels at least 2 standard deviations below the mean for age and a decrease of at least one of the IgM or IgA isotypes, impaired Ab response to vaccines, absent/low isohemagglutinins, and exclusion of defined causes of hypogammaglobulinemia. 27 out of 29 CVID patients were under long-term IgG replacement therapy, adjusted to guarantee pre-infusion Ig levels above 650mg/dL. The two other CVID patients not receiving IgG had levels of total serum IgG of 227 and 473 mg/dL. 4 CVID patients were enrolled in a longitudinal study prior to and after starting IgG replacement therapy. The collection of the blood samples was always performed immediately before the immunoglobulin infusions in the patients under intravenous administration. 5 CVID patients were under subcutaneous IgG administration.

Cell culture

Fresh peripheral blood mononuclear cells (PBMCs) were isolated through Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech), and cultured in RPMI1640 medium (Gibco-Invitrogen) supplemented with 10% (vol/vol) human AB serum (Sigma-Aldrich), 100 U/ml penicillin/100 μg/ml streptomycin and 2 mM glutamine (Gibco-Invitrogen).

Cell stimulation and treatment

Recombinant human BAFF and recombinant human APRIL (PeproTech) were used at concentrations of 100ng/mL, unless otherwise indicated. Serum from healthy donors or CVID patients was added at a final concentration of 10% (vol/vol) for medium supplementation, where indicated.

B cell purification

B lymphocytes were purified from PBMCs by negative selection (STEMCELL Technologies). The purity of isolated $CD19^+$ B cells was routinely over 95%.

Cell staining, flow cytometric analysis and monoclonal Ab (mAb) used in flow cytometry

Phenotypic analysis was performed in whole blood samples, after staining with mAb for 20 minutes and red blood cells lysis using BD FACS Lysing Solution (BD Biosciences). Cultured PBMCs were stained for surface markers, followed by fixation with 2% formaldehyde. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) and data analysed using CellQuest (BD Biosciences) and FlowJo (Tree Star Inc.) softwares. Results are presented as a proportion of total lymphocytes or of a given cell population. The following anti-human mAb were used, with clone and the directly conjugated fluorochrome specified in brackets: CD3 (UCHT1; V500), CD11c (B-ly6; APC), CD38 (HB7; PE), IgD (IA6-2; PE), IgM (G20-127; APC), HLA-DR (L243; PerCP-Cy5-5), from BD Biosciences; CD3 (OKT3; PerCP-Cy5.5), CD8 (RPA-T8; FITC, PE and APC), CD14 (61D3; Efluor 450), CD16 (eBioCB16; FITC), CD19 (HIB19; PerCP-Cy5.5, PE-Cy7 and APC efluor 780), CD123 (6H6; PE-Cy7), CD23 (EBVCS2; APC), CD27 (O323; FITC, PE, and APC), CD38 (HB7; PE), CD56 (MEM188; FITC), BAFFR (8A7; FITC and PE), TACI (11H3; PE) from eBiosciences; CD4 (S3.5; PE) from Caltag; CD21 (BL13; FITC) from IO Test, Beckman Coulter; BAFFR (11C1; Alexa Fluor 647) from Biolegend.

Quantification of serum levels of BAFF and APRIL

Serum concentrations of BAFF and APRIL were quantified by Enzyme Linked Immunosorbent Assay (ELISA) using the BAFF Immunoassay Kit (R&D Systems) and the human APRIL ELISA (IBL International), respectively, according to the manufacturer's instructions. Samples were assayed in duplicate.

Isolation of mRNA and quantitative RT-PCR

mRNA was extracted using either the AllPrep DNA/RNA Micro Kit or the RNeasy Kit (both from Qiagen), according to cell numbers, following the manufacturer’s instructions. Single-stranded cDNA was produced by reverse transcription of 50ng of mRNA with the Superscript III First-Strand Synthesis System and oligo (dT) (Invitrogen). The concentration of mRNA was measured for normalization of all amounts used for reverse transcription. *Power* SYBR Green PCR Master Mix (Applied Biosystems) and Applied Biosystems 7500 Fast (Applied Biosystem) were used for quantitative RT-PCR. Samples were amplified in duplicate for 40 cycles and target gene expression was normalized to the expression of *GAPDH* (encoding Glyceraldehyde 3-phosphate dehydrogenase). Primer pairs for quantitative RT-PCR were as follows: 5’-GGTGGTCTCCTCTGACTTCAACA-3’ and 5’-GTTGCTGTAGCCAAATTCGTTGT-3’ for *GAPDH* and QuantiTect Primer Assay for *TNFRSF13C* (Qiagen).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc.). Two group comparisons were performed using Mann-Whitney test. Analysis of dependent samples was performed using Wilcoxon matched-pairs signed rank test. Spearman's coefficient was used to determine the significance of the correlation between two variables. Results are expressed as mean±SEM, and *P*-values <0.05 were considered to be significant.

Results

Reduced ex vivo BAFF-R expression and increased TACI expression were associated with high serum levels of BAFF in CVID patients

The first aim of this work was to evaluate the expression of BAFF-R and TACI on B cells from CVID patients, and to study its relationship with BAFF and APRIL serum levels.

BAFF-R expression was uniformly reduced on B cells from CVID patients as compared to healthy individuals, and this was observed for both naïve and memory B cells (Figure 1A).

In order to evaluate if decreased BAFF-R protein expression on B cells from CVID patients was a result of decreased gene expression, we assessed the mRNA levels of the *TNFRSF13C* gene, which encodes for BAFF-R, in purified B cells from CVID patients. Given the fact that we observed differential BAFF-R expression on memory and naïve B cells, and in order to be better able to overcome the possible impact of the imbalances of memory B-cell development in CVID patients, we selected to include in this study 3 CVID patients with frequencies of memory B cells within the normal range ("mem+") and 3 CVID patients with very low memory B-cell frequencies ("mem-"), as well as 6 healthy controls. As expected, these selected CVID patients had lower BAFF-R protein expression than healthy individuals (Figure 1B). In addition, "mem-" CVID patients clearly expressed less BAFF-R on their B cells as compared to "mem+" CVID patients, who presented BAFF-R protein levels similar to healthy controls (Figure 1B). The same trend was observed in relation to *TNFRSF13C* mRNA levels, although not as striking (Figure 1C). Nevertheless, BAFF-R protein levels were directly associated with the levels of *TNFRSF13C* mRNA (Figure 1D), suggesting that the events of gene and protein expression are not uncoupled in CVID patients.

Importantly, when CVID were stratified according to clinical manifestations, such as autoimmunity, splenomegaly, adenopathies, lymphoid proliferation, or chronic diarrhoea, we observed that BAFF-R protein expression was uniformly decreased in all subgroups of patients, irrespective of clinical complications.

On the other hand TACI expression was significantly increased on B cells from CVID patients, both in the naïve and memory subsets (Figure 2A). In addition, TACI levels were significantly increased in CVID patients with splenomegaly, as compared to those that do not present this clinical manifestation, when CVID patients were stratified according to clinical complications (Figure 2B).

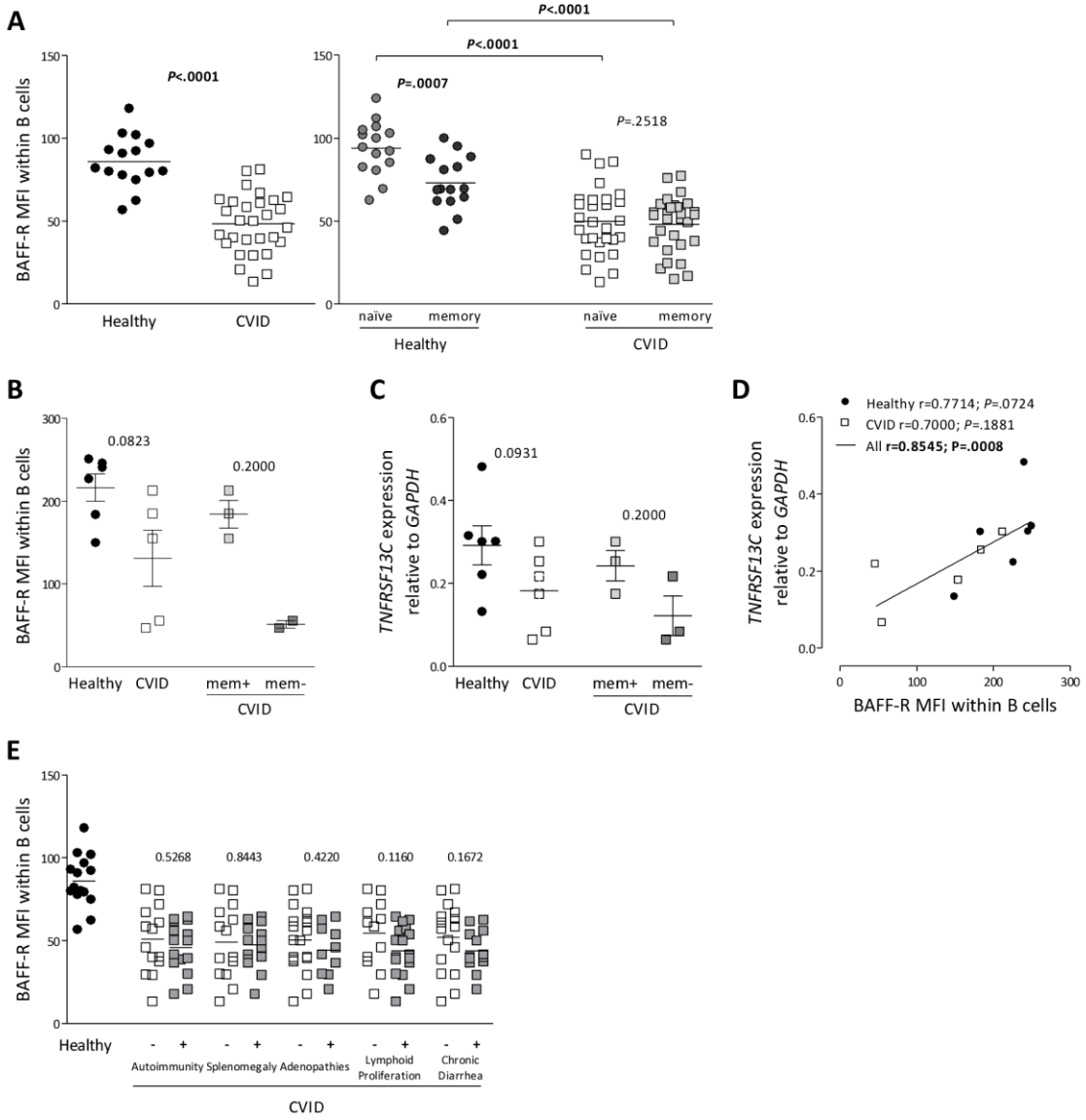


Figure 1. B-cell expression of BAFF-R was decreased in CVID patients, irrespective of clinical phenotype. Mean fluorescence intensity (MFI) of BAFF-R (A) in CVID patients and healthy individuals, within total B cells and within naïve and memory B-cell subsets. Analysis of healthy subjects and of CVID patients further divided into two subgroups, namely "mem+" for patients presenting more than 25% memory B cells and "mem-" for patients having less than 7% memory B cells, in relation to: (B) the mean fluorescence intensity (MFI) of BAFF-R expression within total B cells, and (C) mRNA transcripts for *TNFRSF13C*, encoding BAFF-R, assessed in purified B cells. (D) Correlation between BAFF-R protein expression and *TNFRSF13C* mRNA levels. (E) Stratification by clinical complications in CVID patients of B-cell expression of BAFF-R. Each dot represents one individual. Bars indicate mean. *P* values are shown.

A negative association between BAFF-R expression and BAFF serum levels was observed in both CVID patients and healthy individuals (Figure 3A, left panel), that was not observed for APRIL (Figure 3A, right panel). On the other hand, TACI expression on memory B cells was directly associated with BAFF serum levels in CVID patients, but not in healthy individuals (Figure 3B, left panel). No relationship was found with the serum levels of APRIL, which is the other described ligand for TACI (Figure 3B, right panel).

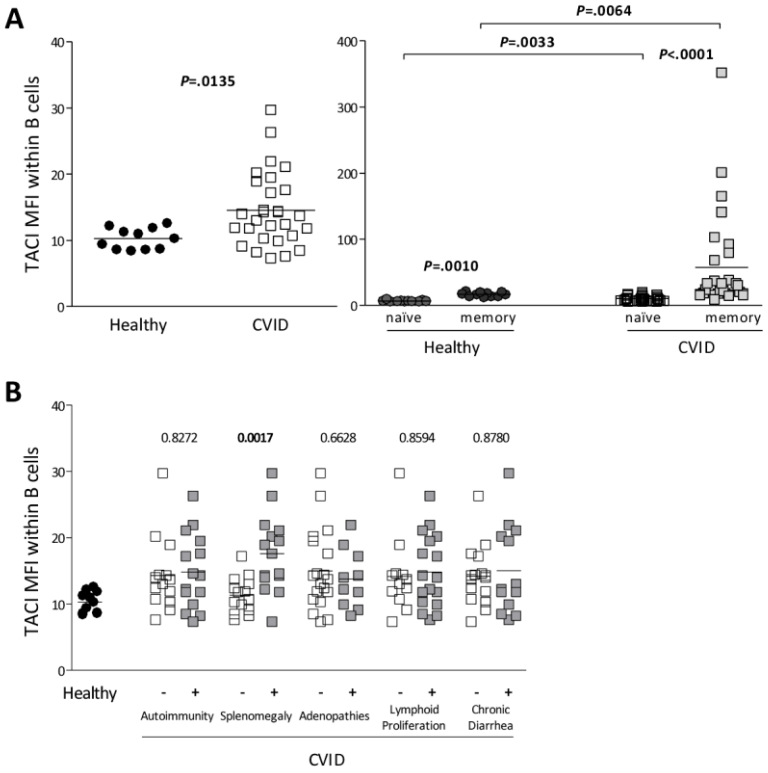


Figure 2. TACI expression was increased in CVID, particularly in patients with splenomegaly. Mean fluorescence intensity (MFI) of TACI (A) in CVID patients and healthy individuals, within total B cells and within naïve and memory B-cell subsets. (B) Stratification by clinical complications in CVID patients of B-cell expression of TACI. Each dot represents one individual. Bars indicate mean. *P* values are shown.

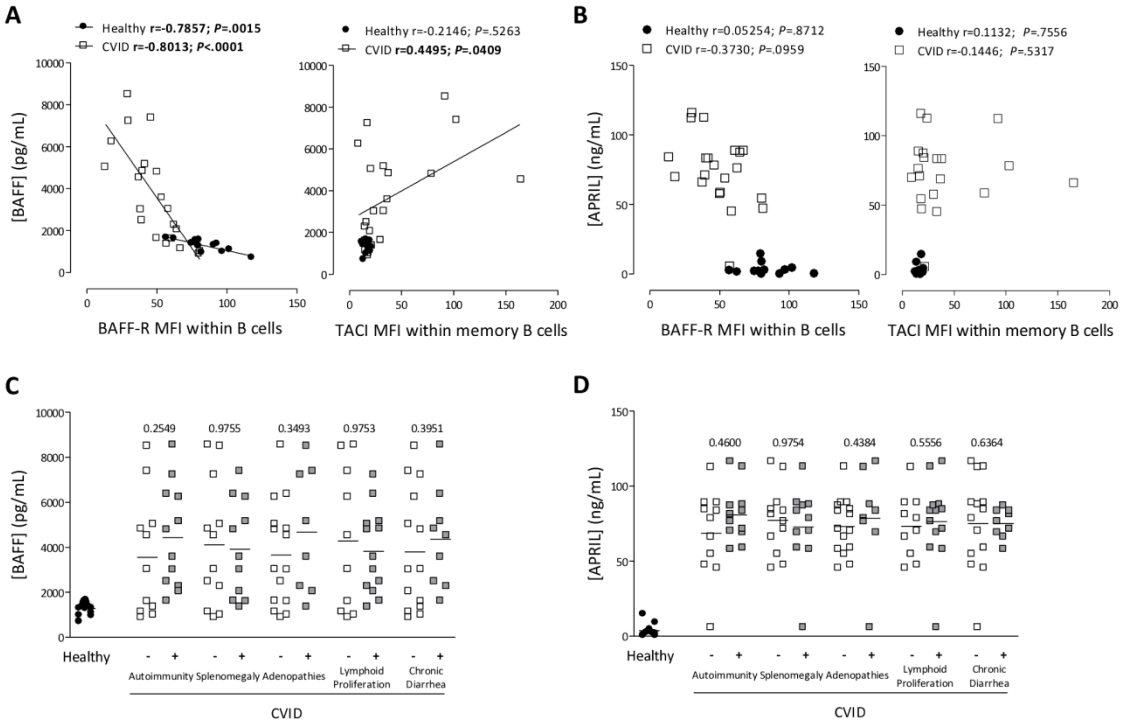


Figure 3. High serum levels of BAFF were associated with reduced BAFF-R expression and increased TACI in CVID.

Our data showing BAFF-R down-regulation in the presence of high levels of BAFF may be particularly relevant in CVID patients, given the increased serum levels of these cytokines that we and others have reported in CVID [29,30,32]. Of note, high serum levels of both BAFF and APRIL were observed in CVID patients, irrespective of clinical manifestations, such as autoimmunity, splenomegaly, lymphoproliferation, adenopathies, or chronic diarrhoea (Figure 3C and 3D). Furthermore, our results raise the possibility that the increased serum BAFF levels may play a role in TACI up-regulation in CVID patients.

BAFF-R protein expression was modulated upon culture in the presence of BAFF

In order to further investigate how BAFF modulates the expression of its receptor BAFF-R, we cultured PBMCs from the selected CVID patients and healthy controls mentioned above in the presence of BAFF or in medium alone, as well as in the presence of APRIL as a negative control, for different time points. In terms of BAFF-R protein expression, we observed that B cells cultured in the presence of BAFF strongly down-regulate its receptor relative to *ex vivo* expression levels, in contrast to B cells cultured in medium alone or APRIL, which gain some BAFF-R expression. Of note, BAFF also induces down-regulation of BAFF-R expression in CVID patients (Figure 4A). However, BAFF-R down-regulation by BAFF on B cells from CVID patients was not as striking as in healthy individuals, which could be related to the fact that B cells from CVID patients already express lower levels of BAFF-R *ex vivo* (Figure 4A). It is also possible that these dynamics of modulation of BAFF-R expression by BAFF are impaired in CVID patients. In line with this view was the fact even though B cells from CVID patients recovered some BAFF-R expression when they are cultured for 24h in the absence of BAFF, the levels attained were always lower than those observed in healthy individuals (Figure 4B).

Next, we asked whether the down-regulation of BAFF-R expression could have an impact in *TNFRSF13C* transcription and in order to do this we quantified its mRNA levels in the different culture conditions previously described. *TNFRSF13C* transcripts did not change significantly upon culture, in either of the time-points assessed (Figure 4C), suggesting that BAFF is not modulating the expression of BAFF-R at the transcriptional level.

Figure 3. (cont.) (A) Correlation between BAFF serum levels and the mean fluorescence intensity (MFI) of BAFF-R or TACI (left and right panels, respectively). (B) Correlation between APRIL serum levels and the MFI of BAFF-R or TACI (left and right panels, respectively). Stratification by clinical complications in CVID patients of BAFF (C) and APRIL (D) serum levels. Each dot represents one individual. Bars indicate mean. P values and Spearman's correlation coefficients are shown.

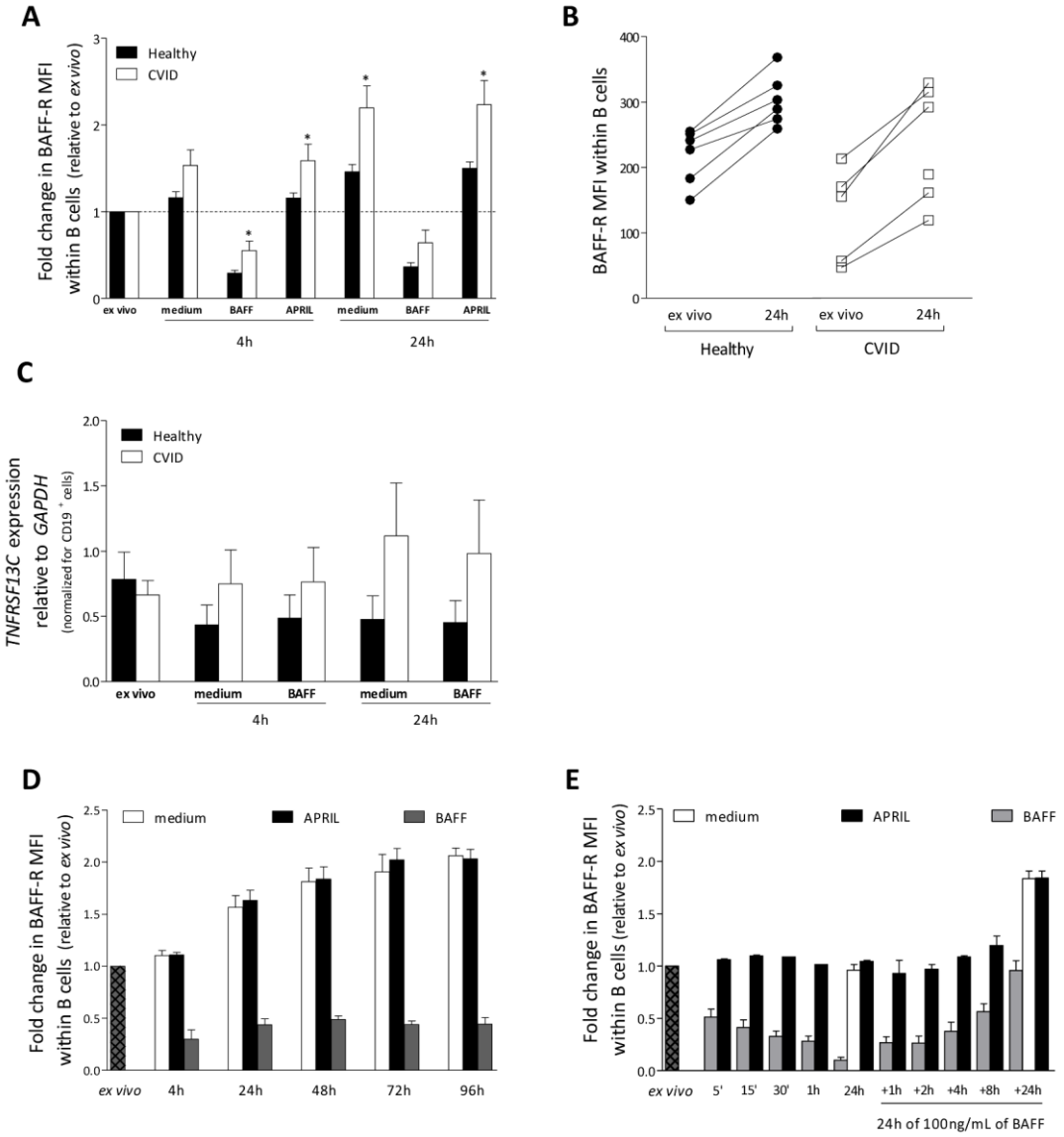


Figure 4. BAFF-R protein expression was modulated upon culture in the presence of BAFF. (A) Fold change in the mean fluorescence intensity (MFI) of BAFF-R expression on B cells upon culture in the presence of either medium alone, BAFF or APRIL, for 4h and 24h relative to *ex vivo* levels, in a group of 6 CVID patients and 6 healthy individuals. (B) Comparison between the MFI of BAFF-R expression on B cells from healthy individuals and CVID patients, either *ex vivo* or after 24h of culture in medium alone. (C) mRNA transcripts of *TNFRSF13C* in cells cultured as described in A. Levels of *TNFRSF13C* expression were normalized to the relative proportion of $CD19^+$ B cells present in culture. (D) Fold change in the MFI of BAFF-R expression on B cells upon culture in the presence of either medium alone, BAFF or APRIL, for 4h, 24h, 48h, 72h, and 96h, relative to *ex vivo* levels. Shown are the results of 3 independent experiments of healthy subjects. (E) Fold change in the MFI of BAFF-R expression on B cells upon short-term stimulation with either BAFF or APRIL. Cells were cultured for 5, 15, 30 minutes, 1 hour or 24h. After 24h of culture, cells were washed and re-plated in medium alone for the indicated period of time. Shown are the results of 2 independent experiments of healthy subjects. Bars indicate mean±SEM. Each dot represents one individual. * indicates *P* value <0.05 relative to healthy individuals.

Further *in vitro* studies in healthy individuals showed that recombinant BAFF induced down-regulation of BAFF-R as early as after 4h of culture, and that this effect was sustained up to 96h of culture, but not increased, suggesting that the maximal response to BAFF in terms of

BAFF-R modulation is attained very early (Figure 4D). In order to assess the possibility of BAFF inducing BAFF-R internalization, we performed short-term cultures with BAFF, from 5 minutes up to 1h. Our results showed that BAFF-R expression is down-modulated as early as after 5 minutes of culture with BAFF (Figure 4E), suggesting that internalization might be occurring. Additionally, removal of BAFF from cultures resulted in BAFF-R time-dependent up-regulation.

Next, we asked whether serum from CVID patients with high BAFF levels would be able to recapitulate the results obtained with recombinant BAFF, i.e., if cultures in the presence of autologous serum would induce *per se* down-regulation of BAFF-R protein expression. B cells from CVID patients cultured with autologous serum expressed lower levels of BAFF-R than those of healthy controls, consistent with the increased amount of BAFF found in the serum (Figure 5A). In addition, this down-regulation was greater in "mem-" than in "mem+" CVID patients, who behaved similarly to healthy individuals (Figure 5A). BAFF-R protein down-regulation was proportional to the level of BAFF found in the serum, as shown in Figure 5B. High concentrations of BAFF in the serum from CVID patients are thus able to recapitulate the modulation of BAFF-R expression driven by the addition of recombinant BAFF to the culture.

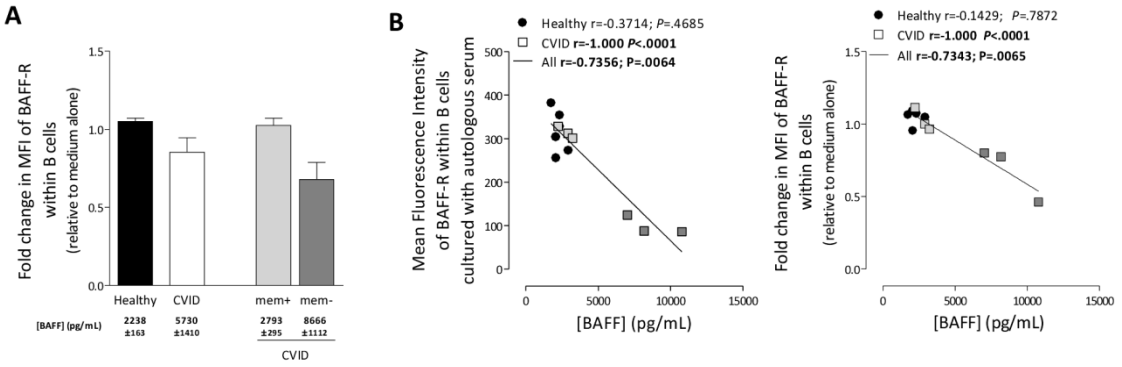


Figure 5. Autologous serum from CVID patients was able to recapitulate the effects of recombinant BAFF in culture. (A) Fold change in BAFF-R MFI expression on B cells cultured in the presence of autologous serum relative to levels expressed on B cells cultured in medium alone. CVID patients were divided into two subgroups: "mem+" for patients presenting more than 25% memory B cells; and "mem-" for patients having less than 7% memory B cells. BAFF serum levels (mean±SEM) for each group of individuals is indicated below the bars. (B) Correlation between BAFF-R MFI expression within total B cells cultured in the presence of autologous serum and the amount of BAFF found in the serum, in CVID patients and healthy controls. Bars indicate mean±SEM. Each dot represents one individual. Spearman's correlation coefficients are shown.

Overall, we show that BAFF directly modulates BAFF-R expression both in healthy subjects and CVID patients.

BAFF-R expression and BAFF serum levels did not change significantly upon starting IgG replacement therapy in CVID patients

CVID patients receive IgG replacement therapy, aiming to restore antibody levels that the patients are not able to produce. BAFF serum levels can change in a variety of settings[29,33], and we hypothesized they may be modulated by the IgG treatment and the control of infections. We thus asked whether the introduction of IgG replacement therapy is able to correct the disturbances in BAFF-R and TACI expression in CVID patients. For this purpose, we performed a longitudinal assessment of serum BAFF levels and BAFF-R protein expression in a group of 4 CVID patients, prior to starting IgG replacement therapy and up to 12 months after commencing it. Even though inter-individual variability was high, we found that BAFF serum levels were remarkably stable over the time-period analyzed (Figure 6A) and that BAFF-R protein levels did not change significantly over time with IgG replacement therapy (Figure 6B). It is important to note that the major B-cell subsets that are considered in CVID, such as the frequency of memory B cells, switched-memory B cells, $CD21^{low}CD38^{low}$ B cells, transitional B cells and plasmablasts, remain virtually unchanged over the time-period analyzed following the introduction of IgG replacement therapy (Supplemental Figure 2A-F).

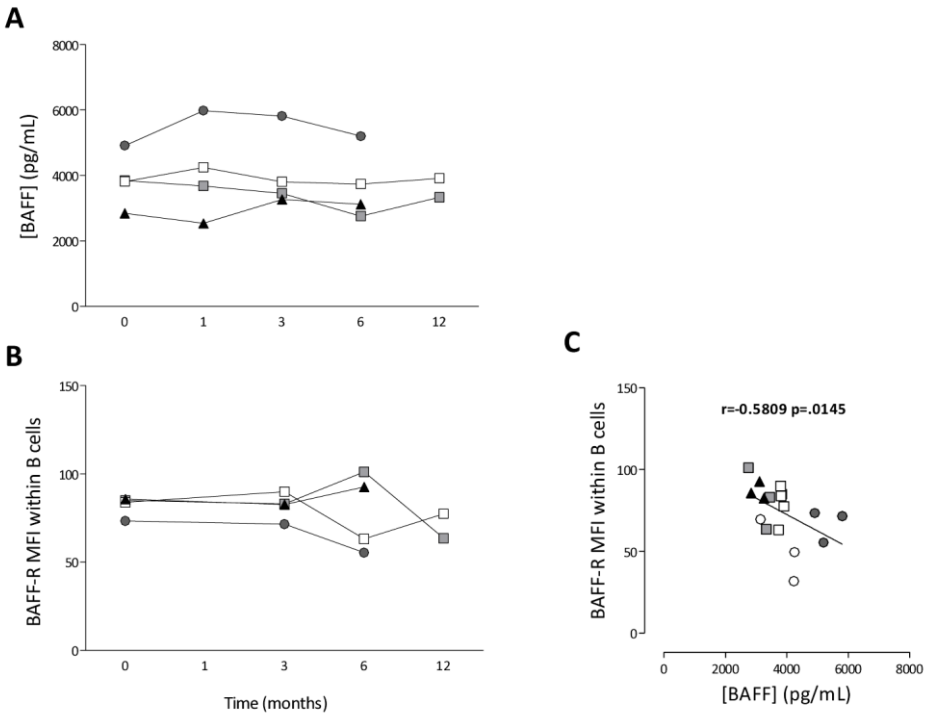


Figure 6. BAFF-R expression and BAFF serum levels did not significantly change upon starting IgG replacement therapy. Time-course analysis of BAFF serum levels (A) and of the mean fluorescence intensity (MFI) of BAFF-R expression on B cells (B) in 4 CVID patients prior to and after starting IgG replacement therapy. (C) Correlation between MFI of BAFF-R expression within total B cells and BAFF serum levels found in the different time-points of the 4 CVID patients. Each dot represents one individual. Spearman's correlation coefficients are shown.

In addition, and in agreement with our previous observations, BAFF-R protein expression was inversely associated with the serum levels of BAFF when all time-points and all individuals were considered (Figure 6C). Thus, the relationship between BAFF serum levels and BAFF-R protein expression also seems to be independent of therapy and of the associated control of infectious events in CVID patients.

Altogether, our data suggest that increased serum BAFF and reduced BAFF-R expression levels are features of CVID that are not modulated by IgG replacement therapy.

Discussion

In this study, we showed that BAFF-R and TACI expression were altered in CVID patients, likely contributing to disease pathogenesis. We found that BAFF-R expression was significantly reduced in all B-cell subsets from CVID patients, both at the protein and the mRNA levels, particularly in patients with very low frequencies of memory B cells. In contrast, TACI expression was increased in CVID patients, especially in the memory B-cell compartment. Relevant polymorphisms in the genes encoding these BAFF-R and TACI were excluded in the studied CVID patients, given that monogenic defects have been described in rare cases (Supplemental Tables 1 and 2). Reduced BAFF-R and increased TACI expression were both directly associated with high BAFF serum levels, but not APRIL. Using an *in vitro* culture approach, we observed that recombinant BAFF, as well as autologous serum, induced down-modulation of BAFF-R protein expression, both in healthy individuals and CVID patients. However, the degree of modulation seemed to be impaired in CVID patients, suggesting that these dynamics are affected. In addition, BAFF did not seem to regulate BAFF-R expression at the transcriptional level in this *in vitro* model. We also observed that BAFF-R expression and BAFF serum levels were rather stable over time in CVID patients starting IgG replacement therapy.

Since the identification of APRIL[34] and BAFF[35,36] as members of the TNF family with B-cell stimulating capacities, and the subsequent discovery of their three receptors, BCMA, TACI[37,38], and BAFF-R[13,14], much knowledge has been gained regarding the specificities of receptor-ligand interactions, signalling pathways involved and biological functions[1]. It has been shown that BCR signals[39,40] and TLR stimulation[41,42] can increase the expression of BAFF receptors, BAFF-R and TACI. However, little is known about the modulation of receptor expression in response to the ligands themselves, particularly in humans. The negative correlation that we observed between BAFF serum levels and BAFF-R expression on B cells, not only in CVID patients, but also in healthy individuals, support the view that BAFF can actually modulate the levels of BAFF-R in a physiological context. It has been previously reported that high BAFF levels are associated with low BAFF-R expression on B cells from SLE and Sjögren's syndrome patients, and that low BAFF-R expression is linked to disease activity[43]. The same study has also shown that the low levels of BAFF-R expression in Sjögren's syndrome patients were not due to transcriptional modulation, since BAFF-R mRNA levels were not reduced in those patients[43], in agreement with the data we obtained in CVID patients. Our *in vitro* studies showed that recombinant BAFF induced down-regulation of BAFF-R as early as 4h of culture. This effect was sustained at later time-points, but not increased, suggesting that the maximal response to BAFF in terms of BAFF-R modulation is

achieved early on. It is possible that BAFF-R may be internalized upon BAFF binding, a process that was demonstrated to occur in other settings, such as in the response of T cells to IL-7, where the IL-7Rα is internalized upon binding of IL-7[44]. This is particularly interesting as BAFF effects on B cells share great similarities to those of IL-7 on T cells. The possible internalization of BAFF-R has been discussed in the past in the contexts of SLE and Sjögren's syndrome, even though hypothesis has not been formally assessed[43,45]. We observed BAFF-R down-regulation as early as after 5 minutes of stimulation with BAFF. Moreover, removal of BAFF from cultures resulted in BAFF-R time-dependent up-regulation. These observations suggest a dynamic mechanism by which BAFF is modulating BAFF-R expression, and not simply a matter of receptor occupancy, as it has been proposed to be underlying the low levels of BAFF-R detection on the surface of B cells from patients with SLE[45]. A component of receptor occupancy does seem to exist in the modulation of BAFF-R expression by BAFF, but only at high concentrations (>10ng/mL) and not at the levels of BAFF found in circulation in either CVID patients or healthy individuals (Supplemental Figure 3). However, receptor occupancy does not explain by itself the modulation of BAFF-R expression that we reported, even with higher concentrations of BAFF (Supplemental Figure 3).

Another possibility that can be responsible for the down-regulation of BAFF-R upon BAFF binding is that BAFF-R is being cleaved and shed into the extracellular space. This is a rather interesting possibility, since it would prevent BAFF from acting on its target B cells. BAFF-R shedding could then act as a buffer in situations where excess BAFF is present. This mechanism has been described to occur for TNF receptors and to be critical for the regulation of TNF activity *in vivo*[46]. Although BAFF-R shedding has also been discussed in the past[43,45], it has not been formally evaluated. TACI has been detected in a soluble form, particularly in the serum of CVID patients[24,30], indicating that some cleavage from the cell surface can occur. Nevertheless, the biological relevance of this phenomenon remains elusive.

It will be important to determine if lower BAFF-R expression on B cells from CVID patients render them less responsive to BAFF signals, as proposed in the case of SLE[45]. A previous study has shown that BAFF induces proliferative and antibody-secreting responses in B cells from some CVID patients[47]. However, the same study claimed that the expression of receptors for BAFF was not altered in those CVID patients[47].

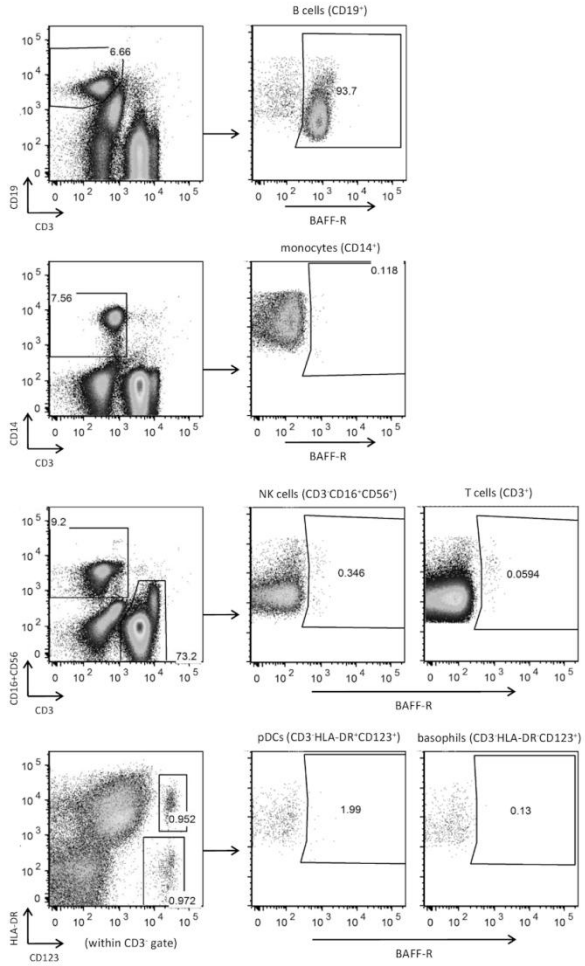
The basis of elevated serum levels of BAFF in CVID patients is a question that remains unclear. It has been recently proposed that steady-state soluble BAFF levels are determined by the size of the B-cell pool and the availability of BAFF receptors, although the actual expression of these BAFF receptors has not been formally addressed[31]. It had already been shown in the

mouse that two distinct pools of BAFF actually exist: a constitutive pool that is produced by radiation-resistant cells and controls the size of the peripheral B cell pool; and a cytokine-induced pool that will act at sites of inflammation[7,8]. We and others have shown that the highest serum BAFF levels are found in individuals lacking B cells, as exemplified by Congenital Agammaglobulinemia patients[29,31,32], supporting a relevance of target availability in the regulation of serum BAFF levels. We could also confirm that BAFF serum levels were inversely correlated with both the percentage and total circulating numbers of B cells (data not shown), as reported by Kreuzaler and coleagues[31]. Several studies have investigated whether high BAFF or APRIL levels could be related to polymorphisms in the coding genes for these cytokines[48,49]. BAFF polymorphisms have been studied in CVID patients, but no mutations were found[49]. One polymorphism in the human APRIL gene has been found to be associated with SLE, even though it is also found in the healthy population and no functional assessment of this polymorphism has been made[48]. Thus, it seems unlikely that the high levels of BAFF and APRIL observed in autoimmune diseases and in CVID are a consequence of genetic alterations in the coding genes for these factors.

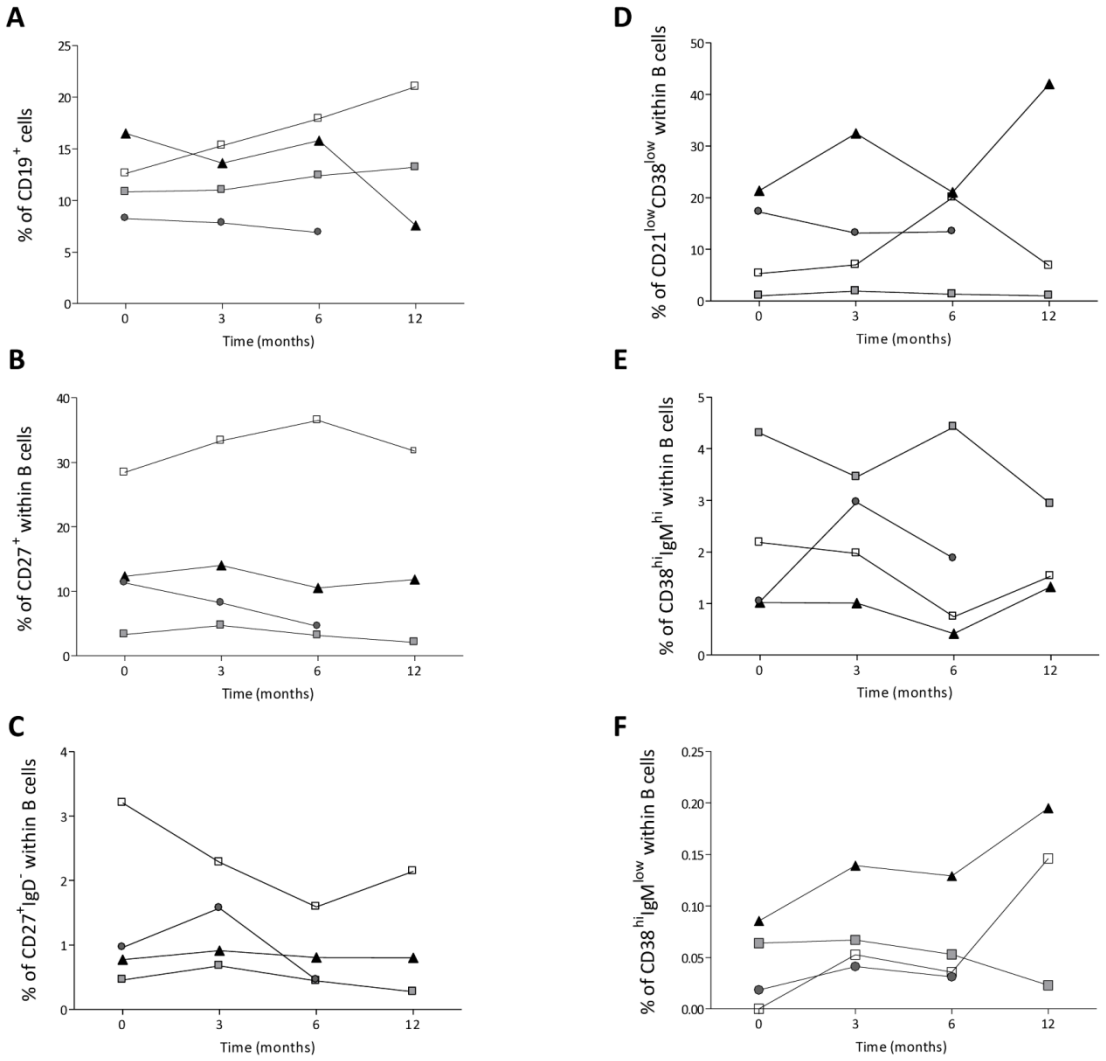
The description of BAFF-transgenic mice as presenting with autoimmune manifestations that include autoantibody production, proteinuria and Ig deposition in the kidneys[10,11] led to intense research about the role of BAFF in human autoimmune diseases, such as SLE, Sjögren's syndrome or rheumatoid arthritis. Several reports have shown that BAFF levels are increased in these conditions[43,50-56], but its association with disease activity is controversial. Some works found a direct association between high levels of BAFF and autoantibody production and disease stage[50-53], while others did not[43,55]. APRIL levels have also been described to be increased in SLE and to be associated with autoimmune features[57]. Our CVID cohort has a high prevalence of autoimmune manifestations, as previously reported[32,58]. Nevertheless, despite the increased circulating levels of BAFF and APRIL observed in CVID patients, as previously reported[29-32], we did not find any relationship between serum BAFF or APRIL levels and the clinical manifestations that CVID patients presented, namely autoimmunity, lymphoid proliferation or splenomegaly.

Our work showed that B-cell homeostasis is likely impaired in CVID, given that expression of BAFF-R is significantly reduced, while TACI expression is increased in CVID patients, both of which in direct correlation with high serum levels of BAFF. These data raise the possibility of BAFF-depleting therapies being envisaged in the treatment of CVID. Understanding the mechanisms involved in the modulation of BAFF-R expression by its ligand BAFF will be crucial to clarify the role of BAFF-R dysregulation in CVID pathogenesis, in particular, and in B-cell biology, in general.

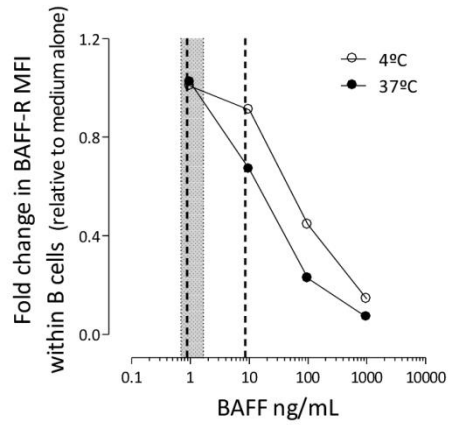
Supplemental Data



Supplemental Figure 1. BAFF-R expression within peripheral blood populations. Given that cultures with BAFF were performed using total PBMCs and not purified B cells, the expression of BAFF-R by other cell populations was assessed, at the protein level. BAFF-R expression assessed by flow cytometry in B cells, monocytes, T cells, NK cells, plasmacytoid dendritic cells (pDCs) and basophils. Gating strategy for each population is shown, within a manually set lymphocyte+monocyte gate. One of two representative experiments is shown.



Supplemental Figure 2. B-cell populations in CVID patients before and up to 12 months after starting IgG replacement therapy. Frequencies of total B cells within lymphocytes (A), memory B cells ($CD27^+$, B), switched-memory B cells ($CD27^+IgD^-$, C), $CD21^{low}CD38^{low}$ B cells (D), transitional B cells ($CD38^{hi}IgM^{hi}$, E), and plasmablasts ($CD38^{hi}IgM^-$, F), in four CVID patients before (time 0) and after starting IgG replacement therapy.



Supplemental Figure 3. Down-regulation of BAFF-R expression upon BAFF binding appeared to occur independently of receptor occupancy. To analyze receptor occupancy by BAFF on BAFF-R expression, peripheral blood mononuclear cells were incubated for 30 minutes with increasing amounts of BAFF, either at 4ºC or 37ºC, and BAFF-R expression was subsequently determined and compared. Receptor occupancy is observed at 4ºC, while the observations at 37ºC reflect both receptor occupancy and modulation of BAFF-R expression. The grey bar represents the range of BAFF serum levels found in healthy individuals, while the dashed lines indicate the range of BAFF serum levels in CVID patients.

Supplemental Table 1. Analysis of sequence variants in the BAFF-R gene in the cohort of CVID patients studied. Work done in collaboration with Professor Lennart Hammarström (Department of Laboratory Medicine, Karolinska Institut, Stockholm, Sweden).

	EXON 1	EXON 2	INTRON 1	INTRON 2	EXON 3
CVID #001*	none	none	none	none	none
CVID #002	none	none	none	none	none
CVID #003	none	none	none	none	none
CVID #004	none	G64V(191G/T;192C/T)het	none	none	none
CVID #005	none	G64V(191G/T;192C/T)het	none	none	none
CVID #006	P21R(het)	none	IVS2+72G/C(het)	IVS2-33T/C(het)	none
CVID #007	P21R(het)	none	IVS2+72G/C(het)	IVS2-33T/C(hom)	none
CVID #008*	none	none	none	none	none
CVID #009	P21R(het)	none	none	IVS2-33T/C(het)	none
CVID #010	none	G64V(191G/T;192C/T)het	none	none	none
CVID #011	none	none	none	none	none
CVID #012	none	none	none	none	none
CVID #013	none	none	none	none	none
CVID #014	none	none	none	none	none
CVID #015	none	none	none	none	none
CVID #016*	none	none	none	none	none
CVID #017	none	none	none	none	none
CVID #018	P21R(het)	none	IVS2+72G/C(het)	IVS2-33T/C(het)	none
CVID #019	none	G64V(191G/T;192C/T)hom	none	none	none
CVID #020*	none	none	none	none	none
CVID #021	none	none	none	none	none
CVID #022	none	none	none	none	none
CVID #023	none	none	none	none	none
CVID #024	P21R(het)	none	IVS2+72G/C(hom)	IVS2-33T/C(hom)	none
CVID #025*	none	none	none	none	none
CVID #026*	none	none	none	none	none
CVID #027	none	none	none	none	none
CVID #028	P21R(het)	none	none	IVS2-33T/C(het)	none
CVID #029	P21R(het)	none	none	IVS2-33T/C(het)	none

* patients selected for the study of *TNFRSF13C* quantification and cultures with BAFF het= heterozygous; hom= homozygous

Supplemental Table 2. Analysis of sequence variants in the TACI gene in the cohort of CVID patients studied. Work done in collaboration with Professor Lennart Hammarström (Department of Laboratory Medicine, Karolinska Institut, Stockholm, Sweden).

	EXON 1	EXON 2	EXON 3	INTRON 3	EXON 4	INTRON 4	EXON 5	3' UTR
CVID #001	none	T27T(hom)	none	none	none	none	none	none
CVID #002	none	T27T(hom)	none	none	none	none	none	none
CVID #003	none	T27T(hom)	none	none	none	none	none	none
CVID #004	none	T27T(het)	none	IVS3+25A>C(het)	none	IVS4+60T>C(het)	S277S(het)	G or A (hom)
CVID #005	none	T27T(het)	none	IVS3+25A>C(het)	none	IVS4+60T>C(het)	S277S(het)	G or A (het)
CVID #006	none	T27T(hom)	none	none	none	none	c.980-982del	none
CVID #007	none	T27T(hom)	none	none	none	none	none	none
CVID #008	none	T27T(hom)	none	none	none	none	none	none
CVID #009	none	T27T(hom)	none	IVS3+25A>C(het)	A181E(het)	IVS4-84G>C(het)	S277S(het)	G or A (het)
CVID #010	none	T27T(het)	none	IVS3+25A>C(hom)	none	IVS4+60T>C(het)	S277S(hom) P251L(het)	G or A (hom)
CVID #011	none	T27T(hom)	none	none	none	none	none	none
CVID #012	none	T27T(het)	none	IVS3+25A>C(het)	none	IVS4+60T>C(het)	S277S(het)	G or A (hom)
CVID #013	none	T27T(het)	none	IVS3+25A>C(het)	none	IVS4+60T>C(het)	S277S(het)	G or A (het)
CVID #014	none	T27T(hom)	none	none	none	none	S277S(het)	G or A (het)
CVID #015	none	T27T(hom)	none	none	none	none	none	none
CVID #016	none	T27T(hom)	none	none	none	none	none	none
CVID #017	none	none	none	IVS3+25A>C(het)	none	IVS4+60T>C(het)	S277S(het)	G or A (het)
CVID #018	none	T27T(hom)	none	IVS3+25A>C(het)	none	none	S277S(het)	G or A (het)
CVID #019	none	T27T(hom)	none	none	none	none	none	none
CVID #020	none	T27T(het)	none	IVS3+25A>C(het)	none	none	S277S(het)	G or A (hom)
CVID #021	none	T27T(hom)	none	none	none	none	none	none
CVID #022	none	T27T(het)	P97P(het)	IVS3+25A>C(hom)	none	IVS4+60T>C(het)	S277S(hom)	G or A (het)

CVID #023	none	T27T(het)	none	IVS3+25A>C(het)	none	IVS4+60T>C(het)	S277S(het)	G or A (het)
CVID #024	none	T27T(het)	none	IVS3+25A>C(het)	none	IVS4+60T>C(het)	S277S(het)	G or A (het)
CVID #025	none	T27T(het)	none	IVS3+25A>C(hom)	none	IVS4+60T>C(het)	S277S(hom) P251L(het)	G or A (hom)
CVID #026	none	T27T(het)	none	IVS3+25A>C(het)	none	IVS4+60T>C(het)	S277S(het)	G or A (het)
CVID #027	none	T27T(hom)	none	none	none	none	none	none
CVID #028	none	T27T(het)	none	none	none	none	none	none
CVID #029	none	T27T(het)	none	none	none	none	none	none

het= heterozygous; hom= homozygous

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3.2 – RELATIONSHIP BETWEEN B- AND T-CELL IMBALANCES IN PRIMARY B-CELL IMMUNODEFICIENCIES

including

Primary B-cell deficiencies reveal a link between human IL-17-producing CD4 T-cell homeostasis and B-cell differentiation

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Abstract

IL-17 is a pro-inflammatory cytokine implicated in autoimmune and inflammatory conditions. The development/survival of IL-17-producing CD4 T cells (T_H17) share critical cues with B-cell differentiation and the circulating follicular T helper subset was recently shown to be enriched in T_H17 cells able to help B-cell differentiation. We investigated a putative link between T_H17-cell homeostasis and B cells by studying the T_H17-cell compartment in primary B-cell immunodeficiencies. Common Variable Immunodeficiency Disorders (CVID), defined by defects in B-cell differentiation into plasma and memory B cells, are frequently associated with autoimmune and inflammatory manifestations but we found no relationship between these and T_H17-cell frequency. In fact, CVID patients showed a decrease in T_H17-cell frequency in parallel with the expansion of activated non-differentiated B cells ($CD21^{low}CD38^{low}$). Moreover, Congenital Agammaglobulinemia patients, lacking B cells due to impaired early B-cell development, had a severe reduction of circulating T_H17 cells. Finally, we found a direct correlation in healthy individuals between circulating T_H17-cell frequency and both switched-memory B cells and serum BAFF levels, a crucial cytokine for B-cell survival. Overall, our data support a relationship between T_H17-cell homeostasis and B-cell maturation, with implications for the understanding of the pathogenesis of inflammatory/autoimmune diseases and the physiology of B-cell depleting therapies.

Introduction

CD4 T cells with the ability to produce the pro-inflammatory cytokine interleukin (IL)-17, designated T_H17[1-3], act as co-ordinators of the innate and adaptive immune responses to bacteria and fungi, in particular *Candida albicans*[4], and have been implicated in several autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and Crohn's disease[5]. Common Variable Immunodeficiency Disorders (CVID) are defined by impaired antibody production and frequently associate with autoimmune and inflammatory manifestations[6-9]. It is thus plausible that IL-17 may play a role in these processes.

The defects in mature B-cell development that characterize CVID mainly result in impaired organization of germinal centres (GC)[10], specialized structures within follicles where antigen-driven somatic hypermutation and class switch recombination occur, and thus the main source of switched-memory B cells and plasma cells[11]. Several molecular cues that are essential for B-cell differentiation in GCs are also required or may contribute to the induction and/or survival of T_H17 cells. IL-6, a major factor for the differentiation of naive CD4 T cells into T_H17 cells[3], also plays a key role in B-cell proliferation and antibody secretion[12]. IL-21 was first described as having a critical role in the regulation of antibody production by B cells[13,14], and was later shown to be involved in T_H17-cell differentiation[15,16]. Furthermore, IL-21 is abundantly produced by T_H17 cells and plays an important autocrine role in their differentiation and maintenance[4]. Several co-stimulatory molecules have also been shown to play roles in both Th17 induction and/or survival as well as in B-cell differentiation into plasma and memory B cells, namely ICOS and CD40L[17-22]. T-cell help is known to be fundamental to the induction and subsequent organization of GCs, enabling an adequate generation of plasma and memory B cells. This help is a characteristic of a particular subset of T cells, follicular helper T cells (T_{FH}), identifiable by the expression of the chemokine receptor CXCR5, which is essential for their specific homing to follicles in lymphoid tissues, and by the production of IL-21[23]. Although T_{FH} cells reside mainly within follicles and GCs, a population of circulating T_{FH} cells has been consistently observed in humans[24,25]. This circulating T_{FH} subset has been recently demonstrated to be a counterpart for T_{FH} cells found in GCs[26], and to be enriched not only in T_H2 but also in T_H17 cells that are able to help B-cell differentiation[26].

We hypothesized that the homeostasis of the circulating T_H17 compartment may be related to B-cell differentiation. Confirming such a relationship would have major clinical implications, given the increasing use of B-cell depleting therapies in many autoimmune and

lymphoproliferative diseases. As a strategy to investigate the contribution of B cells to the T_H17 subset, we studied this population in CVID patients as well as in patients lacking B cells due to Congenital Agammaglobulinemia. This latter condition is associated with impaired early B-cell development in the bone marrow as a result, in the majority of cases, of mutations in the Bruton's tyrosine kinase gene, usually leading to a complete lack of circulating B cells[27]. The evaluation of these primary B-cell deficiencies combined with the study of healthy individuals supports a link between the homeostasis of the circulating T_H17-cell pool and B-cell differentiation.

Materials and Methods

Ethics Statement

All subjects gave written informed consent for blood sampling and processing. The study was approved by the Ethical Board of the Faculty of Medicine of Lisbon.

Participants

The study involved 30 healthy individuals, 31 patients with CVID, and 6 patients with Congenital Agammaglobulinemia. The clinical-epidemiological data of the three groups are summarized in Table 1. CVID patients were diagnosed according to the European Society for Immunodeficiency criteria (www.esid.org), namely decreased serum IgG as well as IgM and/or IgA levels at least 2 SD below the mean for age, impaired antibody response to vaccines, absent/low isohemagglutinins, and exclusion of defined causes of hypogammaglobulinemia. The 31 CVID patients were not related, with the exception of two homozygous twins. All the patients with Congenital Agammaglobulinemia had less than 1% B cells within total peripheral lymphocytes. 29 CVID and all Congenital Agammaglobulinemia patients were under IgG replacement therapy, adjusted to guarantee pre-infusion Ig levels above 650mg/dL. The two CVID patients not receiving IgG had levels of total serum IgG of 227 and 473 mg/dL.

Monoclonal antibodies used in flow cytometry

The following anti-human monoclonal antibodies were used, with clone and the directly conjugated fluorochrome specified in brackets: CD3 (SK7; peridinin chlorophyll protein (PerCP)), CD4 (SK3; PerCP), CD8 (SK1; PerCP and allophycocyanin (APC)-Cy7; RPA-T8; APC), CCR6 (11A9; phycoerythrin (PE)), CD38 (HB7; PE), CD45RA (L48; PE-Cy7), IgD (IA6-2; PE), IgM (G20-127; APC), HLA-DR (L243; fluorescein isothiocyanate (FITC)), IFN-γ (4S.B3; FITC), IL-2 (MQ1-17H12; PE), IL-4 (MP4-25D2; PE), from BD Biosciences, San Jose, CA; CD3 (UCHT1; APC-eFluor780), CD4 (RPA-T4, FITC, PerCP-Cy5.5 and PE-Cy7), CD8 (RPA-T8; FITC and PE), CD19 (HIB19; PerCP-Cy5.5 and PE-Cy7), CD27 (O323; FITC, PE, APC and PE-Cy7), CD45RO (UCHL1; PE), CD69 (FN50; FITC), CD45RA (HI100; FITC and APC), IL-17A (eBio64DEC17; PerCP-Cy5.5 and Alexa Fluor 647), TNF-α (MAb11; PE), from eBiosciences, San Diego, CA; CD4 (S3.5; PE) from Caltag, Buckingham, UK; CCR7 (150503; FITC), CXCR5 (51505.111; PE), from R&D Systems, Minneapolis, MN; CD21 (BL13; FITC) from IO Test, Beckman Coulter, Brea, CA.

Table 1. Clinical and epidemiological data of the cohorts studied

	Healthy	CVID	Congenital Agammaglobulinemia[a]
Number (male/female)	30 (9/21)[d]	31 (11/20)	6 (6/0)
Age (*yrs.*)	41±15[d]	40±13	24±5
Clinical manifestations[b,c]			
Autoimmune disease	n.a.	17/31 (55%)	0
Adenopathies	n.a.	10/31 (32%)	0
Lymphoid proliferation	n.a.	18/20[e] (86%)	0
Granulomas	n.a.	3/20[e] (15%)	0
Chronic diarrhoea	n.a.	15/31 (48%)	0
Splenomegaly	n.a.	16/31 (52%)	0
IgG replacement therapy[c]			
intravenous	n.a.	24/31 (77%)	6 (100%)
subcutaneous	n.a.	5/31 (16%)	0
Length of IgG therapy (*yrs.*)	n.a.	7±6	15±9

n.a. not applicable, CVID: Common Variable Immunodeficiency Disorders [a] Genetic defects in the *Btk* gene were identified in 4 of the congenital agammaglobulinemia patients, namely *IVS17-1G→C*, R288Q, *IVS8-2A→G*, and Y375X mutations; in the other 2 patients, *Btk* mutations have been excluded and other genes are currently being evaluated. [b] Diagnostic criteria: Autoimmune disease - clinical data, given the impairment in Ab production; Adenopathies - lymph node larger than 1cm diameter in 2 or more lymphatic chains in clinical and/or imaging exams; Lymphoid proliferation and Granulomas - diffuse lymphocytic infiltrates or granulomas on gastrointestinal, lymph node or pulmonary biopsies; Splenomegaly - longitudinal spleen diameter superior to 15cm (computed tomography or ultrasonography). [c] Percentage within total cohort evaluated in brackets. [d] 15/30 healthy subjects were included in detailed immunological studies (10 female; age 39±11 years). [e] Total number of individuals with biopsies.

Cell staining and flow cytometric analysis

Phenotypic analysis was performed in whole blood samples collected immediately before IgG administration, after staining with monoclonal antibodies and red blood cells lysis using BD FACS Lysing Solution (BD Biosciences). Samples were acquired on a FACSCalibur or on a FACSCanto flow cytometer (BD Biosciences). A minimum of 100,000 lymphocytes were acquired per sample. Data were analysed using CellQuest Software (BD Biosciences) and FlowJo Software (Tree Star Inc., Ashland, OR). Total cell numbers were calculated by multiplying the percentage of each population within total lymphocytes by the peripheral blood lymphocyte count obtained at the clinical laboratory on the day of sampling.

Analysis of cytokine production

Freshly isolated peripheral blood mononuclear cells (PBMC) by Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) were assessed for cytokine production at the single-cell level, as previously described[28]. Briefly, after a 4-hour culture with phorbol myristate acetate (PMA) (50ng/mL, Sigma-Aldrich) plus ionomycin (500ng/mL; Calbiochem, Merck Biosciences, Nottingham, U.K.), in the presence of brefeldin A (10μg/mL; Sigma-Aldrich), PBMC were surface stained and then fixed (2% formaldehyde; Sigma-Aldrich, St Louis, MO), permeabilized (phosphate buffered saline/1% bovine serum albumin/0.5% saponin) (Sigma-Aldrich) and stained intracellularly with monoclonal antibodies against IL-2, IL-4, IFN-γ, TNF-α and IL-17. Flow cytometric analysis was subsequently performed as described above.

Quantification of serum levels of BAFF

Serum concentrations of BAFF (BlyS/TNFSF13B; B-cell activating factor) were quantified by Enzyme Linked Immunosorbent Assay (ELISA) using the BAFF Immunoassay Kit (R&D Systems), according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc, SD). Two group comparisons were performed using Mann-Whitney test. Spearman's coefficient was used to determine the significance of the correlation between two variables. Results are expressed as mean±SEM, and *P*-values <0.05 were considered to be significant.

Results

T_H17 cells in patients with CVID

We evaluated, for the first time, the frequency of circulating T_H17 cells in CVID. Our CVID cohort (Table 1) featured the characteristic impairment of GC organization and generation of B-cell memory, demonstrated by the striking decrease in the frequency of switched-memory B cells, accumulation of $CD38^{hi}IgM^{hi}$ transitional B cells, and expansion of $CD21^{low}CD38^{low}$ B cells, a population believed to be related to continuous B-cell activation in the presence of impaired GC function[29,30] (Supplemental Figure S1A and S1B). No association was observed between the frequency of T_H17 cells and transitional B cells, a pre-GC B-cell population (Figure 1A). In contrast, we found a statistically significant negative correlation between T_H17 cells and $CD21^{low}CD38^{low}$ B cells, in CVID patients (Figure 1A),

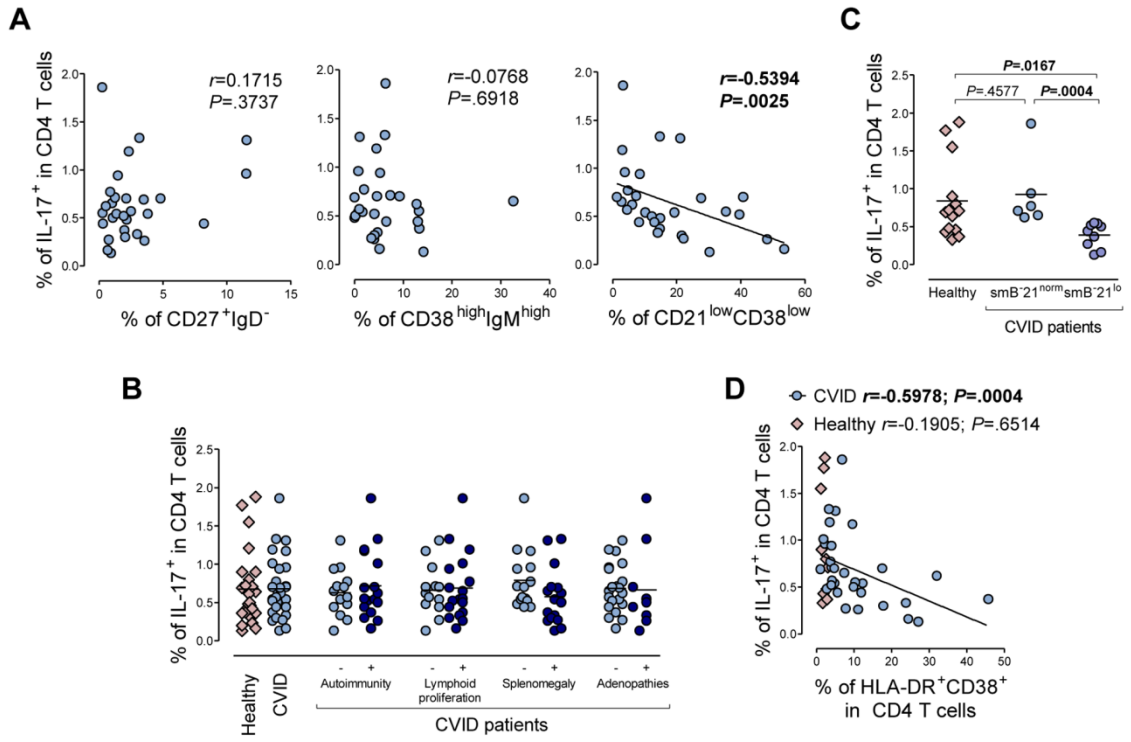


Figure 1. T_H17 cells in patients with CVID. (A) Correlation between T_H17 frequency and frequency of switched-memory B cells (left), transitional B cells (middle) or $CD21^{low}$ B cells (right), in CVID patients. Switched-memory B cells were defined as $CD27^+$ IgD^- cells, transitional B cells as $CD38^{high}$ IgM^{high} cells and $CD21^{low}$ B cells were defined as $CD21^{low}$ $CD38^{low}$ cells within gated B cells ($CD19^+$) after surface staining of whole blood samples. IL-17 expression was assessed at the single-cell level by intracellular staining following short-term stimulation of PBMC with PMA and ionomycin. (B) T_H17 frequency in CVID individuals stratified according to their clinical manifestations, namely autoimmunity, lymphoid proliferation, splenomegaly, and adenopathies. (C) T_H17 frequency in healthy controls and CVID patients grouped according to EUROclass. (D) Correlations between frequencies of activated CD4 T cells, defined by concurrent expression of HLA-DR and CD38, and of T_H17 cells in CVID and healthy individuals. Each symbol represents one individual. Bars represent mean. Data were compared using Mann-Whitney test, and *P* values are shown. Correlation significance was assessed using Spearman coefficient test, and *r* and *P* values are shown.

suggesting a decline in the frequency of circulating T_H17 cells that matched the B-cell disturbances indicative of GC disruption. The fact that we did not observe a relationship with the frequency of switched-memory B cells is likely related to the heterogeneity of blocks in GC differentiation that CVID patients present[10]. Our CVID cohort presented an unusually high proportion of autoimmune and lymphoproliferative manifestations (Table 1), possibly related to a reference bias associated with an immunology department in a central hospital. We next assessed whether these inflammatory and autoimmune manifestations could be related to an expansion of T_H17 cells, as reported in other autoimmune settings[5]. We found no increase in the frequency of T_H17 cells in CVID patients with autoimmunity (Figure 1B), even when CVID patients were split according to the type of autoimmune manifestation, namely autoimmune cytopenias and organ specific autoimmunity (data not shown). In contrast, the $CD21^{low}CD38^{low}$ B-cell subset was significantly expanded in the subgroup of CVID patients with autoimmunity (Supporting Information, Figure S1C).

CVID patients have been classified according to the previously mentioned B-cell sub-populations[9]. The stratification of our CVID cohort according to the EUROclass classification[9] revealed that a statistically significant decrease in T_H17 frequency was restricted to the group with less than 2% switched-memory B cells and more than 10% $CD21^{low}CD38^{low}$ B cells (Figure 1C), in agreement with the above results.

Several T-cell imbalances were also observed in the CVID cohort, as previously reported[31], namely an expansion of effector-memory T cells, increased production of the pro-inflammatory cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α, and up-regulation of activation markers within both CD4 and CD8 T cells (Supplemental Figure S2). Of note, the frequency of T_H17 cells was found to negatively correlate with CD4 T-cell activation in CVID patients (Figure 1D), with no relationship with naïve/memory T-cell imbalances (correlation with the frequency of naïve cells within CD4 T cells, r=0.3065, P=.1058). In contrast, the frequency of IFN-γ-producing cells was found to negatively correlate with naïve CD4 T cells (r=-0.8199, P<.0001) and positively with the levels of CD4 T-cell activation in CVID patients (r=0.6098, P=.0003). Similar relationships were observed with the expression levels of activation markers within the CD8 T-cell subset (data not shown). We also investigated whether the increased production of IFN-γ observed in CVID patients had an impact on the concomitant production of this cytokine by T_H17 cells. We found no significant differences in the proportion of IFN-γ^+ cells within the IL-17-producing CD4 subset as compared to healthy subjects (22.55%±2.34% in CVID and 14.45%±1.70% in healthy, P=.0567) and no relationship between the frequencies of IFN-γ- and IL-17-producing CD4 T cells (r=-0.2504, P=.1821). Moreover, in contrast to IFN-γ-producing

CD4 T cells, no association was found between the frequency of IL-17^{+}IFN-γ^{+} CD4 T cells and the naïve/memory imbalances (*P*>.1500).

In conclusion, no association between T_H17 cells and autoimmune manifestations or production of other pro-inflammatory cytokines was observed in CVID patients. The levels of T_H17 cells were apparently more directly related to B-cell differentiation than to T-cell disturbances, showing a negative correlation with the pathological expansion of a B-cell population associated with impaired GC function (CD21^{low}CD38^{low} B cells) in CVID.

Decreased frequency of T_H17 cells in individuals without B cells

In order to further investigate the relationship between B cells and the IL-17-producing CD4 subset, we evaluated this population in individuals with Congenital Agammaglobulinemia, who lack mature B cells due to genetic defects impairing early B-cell development[27] (Table 1). A marked reduction in the frequency of T_H17 cells was found in these patients (Figure 2A and 2B).

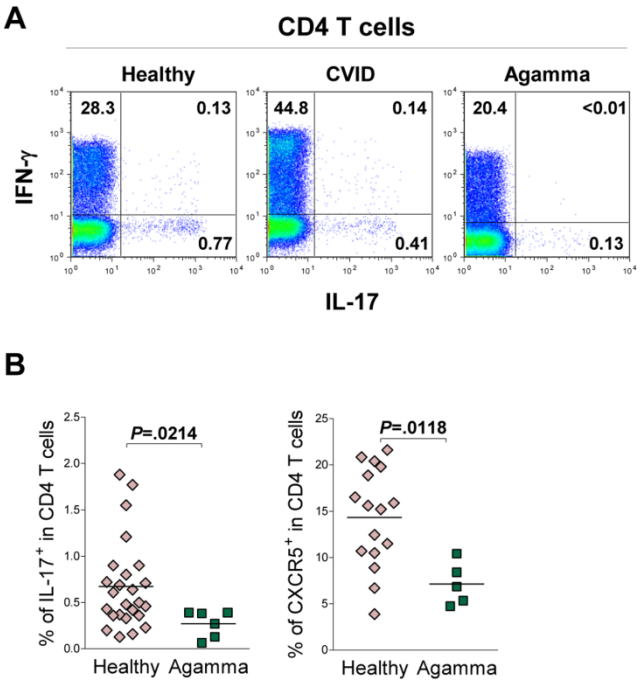


Figure 2. Decreased frequency of T_H17 cells in individuals lacking B cells. (A) Representative dot-plots of the analysis of IFN-γ and IL-17 production by CD4 T cells, determined by intracellular staining, for a healthy individual (left), a CVID patient (middle) and a Congenital Agammaglobulinemia (Agamma) patient (right). Numbers inside dot-plots represent the proportion of cells expressing the markers. (B) Frequency of T_H17 cells (left) and CXCR5^{+} CD4 T cells (right) in healthy individuals and Congenital Agammaglobulinemia patients (Agamma). Each symbol represents one individual. Bars represent mean. Data were compared using Mann-Whitney test, and *P* values are shown.

In spite of the reduced T_H17 frequency, these patients do not exhibit a major increase in the frequency of infections with *Candida albicans*, which may be related to the preservation of innate sources of IL-17, such as $\gamma\delta$ T cells, natural killer T cells or myeloid cells.

Of note, these individuals featured neither significant naïve T-cell imbalances, nor major alterations in the levels of T-cell activation nor frequencies of IFN-γ-, TNF-α-, IL-2- or IL-4-producing cells, as compared to healthy subjects (Supplemental Figure S2).

Importantly, a significant reduction of circulating T_{FH} cells was found in these patients (Figure 2B), likely related to decreased GC generation in the context of Congenital Agammaglobulinemia, as recently reported[32]. A decrease in this circulating CD4 T-cell population has also been described in the context of human ICOS-deficiency, a situation in which the generation of GCs is known to be severely perturbed[33].

In conclusion, we report here a major reduction in the T_H17-cell pool in patients lacking B cells.

Direct correlation between the frequency of circulating T_H17 cells and switched-memory B cells in healthy individuals

Our findings suggesting a relationship between the homeostasis of the circulating T_H17 compartment and B-cell disturbances led us to investigate this putative relationship in healthy individuals. We found a direct correlation between the frequency of CD4 T cells able to produce IL-17 and the frequency of B cells exhibiting a switched-memory phenotype in these individuals (Figure 3). This relationship was also observed when absolute numbers of the circulating populations were considered (r=0.4565, *P*=.0249).

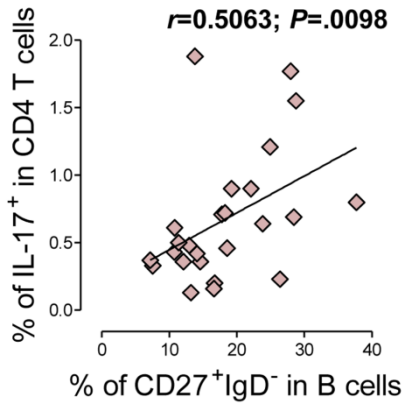


Figure 3. Direct correlation between the frequencies of circulating T_H17 cells and switched-memory B cells in healthy individuals. Correlation between the frequencies of switched-memory ($CD27^+IgD^-$) B cells and T_H17 cells in healthy individuals. Each symbol represents one healthy individual. Correlation significance was assessed using Spearman coefficient test, and *r* and *P* values are shown.

In addition, healthy individuals exhibited a negative correlation between the frequency of T_H17 cells and the serum levels of B-cell activating factor (BAFF) (Figure 4A), a critical cytokine for B-cell differentiation and survival[34]. In contrast, we did not observe any relationship between

BAFF serum levels and the production of other pro-inflammatory cytokines, such as IFN-γ and TNF-α, or IL-4 (data not shown), thus highlighting the specificity of the link to T_H17 cells. These results are in conformity with our previous observation of a relationship between IL-17 production by CD4 T cells and B-cell maturation in the context of primary B-cell deficiencies.

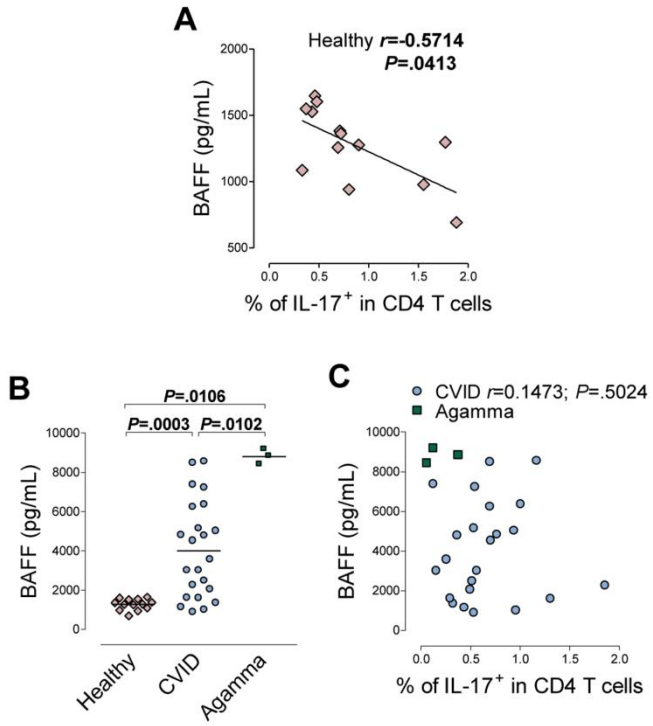


Figure 4. Negative correlation between the frequency of T_H17 cells and serum BAFF levels in healthy subjects. (A) Correlation between the frequency of T_H17 cells and serum levels of the cytokine BAFF, as determined by ELISA, in healthy individuals. (B) Analyses of the serum levels of BAFF in healthy individuals, CVID and Congenital Agammaglobulinemia patients. Each symbol represents one individual. Data were compared using Mann-Whitney test, and *P* values are shown. (C) The same correlation described in (A) for CVID patients and Congenital Agammaglobulinemia patients. Each symbol represents one individual. Correlation significance was determined using Spearman coefficient test, and *r* and *P* values are shown.

It has recently been demonstrated in mice that local BAFF-gene targeting in dendritic cells suppressed both the generation of plasma cells and T_H17 cells[35], suggesting that BAFF promotes T_H17-cell proliferation and expansion, possibly through the modulation of the cytokine milieu. However, providing our finding of a negative association between BAFF and IL-17 we hypothesize that BAFF effects may be indirectly mediated by the B-cell subset. In order to further investigate this possibility we quantified serum BAFF levels in the CVID and Congenital Agammaglobulinemia cohorts. In agreement with recent reports[36,37], serum BAFF levels were significantly increased in CVID patients, and were even higher in Congenital Agammaglobulinemia, reaching statistical significance in comparison to both healthy individuals and to CVID patients (Figure 4B). However, no correlation was found between BAFF serum levels

and the frequency of T_H17 cells in CVID and Congenital Agammaglobulinemia patients (Figure 4C). Notably, in the absence of B cells as illustrated by Congenital Agammaglobulinemia patients, very high levels of BAFF may be associated with very low frequencies of T_H17 cells (Figure 4C), not supporting a direct role of BAFF in the induction/survival of T_H17 cells. Overall, our data suggest that the association between IL-17-producing CD4 T cells and BAFF is likely related to its effects on B cells.

Discussion

We report here an association between the frequencies of T_H17 cells and switched-memory B cells in healthy individuals, and a marked reduction of this CD4 subset in patients with congenital absence of peripheral B cells. Additionally, in patients with primary defects in mature B-cell differentiation, the frequency of T_H17 cells was inversely correlated with markers of GC impairment, further supporting the link between B cells and the differentiation or maintenance of T_H17 cells.

Our data generated in human B-cell deficiencies suggest, for the first time, that B cells play a role in the homeostasis of the T_H17 subset. On the other hand, several previous reports have implicated IL-17 in B-cell differentiation and function. A recent report[38] suggested that IL-17 and possibly T_H17 cells can contribute to GC function in general. This work using BXD2 mice, which develop spontaneous erosive arthritis associated with auto-antibody production, showed that the enhanced somatic hypermutation and class-switch recombination found in these animals resulted from the direct action of IL-17 on B cells, leading to increased frequency and duration of GCs[38]. Recent data from human studies also suggest that T_H17 cells may contribute to the generation of ectopic GCs within kidney allografts through the production of IL-21[39], further establishing a link between IL-17 and B-cell function. Importantly, IL-17 alone has been described to promote human B-cell survival and to synergize with BAFF to induce B-cell proliferation and differentiation into antibody-secreting plasma cells[40]. Recent reports have, in this sense, established a direct impact of IL-17 on B cells[38,40], with respect to proliferation, survival and antibody production, and have shown that T_H17 cells can act as B-cell helpers[41]. Our observation of markedly reduced levels of T_H17 cells in the absence of other major T-cell imbalances in patients with congenital absence of B cells suggests an important contribution of B cells to the homeostasis of T_H17 cells. Although the number of patients with this rare immunodeficiency under follow-up is small, the remarkable consistency of the results generated in these patients strengthens our finding of a reduced T_H17 compartment in Congenital Agammaglobulinemia. Such a relationship was further supported by the negative correlation between T_H17 frequency and the expansion of the $CD21^{low}CD38^{low}$ B-cell subset in CVID patients, a pathological B-cell population resulting from altered B-cell activation associated with defects in GC function[29,30].

Our results raise the possibility of a follicular contribution to the maintenance of the T_H17-cell subset, a notion further supported by a recent report[26]. We were able to reveal a fraction of T_H17 cells expressing both CXCR5 and CCR7, in addition to CCR6, likely having the

potential to home to follicles (data not shown). This is particularly relevant given the fact that circulating T_{FH} cells were reported to be enriched in T_H17 cells that are able to help B-cell differentiation[26]. We have studied this population in patients without peripheral B cells due to early defects in B-cell development, which, as described in ICOS-deficient patients[33,42], are expected to have reduced generation of GCs. We found the circulating $CXCR5^+$ CD4 T-cell population to be significantly diminished in these patients, as compared to healthy subjects, in agreement with a recent report[32], further supporting the notion of circulating T_{FH} cells being counterparts of the follicular helper T cells found in GCs. Our results suggest that the impairment in GC generation may underlie the reduced frequencies of both T_{FH} and T_H17 cells.

Contrary to what we observed in Congenital Agammaglobulinemia, CVID patients presented with several T-cell imbalances[31], namely an increase in the production of the pro-inflammatory cytokines IFN-γ and TNF-α, in direct correlation with the up-regulation of activation markers. We found that the T_H17-cell population decreased concurrently with the hyper-activated state, suggesting that a different mechanism is involved in its regulation in CVID patients. Furthermore, a significant proportion of our CVID cohort featured autoimmune manifestations and some patients exhibited inflammatory processes that shared similarities with the pathology of Crohn's disease, a condition in which T_H17 cells have been implicated[5,43]. However, in contrast to Crohn's disease, it has been shown that CVID patients with symptomatic gut inflammation exhibit a reduced ability to produce IL-17 and IL-23 by the lamina propria mononuclear cells, in spite of increased IL-12 and IFN-γ secretion[43]. Importantly, we found no increase in the frequency of CD4 T cells able to produce IL-17 in CVID patients presenting with autoimmune disease, either with autoimmune cytopenia or other autoimmune disorders. In fact, an inverse correlation was found with the expansion of the $CD21^{low}CD38^{low}$ B-cell subset that has been implicated in autoimmunity. These findings are particularly relevant given that our cohort presents an exceptionally high prevalence of autoimmune manifestations.

A recent report suggested that the commercial immunoglobulin (Ig) used in intravenous therapy has the ability to inhibit the differentiation, amplification and function of human T_H17 cells *in vitro*[44]. However, such an effect is unlikely to account for the observations described here, since both Congenital Agammaglobulinemia and CVID patients undergo replacement therapy with Ig (Table 1).

The development and homeostasis of T_H17 cells and memory B cells share several determinants. Tumour growth factor (TGF)-β has a unique role in driving IgA isotype switching, which is the major Ig type produced at mucosal sites[45], and is also critical for T_H17-cell differentiation[15,16]. Both IgA and IL-17-producing CD4 T cells have an important function in the

modulation of gut flora and mucosal immunity[45]. It is thus plausible that the link between IL-17 production and B-cell function could mainly lie in the isotype switch to IgA, a notion further supported by the fact that patients with Congenital Agammaglobulinemia or CVID have impaired IgA production. This possibility led us to investigate the frequency of circulating T_H17 cells in patients with selective IgA deficiency. However, no imbalances in this CD4 subset were found in patients with undetectable serum levels of IgA as compared to healthy individuals (data not shown), suggesting that the link between B cells and IL-17 production is not dependent of the development of IgA-producing cells. It is unlikely that a unique molecule or pathway determine the impact of B cells in the homeostasis of the T_H17 subset, being more plausible that several mechanisms are involved, either through direct interactions or through the influence of factors modulated by B cells.

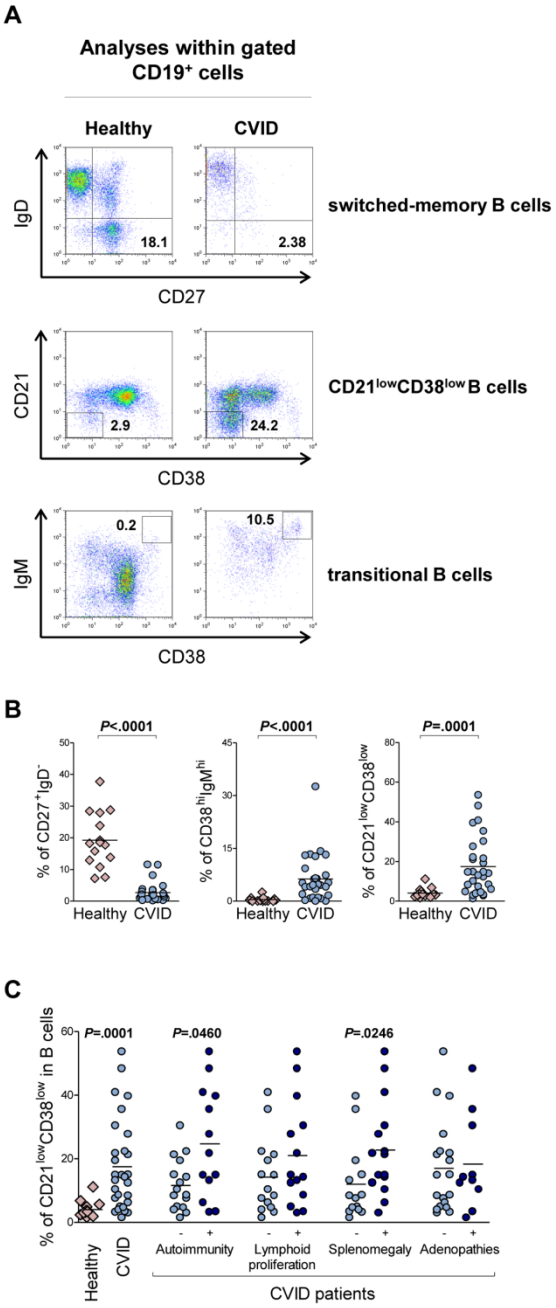
BAFF is a crucial survival factor for peripheral B cells. As expected from previous reports, both CVID and Congenital Agammaglobulinemia patients presented highly increased serum levels of this cytokine[36,37]. Although increased BAFF production by cells of the innate immune system, such as neutrophils, macrophages, monocytes and dendritic cells, could be enhanced in these patients, contributing to these increased levels, it is more likely that they resulted from reduced consumption of BAFF due to the decreased number of target cells in both conditions. This latter hypothesis is supported by the fact that B-cell depletion upon rituximab treatment, a monoclonal antibody targeting CD20, results in significantly increased levels of BAFF that return to baseline levels upon B-cell re-population[46]. Thus, high BAFF levels may be considered a measure of B-cell dysfunction and, in this sense, the negative correlation we report in healthy individuals between the frequency of T_H17 cells and the serum levels of BAFF further strengthens the link between IL-17 production and B-cell maturation.

B cell-depleting therapies have been increasingly used in various clinical settings, including pathologies where IL-17 has been implicated[46-48]. Additionally, BAFF-targeting therapies offer a promising approach for autoimmune/inflammatory disorders[46,48]. Our data raise the possibility that these treatments may have an impact on T_H17 cells and, in this way, influence the clinical outcome mediated by these therapeutic strategies. In line with these observations, a very recent report has shown that rituximab treatment selectively reduces the T_H17-cell response in rheumatoid arthritis patients[49]. Nevertheless, such an inhibition does not seem to lead to an increased frequency of mucocutaneous candidiasis in rituximab-treated patients[49], an infection associated with T_H17 deficiency.

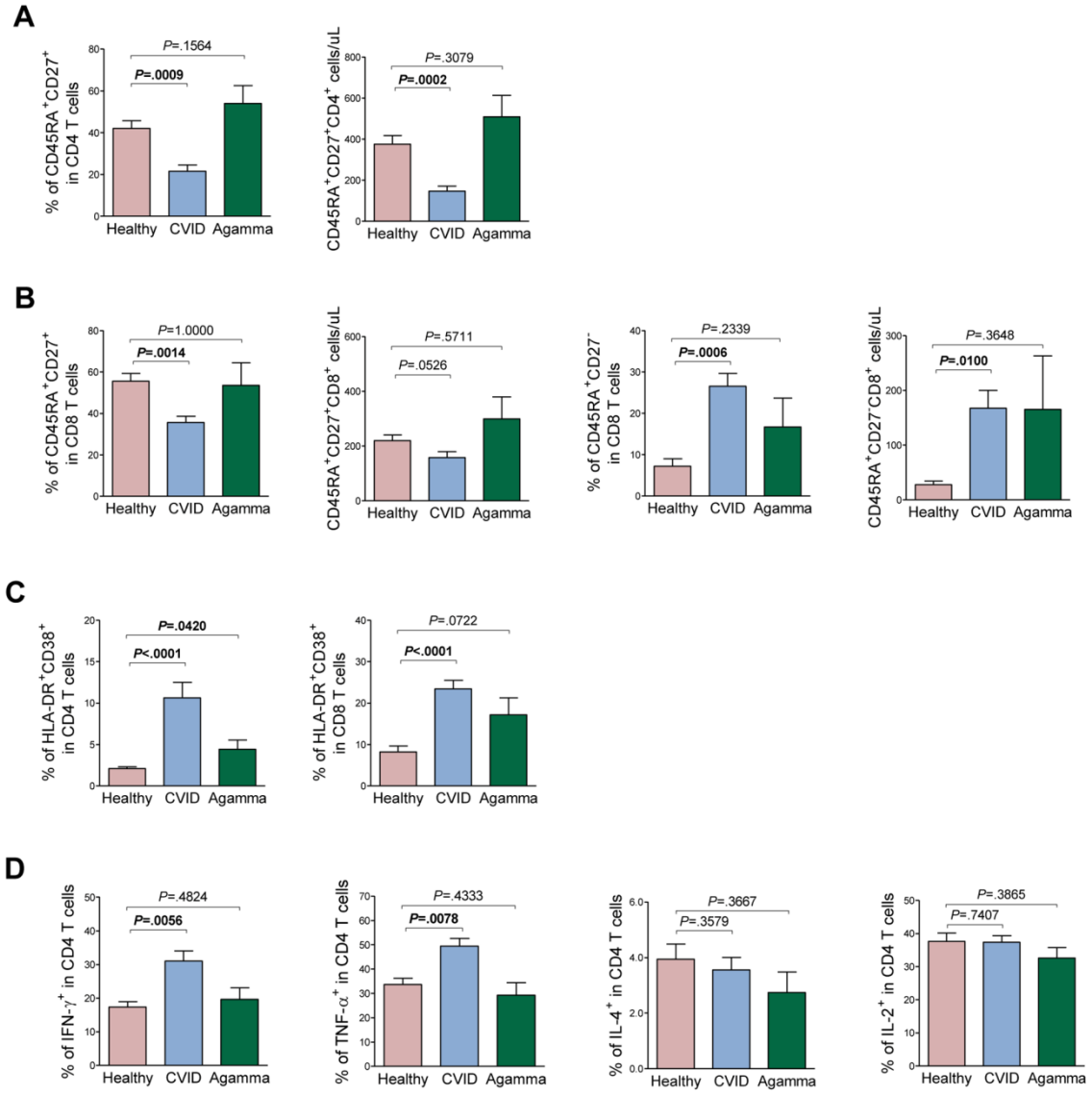
In conclusion, our data show, for the first time, that the induction/proliferation/survival of T_H17 cells is related to B-cell function. These results provide support for a link between T_H17

and B cells that is relevant for the understanding of the pathogenesis of inflammatory/autoimmune diseases as well as the mechanisms underlying the effects of therapeutic strategies targeting B cells.

Supplemental Data



Supplemental Figure 1. B-cell disturbances in CVID patients. (A) Representative plots of the flow cytometry analysis of switched-memory B cells (top), $CD21^{low}CD38^{low}$ B cells (middle) and transitional B cells (bottom), in healthy individuals (left panels) and CVID patients (right panels). Numbers represent the percentage of the given population within $CD19^{+}$ cells. (B) Comparison of the frequencies of these B-cell subsets in CVID and in healthy individuals. (C) Frequency of $CD21^{low}CD38^{low}$ within B cells in healthy controls and CVID individuals stratified according to their clinical manifestations, namely autoimmunity, lymphoid proliferation, splenomegaly, and adenopathies. Each symbol represents one individual. Bars represent mean. Data were compared using Mann-Whitney test, and *P* values are shown.



Supplemental Figure 2. T-cell disturbances in CVID and Congenital Agammaglobulinemia patients. Analysis of: (A) frequency and absolute numbers of naïve ($CD45RA^+CD27^+$) within CD4 T cells; (B) frequency and absolute numbers of naïve ($CD45RA^+CD27^+$) and terminally-differentiated ($CD45RA^+CD27^-$) within CD8 T cells; (C) frequency of activated ($HLA\text{-}DR^+CD38^+$) within CD4 T cells and CD8 T cells; and (D) frequencies of IFN-γ-, TNF-α-, IL-4-, and IL-2-producing CD4 T cells assessed at the single-cell level by intracellular staining following short-term PBMC stimulation with PMA and ionomycin. Bars represent mean±SEM. Data were compared using Mann-Whitney test, and *P* values are shown.

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3.2 – SUPPLEMENT A

EVALUATION OF THE FREQUENCY OF T_H17 CELLS IN PATIENTS WITH SELECTIVE IgA DEFICIENCY

As mentioned previously, the development and homeostasis of T_H17 cells and B-differentiation share several molecular factors. Both patients with Congenital Agammaglobulinemia or CVID have impaired IgA production. TGF-β has a crucial role in B-cell function by driving IgA isotype switching, which is the major Ig type produced at mucosal sites[1]. TGF-β is also critical for T_H17-cell differentiation[2,3]. Given that both IgA and T_H17 cells have an important function in the modulation of gut flora and mucosal immunity[1], it is thus reasonable to speculate that the link between IL-17 production and B-cell function that we described could primarily lie in the isotype switch to IgA. This reasoning led us to investigate the frequency of circulating T_H17 cells in patients with selective IgA deficiency (sIgAD). We have used strict criteria to define selective IgA deficiency, including undetectable serum levels of IgA, and we evaluated the frequency of circulating CD4 T cells able to produce IL-17, using the protocols described in Chapter 3.2.

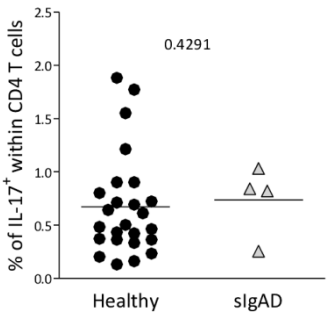

Figure 1. Frequency of T_H17 cells in patients with selective IgA deficiency (sIgAD). Comparison of the frequency of IL-17-producing CD4 T cells between healthy individuals (filled circles) and sIgAD patients (grey triangles), as determined by intracellular staining at the single-cell level. Each symbol represents one individual. Bars represent the mean value. Data were compared using the Mann-Whitney test, and *p* values are shown.

We found no difference between patients with sIgAD and healthy individuals in what concerns the frequency of IL-17-producing CD4 T cells (Figure 1). Our data thus suggest that the relation between B cells and IL-17 production is not dependent on the isotype switch to IgA.

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3.2 – SUPPLEMENT B

DIFFERENTIATION OF T_H17 CELLS FROM NAÏVE CD4 T CELLS FROM CONGENITAL AGAMMAGLOBULINEMIA PATIENTS

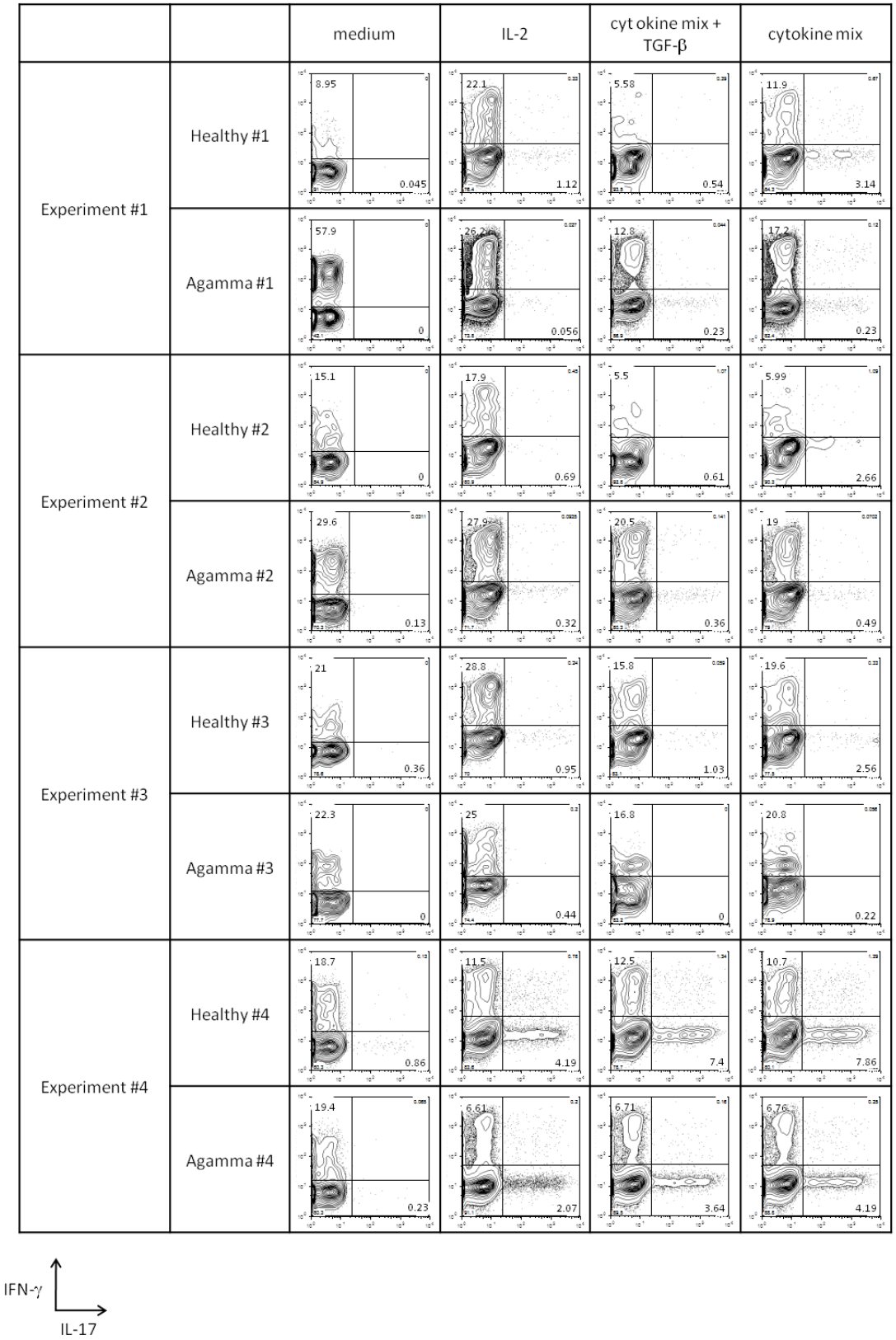
Our observation of markedly reduced frequencies of IL-17-producing CD4 T cells in patients with Congenital Agammaglobulinemia (see Chapter 3.2), who lack circulating B cells, has led us to question whether T_H17 differentiation was affected in these patients.

In order to assess this possibility, we have optimized an *in vitro* T_H17 differentiation protocol using isolated naive CD4 T cells from Congenital Agammaglobulinemia patients with defined mutations in the *Btk* gene (X-Linked Agammaglobulinemia patients).

Human T_H17 differentiation was first described to depend on IL-23 and IL-1β[1] or IL-1β and IL-6[2]. In these reports, both TGF-β and IL-12 were shown to inhibit T_H17 differentiation[2]. However, later on it has been shown that TGF-β is actually required for T_H17 differentiation, although at very low levels, while it can have an inhibitory effect when present at higher concentrations[3]. We have chosen to employ a protocol[4] that combines the use of special culture medium (IMDM - Iscove's modified Dulbecco's medium), in the presence or absence of low concentrations of TGF-β (0.5ng/mL), and a cytokine mix composed of IL-6, IL-1β and IL-23, in the presence of blocking antibodies against IFN-γ and IL-4, to help prevent potential T_H1 and T_H2 polarization, respectively. Naive $CD45RA^+CD45RO^-$ CD4 T cells were isolated using an EasySep magnetic kit and cultured for 5 days with α-CD3 and α-CD28 antibodies to provide TCR and co-stimulation, and IL-2, in the described conditions. Five healthy controls and four XLA patients were studied in parallel.

Figure 1A illustrates the variability of T_H17 polarization between individuals and different experiments after five days of culture. Nevertheless, T_H17 polarization was always more effective in the condition where TGF-β was not added to the culture medium ("cytokine mix").

A


medium
IL-2
cyt okine mix + TGF-β
cytokine mix
Experiment #1
Healthy #1
8.95
0.045
22.1
1.12
5.58
0.54
11.9
3.14
Agamma #1
57.9
0
26.2
0.056
12.8
0.23
17.2
0.23
Experiment #2
Healthy #2
15.1
0
17.9
0.69
5.5
0.61
5.99
2.66
Agamma #2
29.6
0.13
27.9
0.32
20.5
0.36
19
0.49
Experiment #3
Healthy #3
21
0.36
28.8
0.95
15.8
1.03
19.6
2.56
Agamma #3
22.3
0
25
0.44
16.8
0
20.8
0.22
Experiment #4
Healthy #4
18.7
0.86
11.5
4.19
12.5
7.4
10.7
7.86
Agamma #4
19.4
0.23
6.61
2.07
6.71
3.64
6.76
4.19
IFN-γ
IL-17

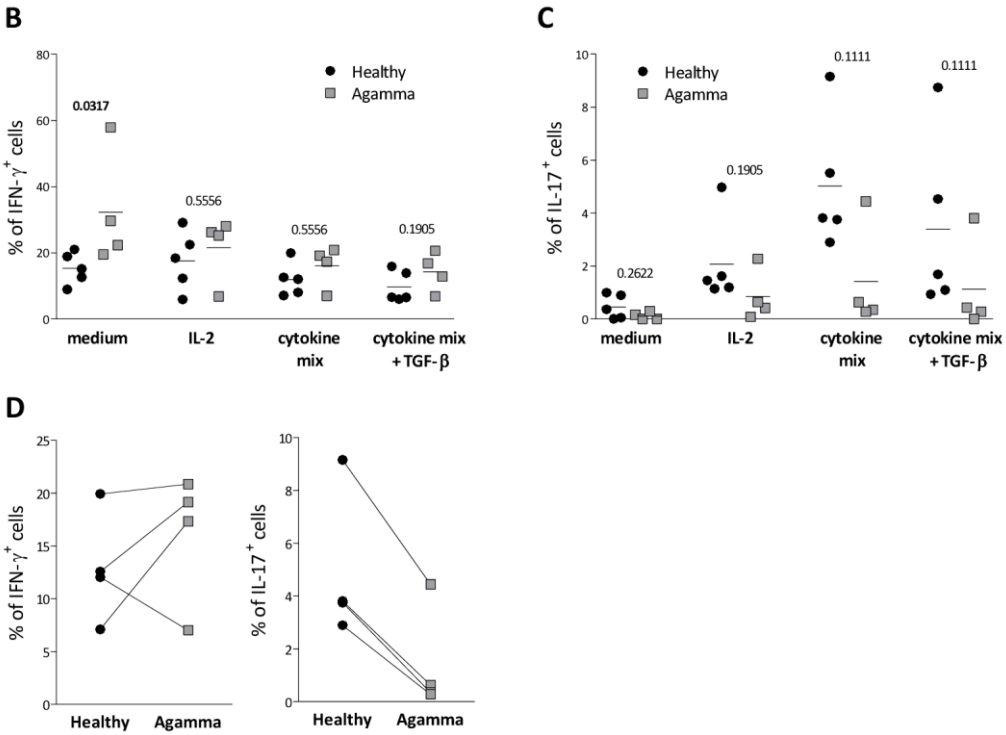


Figure 1. T_H17 differentiation in naïve CD4 T cells from Congenital Agammaglobulinemia patients. (A) Flow cytometry plots representing IL-17 and IFN-γ expression in naïve CD4 T cells cultured for five days in different conditions: medium alone ("medium"), α-CD3/CD28 plus IL-2 ("IL-2"), α-CD3/CD28 plus IL-2 and cytokine cocktail for T_H17 differentiation in the presence ("cytokine mix+TGF-β") or not ("cytokine mix") of TGF-β. Shown are the different pairs of healthy (Healthy) and Congenital Agammaglobulinemia (Agamma) individuals studied in parallel. (B and C) Frequency of IFN-γ^+ (B) and IL-17^+ (C) cells recovered from each culture condition, as described in (A). Healthy individuals are shown as filled circles and Congenital Agammaglobulinemia patients as grey squares. Each symbol represents one individual. Bars represent mean value. Data were compared using the Mann-Whitney test, and *p* values are shown. (D) Comparison between healthy individuals and Congenital Agammaglobulinemia patients studied in parallel, in what concerns IFN-γ (left panel) and IL-17 (right panel) expression.

T_H17 polarization appeared to be defective in XLA patients, while T_H1 polarization was normal (Figure 1A-D).

Even though our results are highly preliminary and need to be further confirmed, they suggest that the reduced *ex vivo* frequency of T_H17 cells found in Congenital Agammaglobulinemia patients can be directly related to a lower cell-specific ability to differentiate into the T_H17 pathway.

Methods

In vitro *differentiation of T_H17 cells*

Naive ($CD45RA^+CD45RO^-$) CD4 T cells were isolated through magnetic negative selection using a kit from EasySep (StemCell Technologies). Cells were cultured in IMDM (Iscove's modified Dulbecco's medium) (Gibco, Invitrogen) supplemented with 5% fetal bovine serum (Gibco, Invitrogen), 1% Pen/Strep (Gibco, Invitrogen), and 1% non-essential aminoacids (Gibco, Invitrogen). Different culture conditions were assessed: medium alone; immobilized α-CD3 (1ug/mL; Ebioscience) and α-CD28 (2.5ug/mL; Ebioscience) plus IL-2 (10U/mL; obtained through the National Institutes of Health (NIH)/AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH [IL-2] from Hoffman-La Roche); α-CD3/CD28 plus IL-2 in the presence of a Th17 cytokine differentiation cocktail [IL-6 (30ng/mL), IL-1β (10ng/mL), IL-23 (10ng/mL); α-IFN-γ (5ug/mL), α-IL-4 (5ug/mL); all from Ebioscience], in the presence or not of TGF-β (0.5ng/mL; Peprotech). Cells were cultured for five days at a concentration of $5x10^5$/mL. Following the culture period, cells were assessed for cytokine production at the single-cell level, as previously described[5]. Briefly, after a 4-hour culture with phorbol myristate acetate (PMA) (50ng/mL, Sigma-Aldrich) plus ionomycin (500ng/mL; Calbiochem, Merck Biosciences), in the presence of brefeldin A (10μg/mL; Sigma-Aldrich), cells were surface stained and then fixed (2% formaldehyde; Sigma-Aldrich), permeabilized (phosphate buffered saline/1% bovine serum albumin/0.5% saponin) (Sigma-Aldrich) and stained intracellularly with monoclonal antibodies against IL-4, IFN-γ, and IL-17. Flow cytometric analysis was subsequently performed. Samples were acquired on a FACSCalibur (BD Biosciences) and data were analysed using FlowJo Software (Tree Star Inc.).

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3.2 – SUPPLEMENT C

REGULATORY T CELLS IN COMMON VARIABLE IMMUNODEFICIENCY

T_{reg} and T_H17 cells are believed to possess reciprocal pathways of differentiation[1]. Several reports have described a decrease in the frequency of T_{reg} cells in of CVID patients[2,3] and a link to autoimmunity has been proposed[4,5]. However, important questions prevail, namely concerning how these alterations are related to the T_H17 compartment. It was thus our aim to determine the relationship between T_{reg} cells and T_H17 cells in CVID patients, for which purpose T_{reg} cells were quantified in terms of FOXP3 and CD25 expression and characterized for the expression of naïve/memory markers and markers of T_{reg}-associated function.

Regulatory T cells in Common Variable Immunodeficiency

We analysed the population of T_{reg} cells in our cohort of CVID patients, using both FOXP3 and CD25 expression to identify these cells. CD25, the alpha-chain of the IL-2 receptor, is up-regulated upon TCR stimulation and is constitutively expressed at high levels by T_{reg} cells[6-10]. It can also be up-regulated upon stimulation with γc cytokines[11-13]. Expression of the FOXP3, a transcription factor associated with T_{reg} differentiation[14-16], is currently considered the best available T_{reg} marker. However, it has also been shown that it may be up-regulated upon T-cell activation[17,18].

We observed that the frequency of $FOXP3^+$ cells within CD4 T cells shows a trend to be increased in CVID patients as compared to healthy individuals (Figure 1B and 1C). However, CD25 expression was significantly decreased in CVID patients, both in terms of total $CD25^+$ within CD4 T cells as well as $CD25^{bright}$ CD4 T cells (Figure 1A, 1D and 1E, respectively). Of note, this reduction of CD25 expression does not seem to have an impact on the frequency of $FOXP3^+CD25^+$ CD4 T cells, since it was similar between CVID patients and healthy individuals (Figure 1F). This decrease in CD25 expression was not apparently related to a decrease in the ability to produce IL-2 since the proportion of IL-2-producing cells was relatively preserved (see Chapter 3.2, Supplemental Figure 2).

Importantly, we observed an expansion of FOXP3$^+$CD25$^-$ CD4 T cells in CVID patients (Figure 1G). This population has been shown to be also expanded in the context of SLE[19,20]. However, its function remains elusive[21], with reports showing that FOXP3$^+$CD25$^-$ CD4 T cells are phenotypically and functionally similar to T_{reg} cells and thus would represent dysfunctional T_{reg}[22], while others concluded that this population most likely represents previously activated conventional T cells, since they are able to produce considerable amounts of cytokines, contrary to true T_{reg} cells[23].

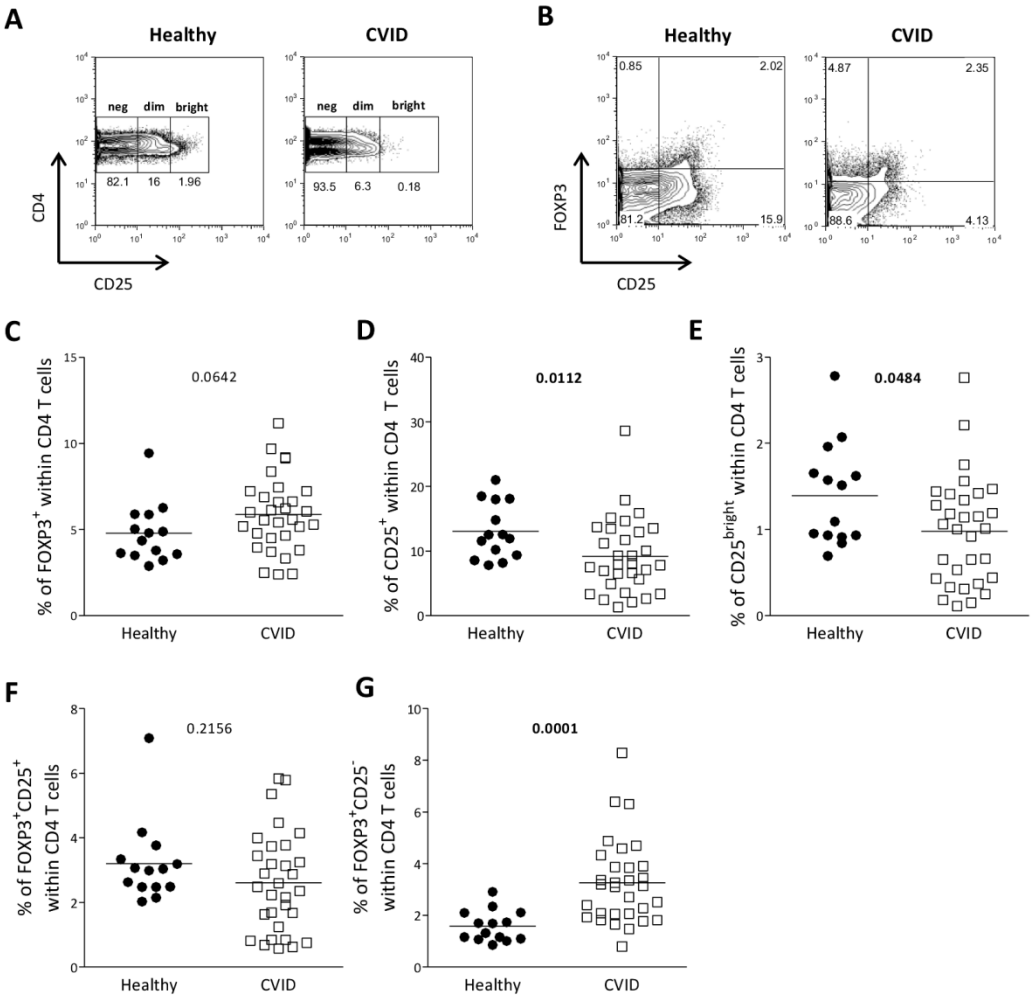

Figure 1. CVID was associated with decreased CD25 expression, while FOXP3 expression was maintained. (A and B) Representative flow cytometric plots of CD25 and FOXP3 expression in a healthy individual and a CVID patient. (C to G) Frequency of different regulatory T-cell populations, as defined by FOXP3 and/or CD25 expression, in healthy controls (filled circles) and CVID patients (open squares). Frequency of FOXP3$^+$ (C), CD25$^+$ (D), CD25^{bright} (E), FOXP3$^+$CD25$^+$ (F) and FOXP3$^+$CD25$^-$ (G) within CD4 T cells. Each symbol represents one individual. Bars represent the mean value. Data were compared using the Mann-Whitney test, and *p* values are shown.

Nevertheless, the expansion of FOXP3$^+$CD25$^-$ CD4 T cells has been associated with disease activity and severity and also with the titres of autoantibodies in SLE patients[20,22], suggesting that

this population can actually have a role in disease pathogenesis. It is still unknown where these cells originate from. Studies have shown that $CD25^-$ cells can give rise to $CD25^+$ cells that are similar to recently isolated T_{reg} cells, upon homeostatic proliferation[24], and it has been further suggested that $FOXP3^+CD25^-$ cells constitute a peripheral reservoir of differentiated T_{reg} cells that can be recruited to the $CD25^+$ pool upon homeostatic expansion[25]. In addition, studies in mice have described an accumulation of a $FOXP3^+$ population within $CD4^+CD25^-$ T cells with suppressive capacity in aged mice, but not in young animals[26]. In this sense, it has been proposed that the increased $FOXP3^+CD25^-$ CD4 T-cell population in SLE patients constitutes a pool of cells that, under the conditions where autoimmune responses are reactivated, can be recruited to expand the T_{reg} population by gaining CD25 expression[27].

Given the apparent dissociation between FOXP3 and CD25 expression in CVID patients, we chose to analyse separately the expression of the two T_{reg} markers. We then analysed the expression of FOXP3 and CD25 in naïve ($CD45RA^+$) and memory ($CD45RA^-$) CD4 T cells (Figure 2).

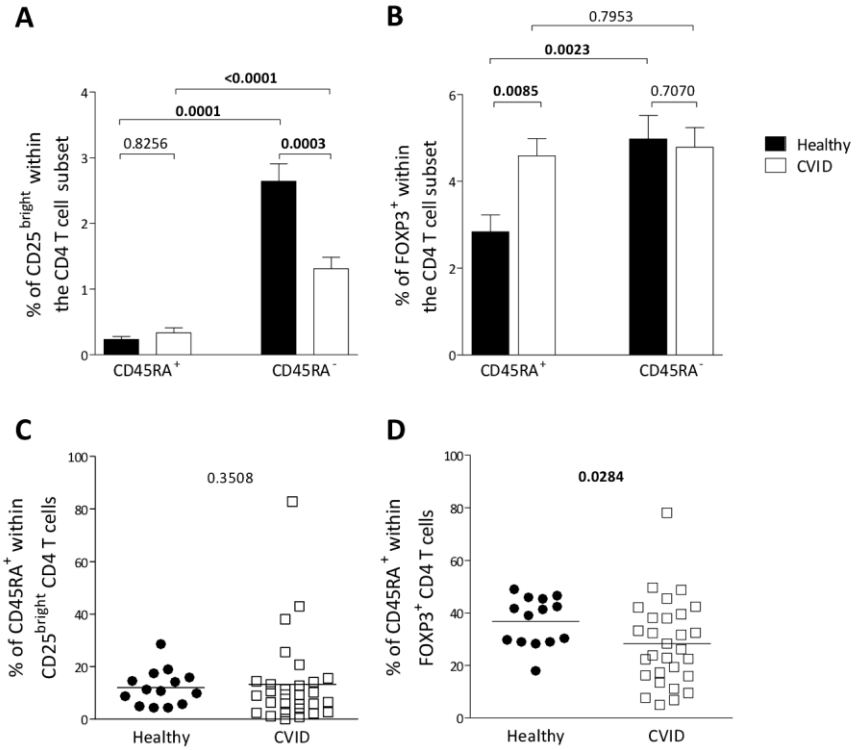

Figure 2. Regulatory T cells in relation to naïve and memory T-cell subsets. Distribution of T_{reg} cells as defined by either $CD25^{bright}$ (A) or $FOXP3^+$ (B) expression within naïve ($CD45RA^+$) and memory ($CD45RA^-$) CD4 T-cell populations. Healthy individuals are represented in black and CVID patients in white bars. Bars represent mean±SEM. Analysis of dependent samples was performed using Wilcoxon matched-pairs signed rank test. (C and D) Quality of T_{reg} cells in terms of the expression of naive cell markers ($CD45RA^+$). Healthy individuals are shown as filled circles and CVID patients as open squares. Each symbol represents one individual. Bars represent the mean value. Data were compared using the Mann-Whitney test, and *p* values are shown.

We found that the frequency of $CD25^{bright}$ cells within naïve CD4 T cells is very small and similar in both healthy individuals and CVID patients (Figure 2A). In the memory CD4 T-cell population, we observed that the frequency of $CD25^{bright}$ cells is significantly decreased in CVID patients as compared to healthy individuals, reflecting the overall loss of CD25 expression we observed in CVID patients (Figure 1A, 1D and 1E). We also found that the frequency of $FOXP3^+$ cells within naïve CD4 T cells is significantly increased in CVID patients, while the frequency in memory CD4 T cells was similar in CVID patients and healthy individuals (Figure 2B). Interestingly, when comparing the two subpopulations within the same cohort, we found that memory CD4 T cells have a higher frequency of $FOXP3^+$ cells than naïve CD4 T cells in healthy individuals, but in CVID patients the frequencies are similar. Again, there seems to be a dissociation between FOXP3 and CD25 expression even within the naïve and memory CD4 T-cell compartments. Conversely, we analysed the frequency of naïve cells within the T_{reg} populations. The $CD4^+CD25^{bright}$ population includes less than 20% of naïve cells, and this percentage is similar in healthy individuals and CVID patients (Figure 2C). Of note, the $CD4^+FOXP3^+$ population includes a significantly lower percentage of naïve cells in CVID patients as compared to healthy individuals (Figure 2D). It should be noted that CVID patients have significantly lower frequencies of naïve CD4 T cells (as shown in Chapter 3.2).

CD39 is an ectoenzyme that degrades adenosine triphosphate (ATP) to adenosine monophosphate (AMP) and has been suggested as an important mechanism of T_{reg}-mediated suppression of inflammatory responses[28]. The frequency of $CD25^+CD39^+$ within CD4 T cells was significantly reduced in CVID patients (Figure 3A), probably reflecting the fact that CD25 expression, as a whole, is prominently diminished in these patients. Supporting this view is the fact that the frequency of $FOXP3^+CD39^+$ within CD4 T cells was similar in healthy individuals and CVID patients (Figure 3B). To overcome the issue of total CD25 or FOXP3 expression, we analysed the expression of CD39 within the differently defined T_{reg} populations.

We observed that CD39 expression in CVID patients and healthy individuals was not significantly different, both in the $FOXP3^+$ CD4 T-cell population as well as in the $CD25^{bright}$ subset (Figure 3C and 3D, respectively). It is worth mentioning that the $CD25^{bright}$ CD4 T-cell population comprised a higher amount of $CD39^+$ cells, which may suggest that high levels of CD25 expression may be a more suitable marker to define T_{reg} cells than FOXP3 expression, as it has actually been proposed in the context of SLE[29].

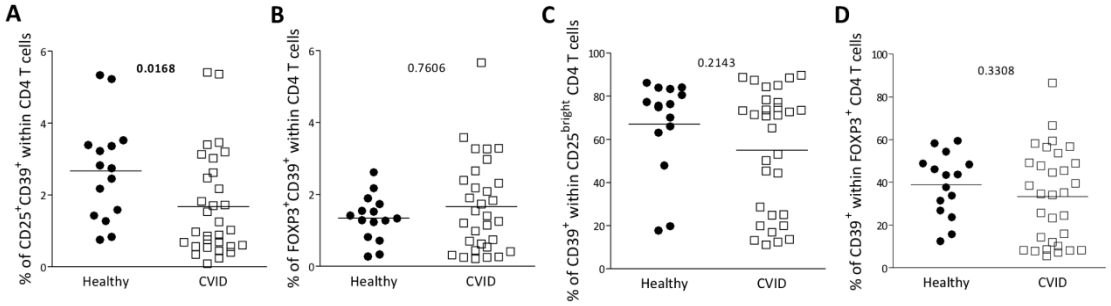

Figure 3. Expression of the ectoenzyme CD39 in regulatory T cells. Frequency of $CD25^{+}CD39^{+}$ (A) and $FOXP3^{+}CD39^{+}$ (B) cells within CD4 T cells. Expression of CD39 within $CD25^{bright}$ (C) and $FOXP3^{+}$ (D) populations. Healthy individuals are shown as filled circles and CVID patients as open squares. Each symbol represents one individual. Bars represent the mean value. Data were compared using the Mann-Whitney test, and *p* values are shown.

Relationship between regulatory T cells and T-cell imbalances

Increased levels of T-cell immune activation are a hallmark of the immunodeficient state observed in HIV-infected patients and are considered to be a good surrogate marker of disease progression[30]. It has been reported that the frequency of T_{reg} cells positively associates with the levels of T-cell activation in HIV infection[31,32], possibly reflecting a self-regulatory mechanism to counterbalance the immune activation. Since our CVID cohort also presents increased levels of T-cell immune activation, we sought to determine if this was associated with the frequency of different subsets of T_{reg} cells and their functional potential. We found no association between the levels of CD4 T-cell activation and the frequency of either $CD25^{bright}$ or $FOXP3^{+}$ CD4 T cells in CVID patients (Figure 4A and 4B, respectively). However, CD4 T-cell activation was inversely correlated with the total frequency of CD25-expressing CD4 T cells (Figure 4C). In order to better understand whether $FOXP3^{+}CD25^{-}$ CD4 T cells can be related to T-cell activation, we analysed the relationship between the frequency of either $FOXP3^{+}CD25^{+}$ or $FOXP3^{+}CD25^{-}$ CD4 T-cell populations and the levels of T-cell activation. We observed that the frequency of the $FOXP3^{+}CD25^{+}$ population was negatively associated with CD4 T-cell activation, in CVID patients (Figure 4D), while the frequency of $FOXP3^{+}CD25^{-}$ CD4 T cells was directly associated with T-cell activation (Figure 4E), further suggesting that the expression of FOXP3 in the $CD25^{-}$ population may be directly related to events of immune activation. As previously mentioned, CVID patients also have naïve/memory T cell imbalances, namely a loss of naïve $CD45RA^{+}$ T cells with a consequent accumulation of memory $CD45RA^{-}CD45RO^{+}$ T cells and increased levels of T-cell activation (see Chapter 3.2)[33].

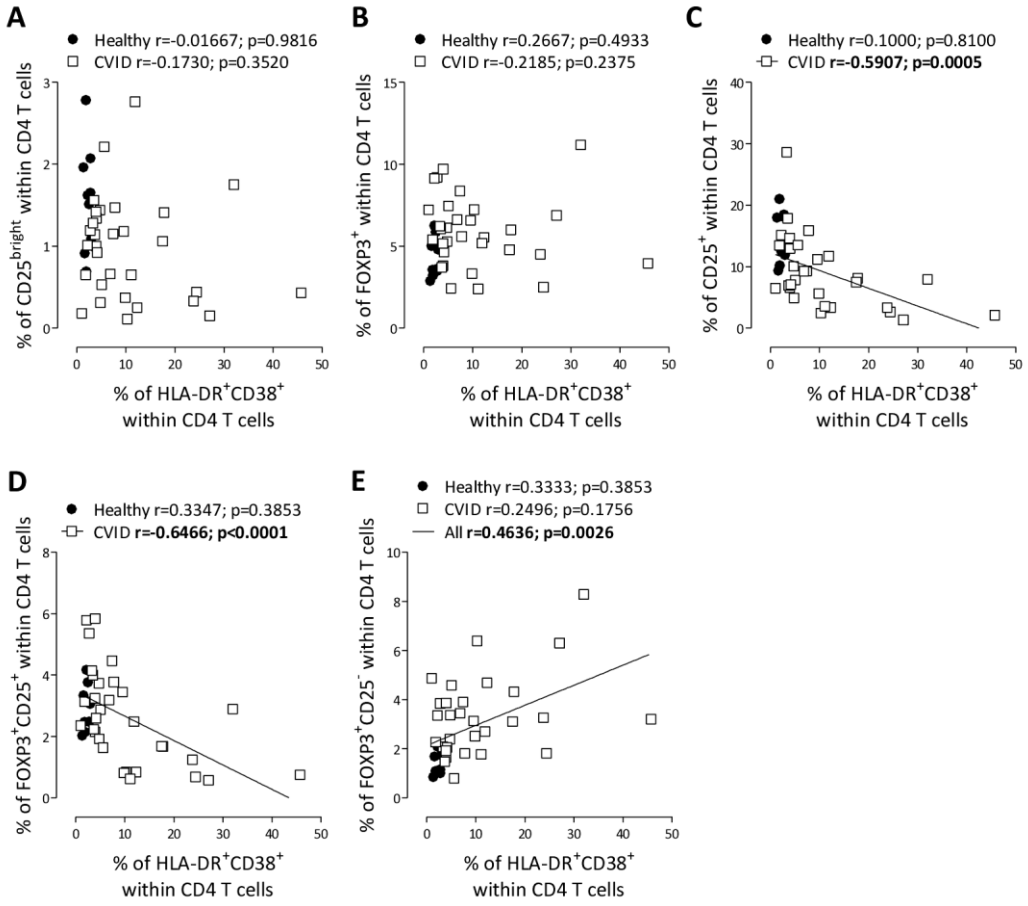

Figure 4. Relationship between regulatory T-cell subsets and T-cell activation in CVID. Correlations between the frequencies of activated CD4 T cells as defined as HLA-DR$^+$CD38$^+$ and CD25 and/or FOXP3-expressing regulatory T-cell subsets, in healthy individuals (filled circles) and CVID patients (open squares). Correlation between activated CD4 T cells and CD25^{bright} (A), FOXP3$^+$ (B), total CD25$^+$ (C), FOXP3$^+$CD25$^+$ (D) and FOXP3$^+$CD25$^-$ (E) within CD4 T cells. Each symbol represents one individual. Correlation significance was determined using the Spearman coefficient test, and *r* and *p* values are shown.

In order to bypass the impact of such imbalances in the frequency of T_{reg} cells, we analysed the frequencies of these cells within the CD45RA$^+$ and the CD45RA$^-$ CD4 T cell subsets and then studied their relationships with the levels of CD4 T cell activation. We observed that, within the naïve CD45RA$^+$ subset, there were no relationship between the frequency of either CD25^{bright} or FOXP3$^+$ within CD4 T cells and the levels of CD4 T cell activation (Figure 5A, left and right panel, respectively). However, within the memory CD45RA$^-$ subset, the frequency of FOXP3$^+$ CD4 T cells was negatively associated with the levels of CD4 T-cell activation in CVID patients (Figure 5B).

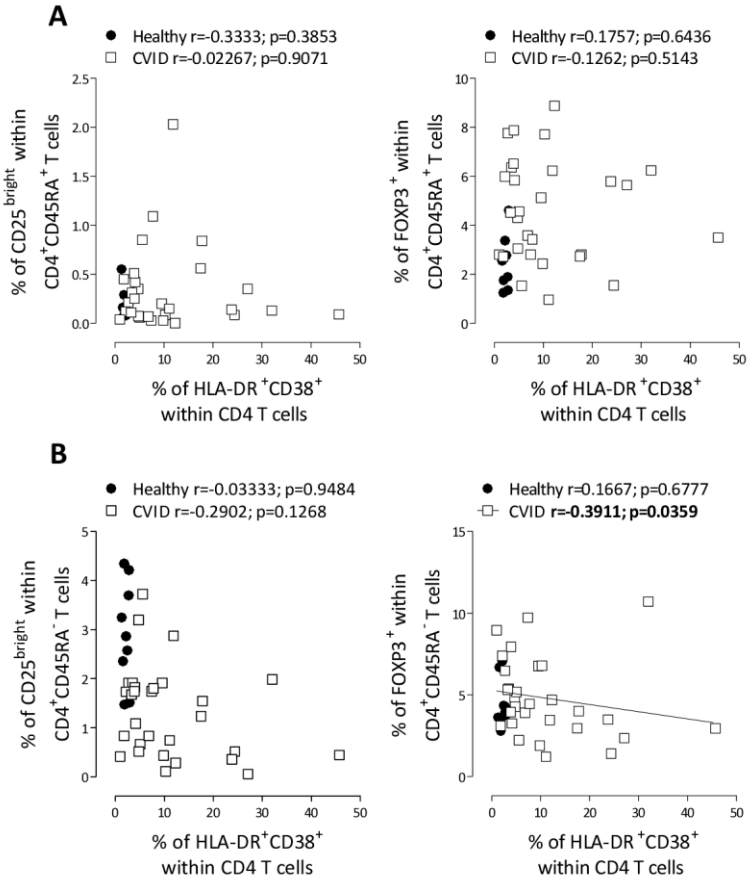

Figure 5. Relationship between naïve and memory regulatory T-cell subsets and T-cell activation in CVID. Correlations between the frequencies of activated CD4 T cells as defined as HLA-DR^{+}CD38^{+} and the distribution of regulatory T cells as defined by either CD25^{bright} (left panels) or FOXP3^{+} (right panels) expression within naive (CD45RA^{+}) (A) and memory (CD45RA^{-}) (B) CD4 T-cell populations. Each symbol represents one individual. Correlation significance was determined using the Spearman coefficient test, and *r* and *p* values are shown.

Interestingly, we observed that the frequency of CD25^{bright} cells within CD4 T cells was directly associated with the frequency of naïve CD4 T cells, in CVID patients (Figure 6A).

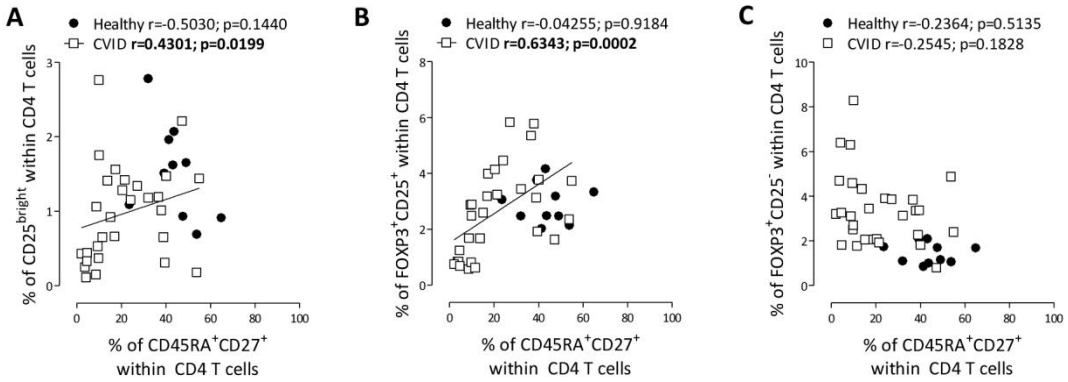

Figure 6. The frequency of regulatory T cells was directly associated with the frequency of naïve T cells in CVID. Correlations between the frequencies of naive CD4 T cells as defined as CD45RA^{+}CD27^{+} and CD25bright (A), FOXP3^{+}CD25^{+} (B), and FOXP3^{+}CD25^{-} (C) CD4 T-cell populations, in healthy individuals (filled circles) and CVID patients (open squares). Each symbol represents one individual. Correlation significance was determined using the Spearman coefficient test, and *r* and *p* values are shown.

No relationship was found between the frequency of naïve CD4 T cells and that of FOXP3$^+$ cells within CD4 T cells. However, the frequency of FOXP3$^+$CD25$^+$ CD4 T cells was also directly associated with the frequency of naïve CD4 T cells, in CVID patients (Figure 6B), while no relationship was found for the frequency of Foxp3$^+$CD25$^-$ CD4 T cells (Figure 6C).

Regulatory T cells and their relationship to IL-17-producing CD4 T cells

As previously mentioned, T_{reg} and T_H17 cells are thought to have reciprocal pathways of differentiation[1], and although human peripheral blood and lymphoid tissue have been shown to contain CD4$^+$FOXP3$^+$ T cells that express CCR6 and have the capacity to produce IL-17 upon activation[34], we observed very low frequencies of FOXP3$^+$IL-17$^+$ cells within circulating CD4 T cells (Figure 7A). We found that the frequency of FOXP3$^+$ cells within CD4 T cells was directly associated with T_H17 cells in CVID patients (Figure 7B).

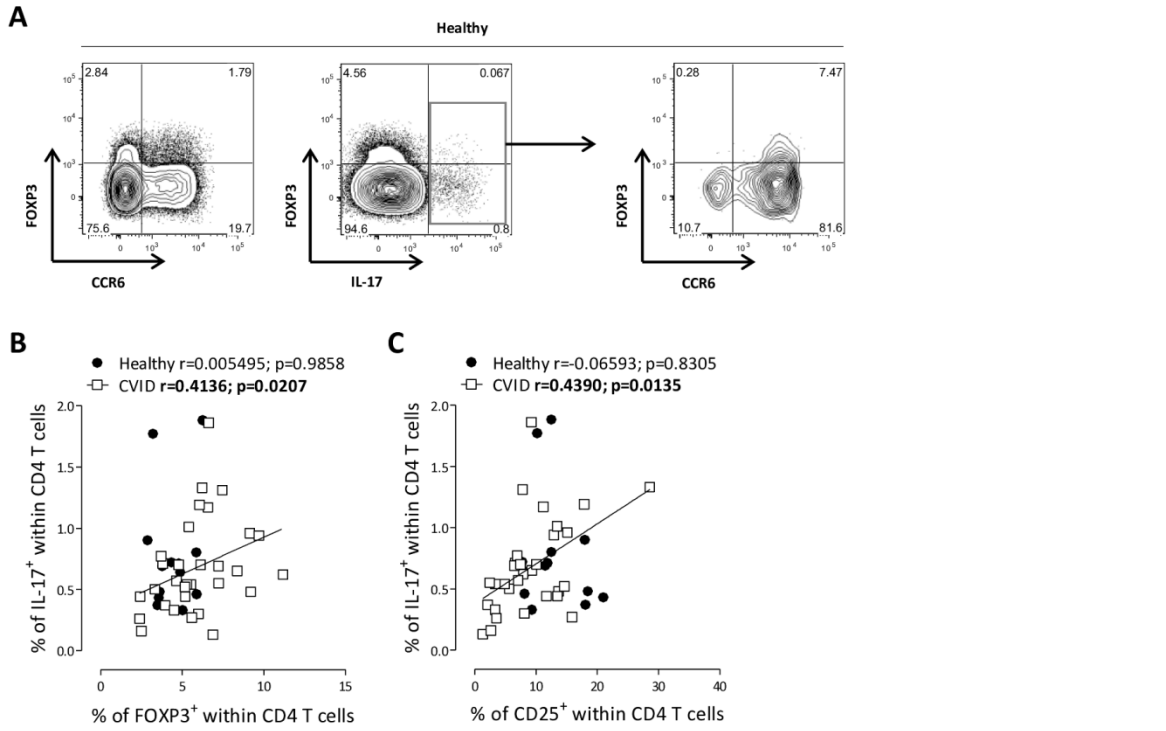

Figure 7. Direct association between the frequency of IL-17-producing CD4 T cells and regulatory T cells in CVID. (A) Representative plots of the analysis of FOXP3 and CCR6, and FOXP3 and IL-17 expression on CD4 T cells, for a healthy individual. In order to analyse concomitant expression of FOXP3 and IL-17, PBMC were intracellular stained after short-term stimulation with PMA and ionomycin, using the FOXP3 Staining Set. The right panel shows the expression of FOXP3 and CCR6 within IL-17$^+$ CD4 T cells. The frequency of FOXP3$^+$IL-17$^+$ cells within CD4 T cells (0.06%±0.04%, n=6) represented around 8% of the total IL-17$^+$ CD4 T-cell population (7.89%±2.50%, n=6) in a subgroup of healthy subjects assessed. Correlations between the frequencies of IL-17-producing CD4 T cells and FOXP3$^+$ (B) and CD25$^+$ (C) CD4 T cells, in healthy individuals (filled circles) and CVID patients (open squares). Each symbol represents one individual. Correlation significance was determined using the Spearman coefficient test, and *r* and *p* values are shown.

We also documented a direct correlation with CD25 expression in CVID (Figure 7C), in clear contrast with the negative correlation found between IL-17 production and markers classically up-regulated in clinical settings associated with T-cell activation (see Chapter 3.2).

Regulatory T cells and their relationship to pro-inflammatory cytokine production by CD4 T cells

Given the results obtained for IL-17 production, we sought to analyse the association between the different T_{reg} subsets and the CD4 T-cell populations that produce other pro-inflammatory cytokines, such as IFN-γ or TNF-α.

We found that both the frequencies of IFN-γ-producing CD4 T cells (Figure 8A) and TNF-α-producing CD4 T cells (Figure 8B) were inversely correlated with the frequency of T_{reg} cells in CVID patients, defined either as $FOXP3^+CD25^+$ CD4 T cells or as $CD25^{bright}$ CD4 T cells. The loss of T_{reg} cells was directly associated to expansions of CD4 T-cell populations that produce the pro-inflammatory cytokines IFN-γ and TNF-α, in contrast to what was found for the production of IL-17.

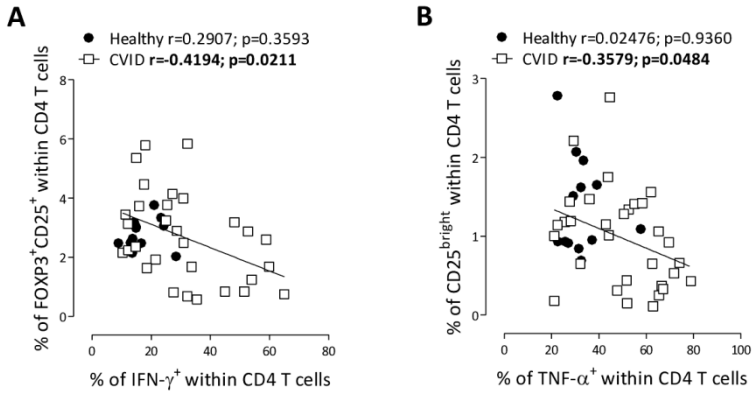

Figure 8. Expansion of T-cell populations producing pro-inflammatory cytokines was directly associated with loss of regulatory T cells in CVID. Correlations between the frequencies of regulatory T (T_{reg}) cells and CD4 T-cell populations producing the pro-inflammatory cytokines IFN-γ and TNF-α, in healthy individuals (filled circles) and CVID patients (open squares). (A) Association between $FOXP3^+CD25^+$ and IFN-γ-producing CD4 T cells. (B) Correlation between the frequency of $CD25^{bright}$ and TNF-α-producing CD4 T cells. Each symbol represents one individual. Correlation significance was determined using the Spearman coefficient test, and *r* and *p* values are shown.

Regulatory T cells and clinical manifestations in CVID patients

We have found that the frequency of $FOXP3^+$ cells within $CD4^+$ T cells was increased in CVID patients as compared to healthy controls. Subsequently, we sought to investigate whether this increase was associated with particular clinical manifestations, namely autoimmune disorders, chronic diarrhea, splenomegaly, lymphoid proliferation and adenopathies. When we

stratified the CVID patients according to these manifestations, no particular pattern was found in what concerns the frequency of FOXP3$^+$ cells within CD4$^+$ T cells (Figure 9A).

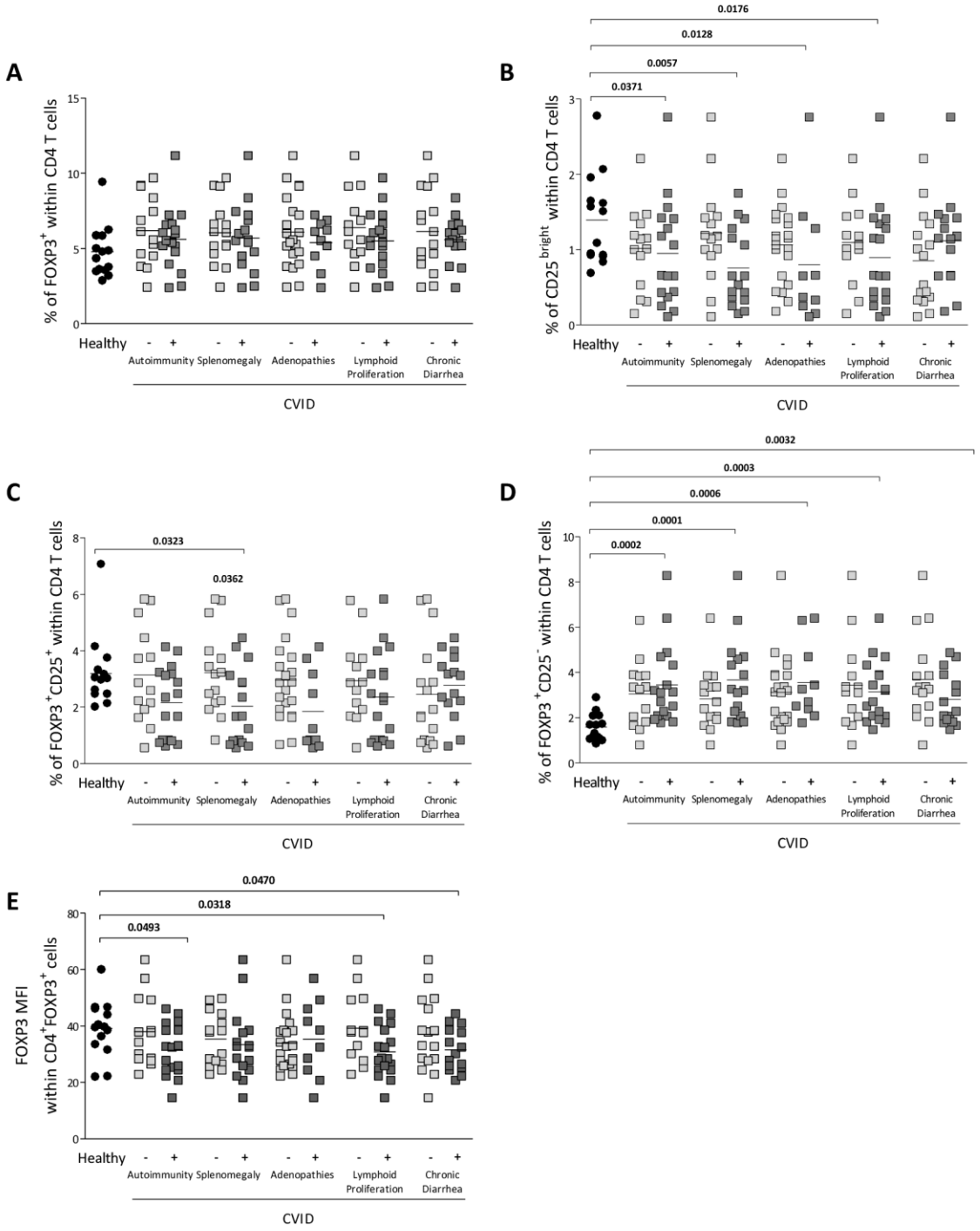

Figure 9. Association of regulatory T-cell imbalances with clinical manifestations in CVID patients. Stratification of different regulatory T (T_{reg}) cell populations in CVID patients, according to the occurrence or not of the following clinical manifestations: autoimmunity, splenomegaly, adenopathies, lymphoid proliferation, and chronic diarrhea. Frequencies of FOXP3$^+$ (A), CD25^{bright} (B), FOXP3$^+$CD25$^+$ (C), and FOXP3$^+$CD25$^-$ (D) CD4 T-cell populations are shown. (E) Mean fluorescence intensity (MFI) of FOXP3 expression within CD4$^+$FOXP3$^+$ cells. Healthy individuals are shown as filled circles and CVID patients as squares with two different shades of grey. Each symbol represents one individual. Bars represent the mean value. Data were compared using the Mann-Whitney test, and *p* values are shown.

While we had observed that the frequency of $CD25^{bright}$ within $CD4^+$ T cells was decreased in total CVID patients when compared to healthy individuals, the stratification upon clinical manifestations showed that CVID patients with autoimmune disorders, splenomegaly, adenopathies, or lymphoid proliferation, exhibited the same decrease in the frequencies of $CD25^{bright}$ CD4 T cells when compared to healthy individuals, at the levels observed in CVID patients without these clinical complications (Figure 9B). No differences have been found in the frequency of $FOXP3^+CD25^+$ cells within CD4 T cells between total CVID patients and healthy controls. However, when we stratified the patients according to clinical manifestations we verified that patients with splenomegaly showed significantly decreased frequencies of this population as compared to patients without splenomegaly, as well as to healthy individuals (Figure 9C). On the other hand, while total CVID patients showed an increased frequency of $FOXP3^+CD25^-$ cells within $CD4^+$ T cells, the stratification of these patients demonstrated that all patients that presented with the clinical manifestations mentioned above had significantly higher frequencies of $FOXP3^+CD25^-$ CD4 T cells as compared to healthy individuals (Figure 9D). It is worth mentioning that the expansion of this population was not significant when compared to CVID patients that did not present the clinical manifestations, showing that the increased frequency of $FOXP3^+CD25^-$ CD4 T cells was common to the majority of CVID patients. Even though we found no difference between healthy subjects and CVID patients in what concerns the levels of FOXP3 expression within $CD4^+FOXP3^+$ cells, when CVID patients were stratified according to clinical complications the picture was quite different. In agreement with the data reported by Yu *et al.*[35], CVID patients with autoimmunity have significantly lower levels of FOXP3 expression than healthy controls (Figure 9E). In addition, CVID patients with lymphoid proliferation and chronic diarrhea also present a reduction in FOXP3 expression on $FOXP3^+$ cells (Figure 9E). This suggests that in a particular subset of CVID patients T_{reg} function may actually be decreased and associated with phenomena of loss of T-cell balance.

Relationship between regulatory T cells and B-cell imbalances in CVID patients

The $CD21^{low}CD38^{low}$ B-cell population is thought to be an activated B-cell population that fails to further differentiate. This population frequently accumulates in CVID patients. Of note, T_{reg} frequency, particularly the $FOXP3^+CD25^+$ CD4 T cell population, inversely correlated with the frequency of $CD21^{low}CD38^{low}$ B cells in CVID patients (Figure 10), in agreement with a previous report[4]. This suggests a possible link between the size of the T_{reg} subset and GC disturbances. The expansion of $CD21^{low}CD38^{low}$ B cells, which has been shown to be associated with autoimmunity[36,37], is associated with a lower frequency of T_{reg} cells, suggesting that a loss of T_{reg}

cells may actually be underlying autoimmune phenomena in CVID patients. It has been described that the frequency of T_{reg} is significantly decreased in CVID patients with autoimmune manifestations as compared with patients without autoimmunity[4,5], but we did not find such clinical correlation in our cohort (Figure 9).

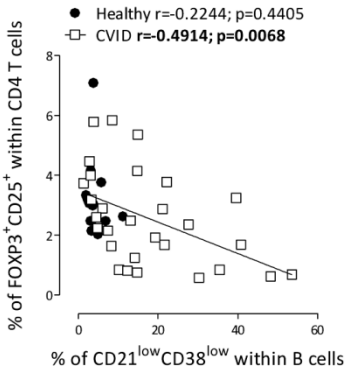

Figure 10. Expansion of the CD21^{low}CD38^{low} B-cell population correlated with loss of regulatory T cells in CVID. Correlation between the frequencies of FOXP3^{+}CD25^{+} CD4 T cells and CD21^{low}CD38^{low} B cells, in healthy individuals (filled circles) and CVID patients (open squares). Each symbol represents one individual. Correlation significance was determined using the Spearman coefficient test, and *r* and *p* values are shown.

Concluding Remarks

We found that CVID patients present a reduction in the frequency of T_{reg} cells in what regards the expression of CD25, while FOXP3 expression appeared to be maintained. We also showed that in CVID patients the frequencies of T_{reg} cells and T_H17 cells were directly associated. In addition, T_{reg} frequency was also inversely associated with the frequency of CD21^{low}CD38^{low} B cells. These data support the view that the impairment of the GCs found in CVID may have an impact in both T_{reg} and T_H17 populations.

It will be important to study the basis of this apparent dissociation between C25 and FOXP3 expression and to clarify whether there is a true loss of regulatory function in these CVID patients, with a possible association with autoimmunity, as previously suggested[35]. This would imply that the maintenance of FOXP3 expression might be related to T-cell activation.

Methods

Cell staining and flow cytometric analysis

PBMCs isolated from heparinized blood, immediately after venipuncture, by Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech), were stained for surface markers, followed by intracellular staining for FOXP3 using the FOXP3 Staining Set (eBiosciences) according to the manufacturer's instructions. A mean of 150.000 PBMCs was analysed. Samples were acquired on a FACSCalibur (BD Biosciences) and data analysed using FlowJo Software (Tree Star Inc.). Results are presented as a proportion of a given cell population.

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3.3 – MONOCYTE IMBALANCES IN PRIMARY B-CELL IMMUNODEFICIENCIES AND THEIR POSSIBLE RELATIONSHIP WITH INCREASED MICROBIAL TRANSLOCATION AND CHRONIC IMMUNE ACTIVATION IN COMMON VARIABLE IMMUNODEFICIENCY

including

Monocyte activation is a feature of Common Variable Immunodeficiency irrespective of plasma lipopolysaccharide levels

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Abstract

Common Variable Immunodeficiency Disorders (CVID), the most frequent cause of symptomatic primary immunodeficiency, are defined by impaired antibody production. Notwithstanding, T-cell activation and granulomatous manifestations represent main causes of CVID morbidity even in patients under immunoglobulin G (IgG) replacement therapy. Additionally, gut pathology is a frequent feature of CVID. Here, we investigated monocyte imbalances and their possible relationship with increased microbial translocation in CVID patients.

Monocyte subsets were defined according to CD14 and CD16 expression levels and evaluated in terms of HLA-DR, CD86 and PD-L1 expression by flow cytometry, in parallel with the quantification of plasma Lipopolysaccharide (LPS), and serum levels of soluble CD14 (sCD14), LPS-binding protein (LBP), and anti-LPS antibodies.

CVID patients (n=31) featured significantly increased levels of serum sCD14 and an expansion of $CD14^{bright}CD16^{+}$ monocytes in direct correlation with T-cell and B-cell activation, the latter illustrated by the frequency of $CD21^{low}CD38^{low}$ subset. Such alterations were not observed in patients lacking B cells due to Congenital Agammaglobulinemia (n=4). Moreover, we found no significant increase in circulating LPS or LBP levels in CVID patients, together with a relative preservation of serum anti-LPS antibodies, in agreement with their presence in commercial IgG preparations.

In conclusion, CVID was associated with monocyte imbalances that directly correlated with T-cell activation markers and with B-cell imbalances, without an association with plasma LPS levels. The heightened monocyte activated state observed in CVID may represent an important target for complementary therapeutic strategies.

Introduction

Common Variable Immunodeficiency Disorders (CVID) represent the most frequent cause of symptomatic primary immunodeficiency[1]. CVID are defined by quantitative and qualitative reduction in antibody production due to heterogeneous defects in mature B cells[1,2]. Besides imbalances in B-cell subsets, CVID are frequently associated with persistent T-cell activation and loss of naive T cells, which are reported even in patients under replacement therapy with immunoglobulin G (IgG)[1-4]. Indeed, non-infectious complications, such as lymphoproliferation and granulomatous disease, are currently main causes of morbidity and mortality in CVID[1,2]. The mechanisms underlying the chronic immune activation associated with CVID remain largely unclear. Another main cause of CVID morbidity is gastrointestinal pathology, with or without mal-absorption[1,2]. The impairment in IgA production and other CVID-associated mucosal alterations have been shown to be often associated with increased intestinal permeability[5]. Thus, it is plausible that increased levels of microbial translocation, particularly of bacterial products such as Lipopolysaccharide (LPS), with consequent monocyte stimulation, may contribute to the chronic immune activation observed in CVID patients, as reported for HIV-1 infected individuals[6].

Monocytes are important orchestrators of the immune system, linking innate and adaptive immune responses. Blood monocytes have the ability to migrate into tissues, where they can differentiate and give rise to distinct functional cell types, such as macrophages and dendritic cells (DCs)[7]. They have long been recognized as the main promoters of inflammatory responses to pathogens and to be involved in many inflammatory diseases[7]. Recent studies have called attention to their suppressive and regulatory functions, suggesting that monocytes play a central role in the modulation of immune responses, particularly in the context of chronic immune activation[8]. These data point to the heterogeneity of monocyte subsets, with distinct functions and phenotypes[9-11]. Recently, an effort was made in putting forward a consensus nomenclature that proposed three main monocyte subsets defined according to the expression levels of CD14 and CD16, as follows: classical $CD14^{bright}CD16^{-}$ monocytes, the most abundant monocyte population found in peripheral blood; intermediate $CD14^{bright}CD16^{+}$, and non-classical $CD14^{dim}CD16^{+}$ subsets[12]. The intermediate $CD14^{bright}CD16^{+}$ monocyte subset has been consistently shown to expand in many pro-inflammatory clinical settings[12,13], and particularly in association with HIV infection[14,15].

CVID has been associated with defective in vitro maturation of DCs from monocytes, at least in a subset of patients[16,17], and with disturbances in the monocyte responses upon LPS

stimulation in vitro[18]. Additionally, CVID patients were shown to have reduced numbers of circulating DCs[19,20], with perturbed differentiation and function, namely reduced expression levels of the co-stimulatory molecules CD86 and CD80, an impaired ability to produce IL-12 upon stimulation[21-23], as well as lower antigen presenting capacity in mixed lymphocyte reactions[22,23].

Monocyte-related alterations, namely an increased frequency of CD16-positive monocytes[24] and decreased numbers of myeloid DCs[25] have also been reported in patients lacking B cells due to blockade in early B-cell development in the bone marrow due to genetic defects in Bruton's tyrosine kinase (Btk) leading to Congenital Agammaglobulinemia[26]. Nevertheless, we and others have shown that, in contrast to CVID, Congenital Agammaglobulinemia is not associated with a significant increase in T-cell activation markers[3,18,27].

Here, we investigated the monocyte compartment in patients with CVID and provide evidence that CVID was associated with increased markers of monocyte activation in direct correlation with the expansion of activated T-cell subsets, irrespectively of plasma LPS levels. Monocytes may thus be important contributors to the inflammatory milieu that leads to T-cell activation, lymphoproliferative manifestations, and granuloma formation associated with CVID[1,28].

Material and Methods

Cohort characterization

The study involved 31 patients with CVID, diagnosed according to the European Society for Immunodeficiencies criteria (www.esid.org), namely IgG and IgA and/or IgM levels at least two standard deviations below the mean for age, impaired antibody response to vaccines, absent/low isohemagglutinins, and exclusion of defined causes of hypogammaglobulinemia. Patients with Congenital Agammaglobulinemia, presenting less than 1% B cells within total peripheral lymphocytes (n=4), were also included. These cohorts have been previously described[3]. The clinical and epidemiological characterization of these cohorts is summarized in Table 1. 29 CVID and all Congenital Agammaglobulinemia patients were under IgG replacement therapy, adjusted to maintain pre-infusion Ig levels above 650 mg/dL. The two CVID patients not receiving IgG featured levels of total serum IgG of 227 and 473 mg/dL. All patients were free from symptomatic infections at the time of sample collection, and the collection of the blood samples was always performed immediately before the immunoglobulin infusions in the patients under intravenous administration. 4 CVID patients were under steroid therapy at the time of the study. 15 healthy individuals were studied in parallel. All subjects gave written informed consent for blood sampling and processing. The study was approved by the Ethical Boards of the Faculdade de Medicina da Universidade de Lisboa and of the Hospital de Santa Maria, and performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

Cell staining and flow cytometric analysis

Phenotypic analysis was performed using whole blood samples collected immediately before IgG administration. After staining with monoclonal antibodies and red blood cells lysis using BD FACS Lysing Solution (BD Biosciences), samples were acquired on a FACSCalibur flow cytometer (BD Biosciences). The following anti-human monoclonal antibodies were used, with the clone and the respective directly-conjugated fluorochrome specified in brackets: CD16 (3G8; FITC), CD3 (SK7; PerCP), CD4 (SK3; PerCP), CD8 (SK1; PerCP), CD8 (RPA-T8; APC), CD38 (HB7; PE), CD45RA (L48; PE-Cy7), CD86 (FUN-1; PE), IgD (IA6-2; PE), IgM (G20-127; APC), HLA-DR (L243; FITC and PerCP), IFN-γ (4S.B3; FITC), from BD Biosciences; CD4 (RPA-T4, FITC, and PerCP-Cy5.5), CD8 (RPA-T8; FITC and PE), CD14 (61D3; PE-Cy7 and APC), CD19 (HIB19; PerCP-Cy5.5 and PE-Cy7), CD27 (O323; FITC, PE, and APC), CD45RA (HI100; FITC and APC), PD-L1 (MIH1; APC), TNF-α (MAb11; PE), from eBiosciences; CD21 (BL13; FITC) from IO Test, Beckman Coulter.

Table 1. Clinical and epidemiological data of the studied cohorts

	Healthy	CVID	Congenital Agammaglobulinemia[a]
Number (male/female)	15 (5/10)	31 (11/20)	4 (4/0)
Age (*yrs.*)	39±11	40±13	26±5
Clinical manifestations[b,c]			
Autoimmune disease	n.a.	17/31 (55%)	0
Adenopathies	n.a.	10/31 (32%)	0
Lymphoid proliferation	n.a.	18/20[d] (86%)	0
Granulomas	n.a.	3/20[d] (15%)	0
Chronic diarrhoea	n.a.	15/31 (48%)	0
Splenomegaly	n.a.	16/31 (52%)	0
EUROclass classification			
smB$^+$21norm	n.a.	5/29 (17%)	n.a.
smB$^+$21lo	n.a.	9/29 (31%)	n.a.
smB$^-$21norm	n.a.	6/29 (21%)	n.a.
smB$^-$21lo	n.a.	9/29 (31%)	n.a.
smB$^-$Trnorm	n.a.	9/29 (31%)	n.a.
smB$^-$Trhi	n.a.	6/29 (21%)	n.a.
IgG replacement therapy[c]			
intravenous	n.a.	24/31 (77%)	4 (100%)
subcutaneous	n.a.	5/31 (16%)	0
Length of IgG therapy (*yrs.*)	n.a.	7±6	16±11

n.a. not applicable, CVID: Common Variable Immunodeficiency [a] Two of the congenital agammaglobulinemia patients had a known genetic defect in the *Btk* gene, namely one had the *IVS17-1G→C* mutation and the other presented with the R288Q mutation; in the other two patients, mutations in the *Btk* gene have been excluded and evaluation of autosomal recessive forms is ongoing. [b] Diagnostic criteria: Autoimmune disease - clinical data, given the impairment in Ab production; Bronchiectasis - computed tomography; Splenomegaly - longitudinal spleen diameter superior to 15cm (computed tomography or ultrasonography); Adenopathies - lymph node larger than 1cm diameter in 2 or more lymphatic chains in clinical and/or imaging exams; Lymphoid proliferation and Granulomas - diffuse lymphocytic infiltrates or granulomas on gastrointestinal, lymph node or pulmonary biopsies. [c] Percentage within total cohort evaluated in brackets. [d] Total number of individuals with biopsies.

Data were analysed using CellQuest (BD Biosciences) and FlowJo (Tree Star Inc.) softwares. Lymphocyte and monocyte subsets were defined within manually set lymphogate/monogate. Results were expressed as percentage of cells that stained positive for a given marker, or as its mean fluorescence intensity (MFI) within the defined population. Total cell numbers were calculated by multiplying the percentage of each population within total lymphocytes/monocytes by the peripheral blood lymphocyte/monocyte count obtained at the clinical laboratory on the day of sampling.

Analysis of cytokine production

Cytokine production was assessed at the single-cell level, as previously described[3]. Briefly, freshly isolated peripheral blood mononuclear cells cultured in the presence of Brefeldin A (10µg/mL; Sigma-Aldrich), for 4h at 37 ºC with 5% CO_2, were stimulated with Lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich) for assessing TNF-α production by monocytes or with Phorbol Myristate Acetate (50ng/mL, Sigma-Aldrich) and Ionomycin (500ng/mL; Calbiochem, Merck Biosciences) for assessing the lymphocyte production of IFN-γ.

Quantification of soluble CD14 (sCD14), LBP, LPS and IgG EndoCAb

The human EndoCAb® Assay Kit and the ELISA Kit for Endoblock Lipopolysaccharide Binding Protein (LBP, HyCult Biotechnology) were used to quantify the serum concentrations of IgG EndoCAb and LBP, respectively. Serum sCD14 levels were quantified by ELISA using the human sCD14 Quantikine (R&D Systems). Plasma LPS levels were quantified using the Limulus Amebocyte Assay (Cambrex); plasma samples were diluted and pre-treated as previously described[6]. All samples were assayed in duplicate, according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software). Two group comparisons were performed using Mann-Whitney test. Spearman's coefficient was used to determine the significance of the correlation between two variables. Results are expressed as mean±SEM, and *P*-values <0.05 were considered to be significant.

Results

CVID was associated with monocyte activation

The monocyte compartment was investigated in a previously described cohort of 31 patients with CVID (Table 1)[3].

CVID patients showed significantly higher levels of sCD14 than healthy subjects (Figure 1a), suggesting significant levels of monocyte activation[6,29].

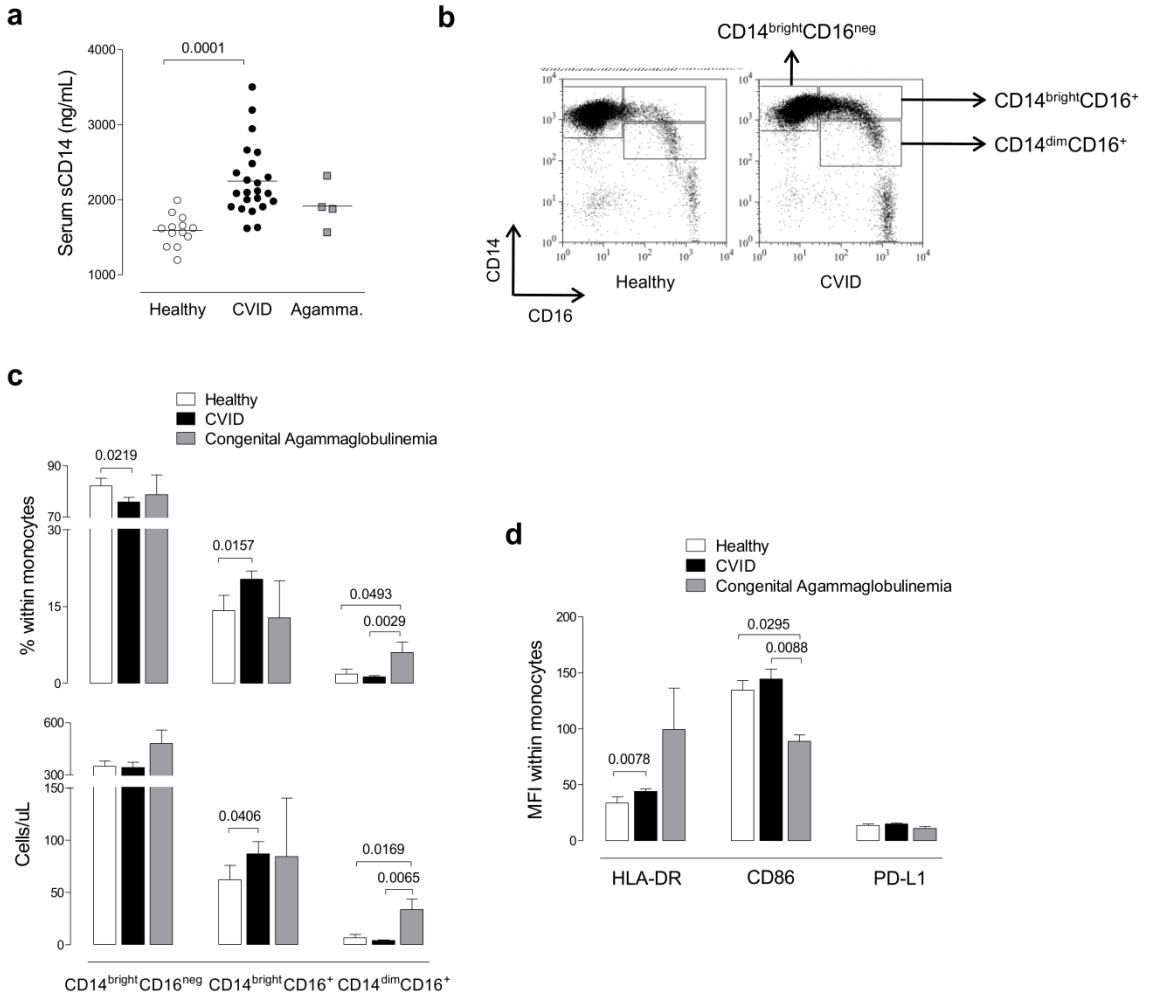

Figure 1. Monocyte activation markers in CVID and Congenital Agammaglobulinemia. (A) Serum levels of soluble CD14 (sCD14). Each dot represents one individual, with bars indicating mean values. Open circles refer to healthy individuals, solid circles to CVID patients, and grey squares to Congenital Agammaglobulinemia (Agamma) patients. (B) Illustrative flow cytometric analysis of monocyte subsets according to CD14 and CD16 expression levels in representative CVID (right panel) and healthy (left panel) individuals. (C) Frequency and absolute counts of monocyte subsets as defined in (B). (D) Mean fluorescence intensity (MFI) of HLA-DR, CD86 and PD-L1 within total monocytes. Healthy individuals are represented in open, CVID patients in solid, and Congenital Agammaglobulinemia patients in grey bars. Bars indicate mean±SEM. P values of statistically significant differences are shown.

Monocyte subsets were defined by flow cytometric whole blood analysis through the differential expression of CD14 and CD16, as illustrated in Figure 1b. Although the number of total circulating monocytes in CVID patients was similar to age-matched healthy individuals (493.3±35.81 cells/uL in controls vs. 504.7±40.25 cells/uL in CVID patients; p=0.9810), we found a significant increase in both the frequency and absolute numbers of $CD14^{bright}CD16^{+}$ monocytes in CVID patients, in comparison with controls (Figure 1c), a subset associated with inflammatory settings[11-15,30].

In agreement with a state of monocyte activation in CVID patients, the levels of expression of HLA-DR within total monocytes were significantly higher than in controls (Figure 1d), mainly due to increased levels of HLA-DR within the expanded $CD14^{bright}CD16^{+}$ monocyte subset (MFI in healthy individuals 133.0±11.34 vs. 187.2±13.28 in CVID patients; p=0.0052). Conversely, no significant alterations in the expression levels of the co-stimulatory molecule CD86 were found in CVID patients (Figure 1d).

We found no increase in the expression levels of PD-L1 on monocytes in CVID patients, as compared to healthy subjects (Figure 1d), suggesting that this inhibitory pathway was probably not significantly induced in CVID, despite the evidence for immune activation.

We also investigated the monocyte compartment in a group of patients with Congenital Agammaglobulinemia (Table 1), that we have previously described[3], who lack circulating B cells due to defects in early B-cell development, and found no significant increase in serum sCD14 or in the frequency of $CD14^{bright}CD16^{+}$ monocytes in these patients (Figure 1a and 1c).

Btk deficiency, a main cause of Congenital Agammaglobulinemia, has been shown to be associated with an increased frequency of CD16-expressing monocytes[24], but no distinction was made, at that time, between $CD14^{brigth}CD16^{+}$ and $CD14^{dim}CD16^{+}$ subsets, which are believed to possess distinct functional properties[30]. We found that the increase in CD16 was restricted to $CD14^{dim}CD16^{+}$ monocytes (Figure 1c). This subset was significantly expanded in patients with Congenital Agammaglobulinemia, both in terms of frequency and absolute counts, in relation to both healthy individuals and CVID patients (Figure 1c).

Although the results should be cautiously interpreted given the small number of patients with Congenital Agammaglobulinemia evaluated, our data showed that, in contrast to CVID, there was no expansion of the $CD14^{bright}CD16^{+}$ subset and no increase in sCD14 levels.

Overall, we provided evidence for significant levels of monocyte activation being a feature of CVID.

Monocyte activation was unrelated to plasma LPS levels in CVID

Our findings of no up-regulation of markers of monocyte activation in patients lacking B cells raise the possibility that factors other than defective mucosal IgA production, and the possible related increase in microbial translocation, are contributing to the CVID-associated monocyte activation.

CVID patients showed low to undetectable levels of plasma LPS, a bacterial product that increases in the peripheral blood as a result of microbial translocation in the gut (Figure 2a). Moreover, CVID patients with gastrointestinal manifestations, such as chronic diarrhea or evidence of mal-absorption, did not feature higher levels of plasma LPS than healthy controls (Figure 2a).

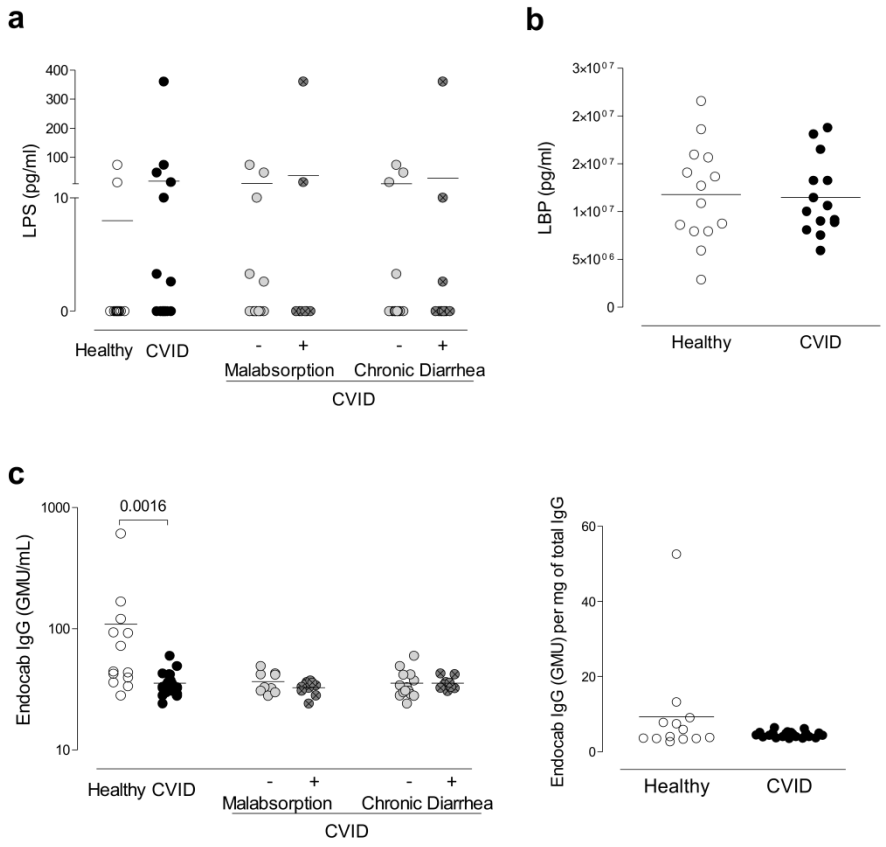

Figure 2. Plasma LPS levels and related molecules in CVID. (A) Plasma LPS levels. (B) Serum LBP levels. (C) Anti-LPS antibodies (left) and its ratio relative to total IgG (right). Each dot represents one individual: healthy individuals are represented in open circles, CVID patients in solid circles. Bars indicate mean. P values of statistically significant differences are shown.

We also confirmed that monocytes from CVID patients were not impaired in their ability to produce TNF-α upon *in vitro* LPS stimulation (percentage of monocytes expressing TNF-α: 55.75±3.56 in 26 CVID patients and 65.96±4.24 in 8 healthy controls; p=0.2820), as previously reported[18]. These data showing that monocytes from CVID patients were not refractory to

additional LPS stimulation *in vitro* further suggest a lack of previous exposure to increased plasma LPS.

We measured the levels of LPS-binding protein (LBP), a molecule that binds circulating LPS[29], and found them to be similar in healthy individuals and CVID patients (Figure 2b), further suggesting that the degree of microbial translocation was not substantial.

Another important pathway contributing to LPS clearance from circulation involves antibodies against the LPS core oligosaccharide (endotoxin core antibodies - EndoCAb)[6]. It is possible that IgG replacement therapy provides adequate amounts of EndoCAb levels, restoring the humoral response to LPS that patients are likely unable to mount.

We measured EndoCAb IgG levels in 5 IgG lots administered to the patients (2 lots of intravenous Octagam® 5%, 2 lots of subcutaneous Vivaglobin® 16%, and 1 lot of subcutaneous Gammanorm® 16,5%) and found these antibodies to be present in significant amounts (values between 120 GMU (IgG median units)/mL and 250 GMU/mL). Additionally, the amount of EndoCAb IgG within total IgG was similar in the serum of CVID patients and healthy individuals, even though total serum IgG was significantly lower in CVID patients than in controls (Figure 2c). All patients assessed were under IgG replacement therapy, with the exception of one, who still presented detectable amounts of EndoCAb IgG (29,78 GMU/mL; total IgG 473 mg/dL and EndoCAb IgG within total IgG 6,13 GMU/mg).

Thus, given the reduced plasma LPS and the unaltered serum LBP levels that we documented in CVID patients, IgG replacement therapy might be sufficient to limit the levels of LPS in peripheral blood. These results are against a major role of the gut-associated microbial translocation to the documented monocyte activation.

In summary, the alterations observed in the monocyte populations from CVID patients seemed to be unrelated to plasma LPS levels.

Monocyte activation was directly associated with T-cell disturbances in CVID patients

We reported above that, in contrast to CVID, patients with Congenital Agammaglobulinemia had no increase in markers associated with monocyte activation. Our results are particularly interesting since we had previously reported the lack of T-cell activation and significant T-cell imbalances in these patients with Congenital Agammaglobulinemia[3].

Accordingly, in CVID patients the expansion of $CD14^{bright}CD16^{+}$ monocytes was directly associated with T-cell activation, and inversely correlated with the frequency of naive CD4 T cells (Figure 3). The expansion of this population in CVID patients was also associated with the frequency of IFN-γ-producing CD4 T cells (Figure 3), another measure of T-cell activation that we

have previously shown to be significantly increased in this CVID cohort in comparison to healthy individuals[3].

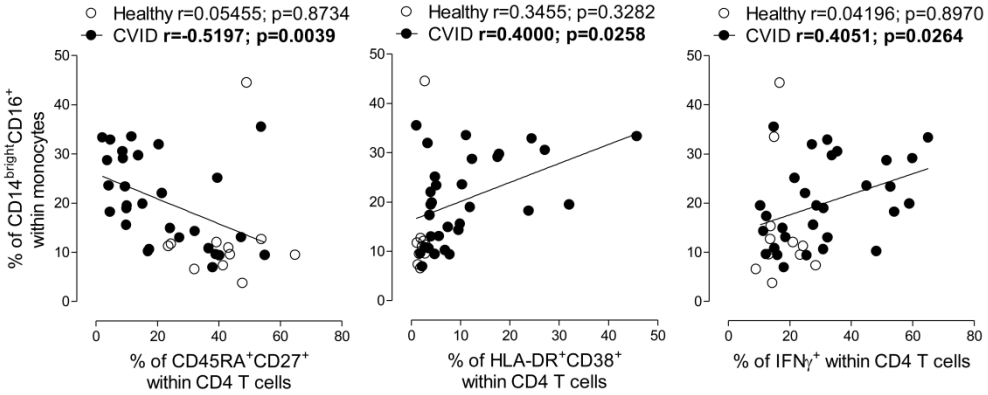

Figure 3. Relationship between markers of monocyte and T-cell activation in CVID. Correlation between the frequency of $CD14^{bright}CD16^{+}$ monocytes and the frequency of naive ($CD45RA^{+}CD27^{+}$, left panel), activated ($HLA\text{-}DR^{+}CD38^{+}$, middle panel), or IFN-γ-producing cells (right panel) within CD4 T cells in CVID patients (solid circles) and in healthy individuals (open circles). Each dot represents one individual. Spearman's correlation coefficients are shown.

Importantly, as described above, we found no significant up-regulation of the inhibitory molecule PD-L1 in CVID patients as compared to healthy subjects, both within whole monocytes (Figure 1d) and in the three monocyte subsets (data not shown). We further investigated a possible association between PD-L1 levels in monocytes and T-cell imbalances and found that PD-L1 levels within the expanded $CD14^{bright}CD16^{+}$ monocyte subset increased in direct correlation with CD4 T-cell activation (r=0.6056, p=0.0003), and the loss of naive CD4 T cells (r=-0.5202, p=0.0038) in CVID patients. Nevertheless, PD-L1 up-regulation in the $CD14^{bright}CD16^{+}$ monocyte subset of CVID patients was apparently not as marked as that observed in other persistent inflammatory settings, like HIV-1 infection[31-33], suggesting an impairment of the PD-L1 pathway in CVID, possibly translating into a reduced ability to control immune activation. This is particularly relevant given the fact that the PD-1/PD-L1 pathway is involved in peripheral tolerance and autoimmunity[34] and that the expansion of $CD14^{brigth}CD16^{+}$ monocytes was significantly higher in CVID patients with autoimmune disease (p=0.0328). Of note, this expansion was not more marked in CVID patients with granuloma or lymphoproliferation (p>0.05). Additionally, significant correlations were also observed between the expression levels of the co-stimulatory molecule CD86 within $CD14^{bright}CD16^{+}$ monocytes and T-cell imbalances (r=0.3706, p=0.0402 with CD4 T-cell activation; r=-0.4300, p=0.0199 with the frequency of naive CD4 T cells) in CVID patients, reinforcing the relevance of addressing the regulation of co-stimulatory and inhibitory pathways in future CVID studies.

Altogether, our data showed that monocyte activation in CVID was directly associated with markers of T-cell activation.

CVID patients grouped according to the EuroClass classification featured distinct monocyte imbalances

B-cell imbalances have been used as an attempt to classify CVID patients into more homogeneous subgroups. The EuroClass classification has thus been proposed based in three main B-cell abnormalities observed in CVID: the reduction in switched-memory B cells, and the expansions of either transitional or $CD21^{low}CD38^{low}$ B cells[35]. When our cohort was stratified according to the EuroClass, we found no differences regarding CD86 expression within $CD14^{bright}CD16^{+}$ monocytes among the different subgroups ($p>0.1900$). However, PD-L1 expression within this subset was significantly higher in patients with reduced switched-memory B cells and expansion of transitional B cells ($smB^{-}Tr^{hi}$) as compared to those that did not present that expansion ($smB^{-}Tr^{norm}$) (Figure 4a).

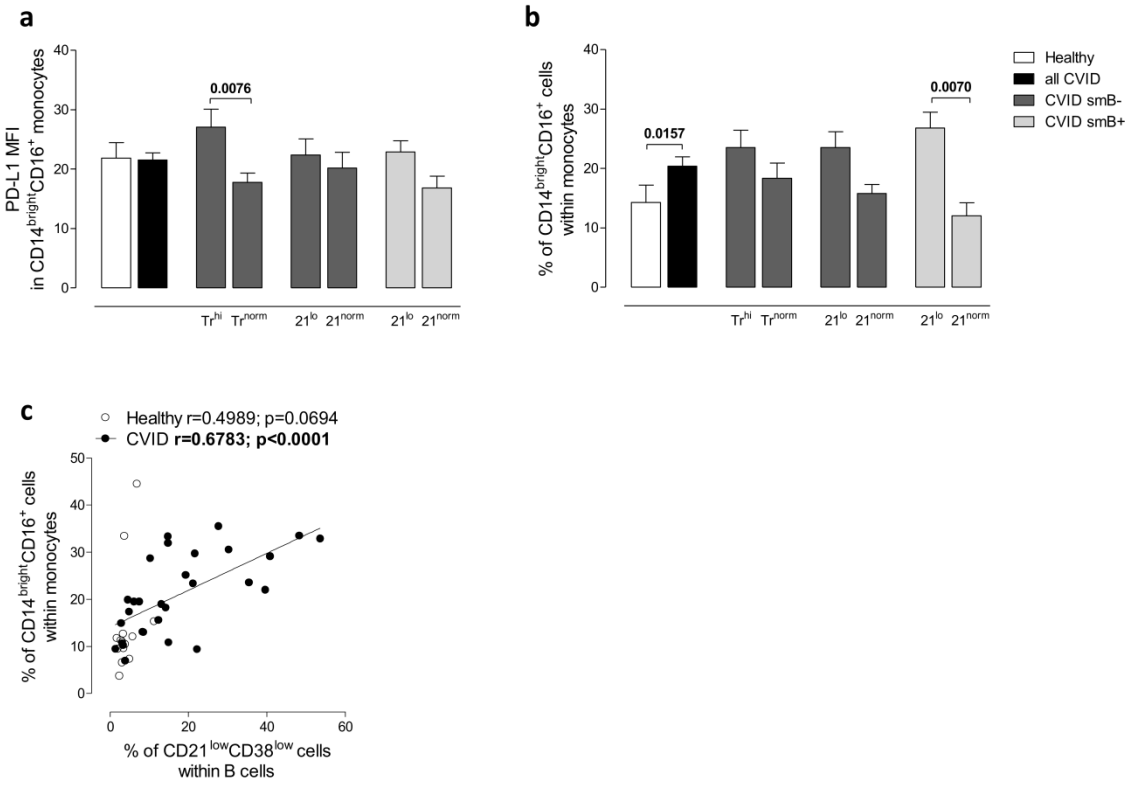

Figure 4. Markers of monocyte activation in CVID grouped according to the EuroClass classification. (A) Mean fluorescence intensity (MFI) of PD-L1 within $CD14^{bright}CD16^{+}$ monocytes. Healthy individuals are represented in open and CVID patients in solid bars. (B) Frequency of $CD14^{bright}CD16^{+}$ monocytes. Bars indicate mean±SEM. P values of statistically significant differences are shown. (C) Correlation between the frequency of $CD14^{bright}CD16^{+}$ monocytes and the frequency of $CD21^{low}CD38^{low}$ B cells in CVID patients (solid circles) and in healthy individuals (open circles). Each dot represents one individual. Spearman's correlation coefficients are shown.

In addition, we also found that the expansion of $CD14^{bright}CD16^{+}$ monocytes was particularly relevant in patients that did not have reduced switched-memory B cells but had expanded $CD21^{low}CD38^{low}$ B cells ($smB^{+}21^{lo}$) as compared to patients that did not ($smB^{+}21^{norm}$) (Figure 4b). In agreement, the frequency of $CD14^{bright}CD16^{+}$ monocytes was directly associated with the frequency of $CD21^{low}CD38^{low}$ B cells in CVID patients (Figure 4c).

In conclusion, we showed that monocyte imbalances were particularly marked in CVID patients lacking switched-memory B cells and/or having an abnormal expansion of the $CD21^{low}CD38^{low}$ B cells.

Discussion

We showed here that CVID is associated with increased serum levels of sCD14 and an expansion of $CD14^{bright}CD16^{+}$ monocytes, in direct correlation with T-cell and B-cell imbalances. Moreover, monocyte hyper-activation occurred irrespective of plasma LPS levels, suggesting that monocytes may represent important targets for therapies complementary to IgG replacement aiming to control the granulomatous and lymphoproliferative manifestations in CVID.

Such monocyte activation markers were not observed in patients lacking peripheral B cells due to Congenital Agammaglobulinemia who, notably, also did not present major T-cell imbalances. Although the number of patients with Congenital Agammaglobulinemia was small, our results are in agreement with a previous study that also reported a lack of major alterations in monocyte function as assessed *in vitro* in patients with X-linked Agammaglobulinemia[18].

CD14 shedding from the membrane of monocytes is generally accepted as a marker of monocyte activation, with levels of sCD14 being increased in inflammatory and autoimmune diseases, in the context of acute inflammation and sepsis, as well as in HIV-infected patients[6,29]. $CD14^{bright}CD16^{+}$ monocytes have been shown to have a high inflammatory potential, based on transcriptome analysis[11,30], and to be expanded in inflammatory conditions[12,13], being associated with disease progression in chronic HIV-1 infection[14,15].

We showed that the expression of HLA-DR within total monocytes was significantly higher in CVID than in controls, compatible with a state of monocyte activation. This is in agreement with the study of Cambronero *et al*, which showed an up-regulation of HLA-DR within the whole monocyte population in CVID patients[18]. It is worth to note that this increase was mainly due to the expression of HLA-DR within the expanded $CD14^{bright}CD16^{+}$ monocyte subset, supporting its enhanced inflammatory capacity. On the other hand, expression levels of the co-stimulatory molecule CD86 were similar in CVID patients and in healthy individuals. CD86, a marker of monocyte differentiation and acquisition of antigen-presentation properties, has been shown to be expressed at reduced levels in monocyte-derived DCs generated *in vitro*, supporting an impaired function as antigen-presenting cells in CVID[16,36].

Monocytes may also up-regulate inhibitory molecules, which are thought to play an important role in the regulation of immune responses and in limiting immunopathology. PD-L1 (B7-H1) binds programmed death-1 molecule (PD-1), an inhibitory receptor expressed on many cells, particularly activated T and B lymphocytes, establishing a pathway of central relevance in inflammatory states[31,32]. Both PD-L1 and PD-1 can be up-regulated upon activation, with PD-1 expression on T cells having been associated with functional exhaustion in the context of chronic

viral infection, due to persistent antigen exposure[37-39]. Of note, PD-L1 expression has been shown to be up-regulated on monocytes from HIV-infected patients[31,32]. We found that monocyte activation in CVID was not accompanied by significant up-regulation of the inhibitory molecule PD-L1, which may favor the deleterious impact of activated monocytes.

In addition, we showed that monocyte imbalances were unrelated to plasma LPS levels in CVID patients, who appear to have a relatively good preservation of the pathways involved in LPS clearance, including anti-LPS antibodies provided by the IgG replacement therapy, and normal levels of LBP. Serum LBP quickly binds LPS molecules in circulation[29]. LBP levels have been shown to increase in sepsis associated with LPS-containing microorganisms (Gram-negative bacteria) and in HIV infection[6,29], in which case a direct correlation with the degree of microbial translocation, estimated by plasma LPS levels, has been reported[14]. Furthermore, both LPS and LBP levels are increased in inflammatory bowel disease, in correlation with disease activity, and recover to normal levels following treatment[40]. The fact that LBP levels were similar in healthy individuals and CVID patients, and that there was no increase in plasma LPS, further suggests that the degree of microbial translocation was not significant. Thus, our data raise the possibility that IgG replacement therapy may provide sufficient antibodies against LPS to control the putative increase in microbial translocation. Longitudinal studies assessing patients prior to and after commencing replacement therapy with IgG will be of great relevance to understand its potential role in regulating microbial translocation, which may have implications in other clinical settings, such as HIV/AIDS.

Additionally, we show that the expansion of $CD14^{bright}CD16^{+}$ monocytes was particularly relevant in CVID patients with autoimmunity and that the frequency of this monocyte population was directly correlated with the frequency of $CD21^{low}CD38^{low}$ B cells. The expansion of this B-cell subset has also been associated with autoimmunity in CVID[35], supporting a role for $CD14^{bright}CD16^{+}$ monocytes in the course of the disease. We should point out that our CVID cohort presents an unusually high frequency of autoimmune manifestations, probably related to a reference bias associated with an immunology department in a central hospital.

Although several studies have acknowledged the relevance of immune activation in CVID pathophysiology, little is known about what drives and sustains it, whether it is related to the increased frequency of infections, or to altered response to pathogens. Our data are in agreement with a possible multi-factorial event impacting on several compartments of the immune system. Irrespective of the monocyte alterations that we describe contributing to the inflammatory process itself or being a marker of underlying inflammation, our finding of major

monocyte imbalances in particular subgroups of CVID patients may help in defining new lines of investigation.

In conclusion, our data show that CVID was associated with monocyte alterations that directly correlated with T-cell activation markers and with B-cell imbalances, without a relationship to plasma LPS levels, supporting a potential clinical relevance of therapeutic strategies targeting monocytes to control the inflammatory manifestations in CVID patients.

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3.3 – SUPPLEMENT A

LONGITUDINAL STUDY OF COMMON VARIABLE IMMUNODEFICIENCY PATIENTS PRIOT TO AND AFTER STARTING IgG REPLACEMENT THERAPY

Our results concerning monocyte activation in CVID patients suggested that IgG replacement therapy could actually play a role in modulating microbial translocation and consequently influence the obtained results. In addition, virtually all published data on CVID immune imbalances relates to patients that are under IgG replacement therapy[1-4]. Therefore, it is very important to perform longitudinal studies in order to assess the possible modulation of different immunological parameters in CVID patients upon starting IgG replacement therapy. With this aim, four CVID patients were followed for a period of 12 months after starting IgG replacement therapy.

We found that the inter-subject variability was high at day 0 in all subjects studied. In relation to the monocyte imbalances that we reported in this chapter 3.3, we observed that the frequency of $CD14^{bright}CD16^{+}$ monocytes, which we found to be increased in treated CVID patients, tended to decrease with the introduction of IgG replacement therapy (Figure 1B). Scattered variations were also noted in $CD14^{bright}CD16^{-}$ and $CD14^{dim}CD16^{+}$ monocyte populations (Figure 1A and 1C). However, it does not seem to have an impact on the expression of HLA-DR by total monocytes (Figure 1D), suggesting that IgG replacement therapy may have an overall limited effect on monocyte activation as measured by these parameters.

In addition to being used as replacement therapy for B-cell immunodeficient patients, intravenous IgG therapy is also used for the treatment of inflammatory and autoimmune

diseases, where it has been shown to play an anti-inflammatory role by decreasing immune activation, among others[5]. Even though this effect has been shown to occur with much higher doses of intravenous IgG therapy than the ones that are given as replacement therapy to CVID patients, we aimed to understand if IgG replacement therapy could have an effect on overall T-cell activation and the naive/memory T-cell imbalances that are usually associated with CVID[6,7].

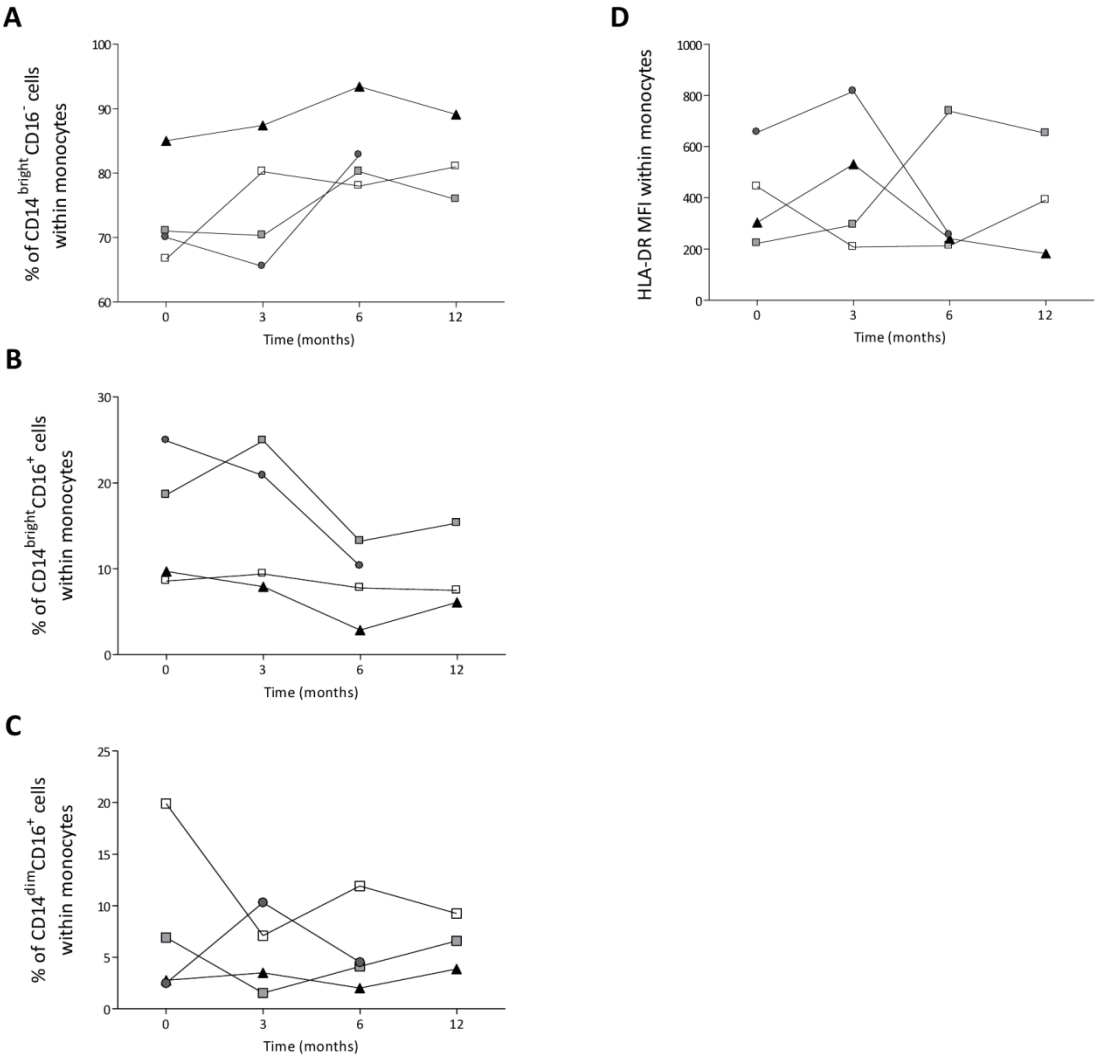

Figure 1. Monocyte populations and HLA-DR expression on monocytes in CVID patients, before and up to 12 months after starting IgG replacement therapy. (A to C) Frequencies of $CD14^{bright}CD16^{-}$ (A), $CD14^{bright}CD16^{+}$ (B), and $CD14^{dim}CD16^{+}$ monocytes (C) and (D) HLA-DR mean fluorescence intensity (MFI) of expression within total monocytes, in four CVID patients before (time 0) and after starting IgG replacement therapy.

We observed that the levels of T-cell activation were unchanged over time, both in CD4 and CD8 T cells (Figure 2A and 2B, respectively). In addition, the frequency of naive T cells, both within CD4 and CD8 T cells, was remarkably stable, regardless of the inter-subject variability (Figure 2C and 2D, respectively). Also, the frequency of terminally-differentiated CD8 T cells ($CD45RA^+CD27^-$), which is significantly increased in CVID patients[6,7] (see Chapter 3.2 of results), is very regular over time in the different CVID patients (Figure 2E). Importantly, the frequency of circulating $CXCR5^+$ CD4 T cells, which we have found to be severely reduced in patients with Congenital Agammaglobulinemia[6], was remarkably constant (Figure 2F) and significantly increased in CVID patients, as compared to healthy individuals (Figure 2G). This raises the hypothesis that, contrary to what happens with Congenital Agammaglobulinemia patients, who have severely impaired GC formation, CVID patients present a significant increase in the circulating population of follicular $CXCR5^+$ CD4 T cells, possibly related to the impaired function of GCs and their hypercellularity[8]. These results are particularly interesting given the recent results on TACI-deficient mice showing that these animals have expanded populations of T_{FH} cells and GC B cells, when immunized with T-dependent antigens[9]. AS previously mentioned, TACI deficiency is reported to be associated with ~10% of CVID cases[10].

Concluding Remarks

Overall, our data on the longitudinal assessment of CVID patients starting IgG replacement therapy suggests that the introduction of therapy was not associated with major immune alterations, and that the immune imbalances were stable and likely related to the clinical course of CVID patients.

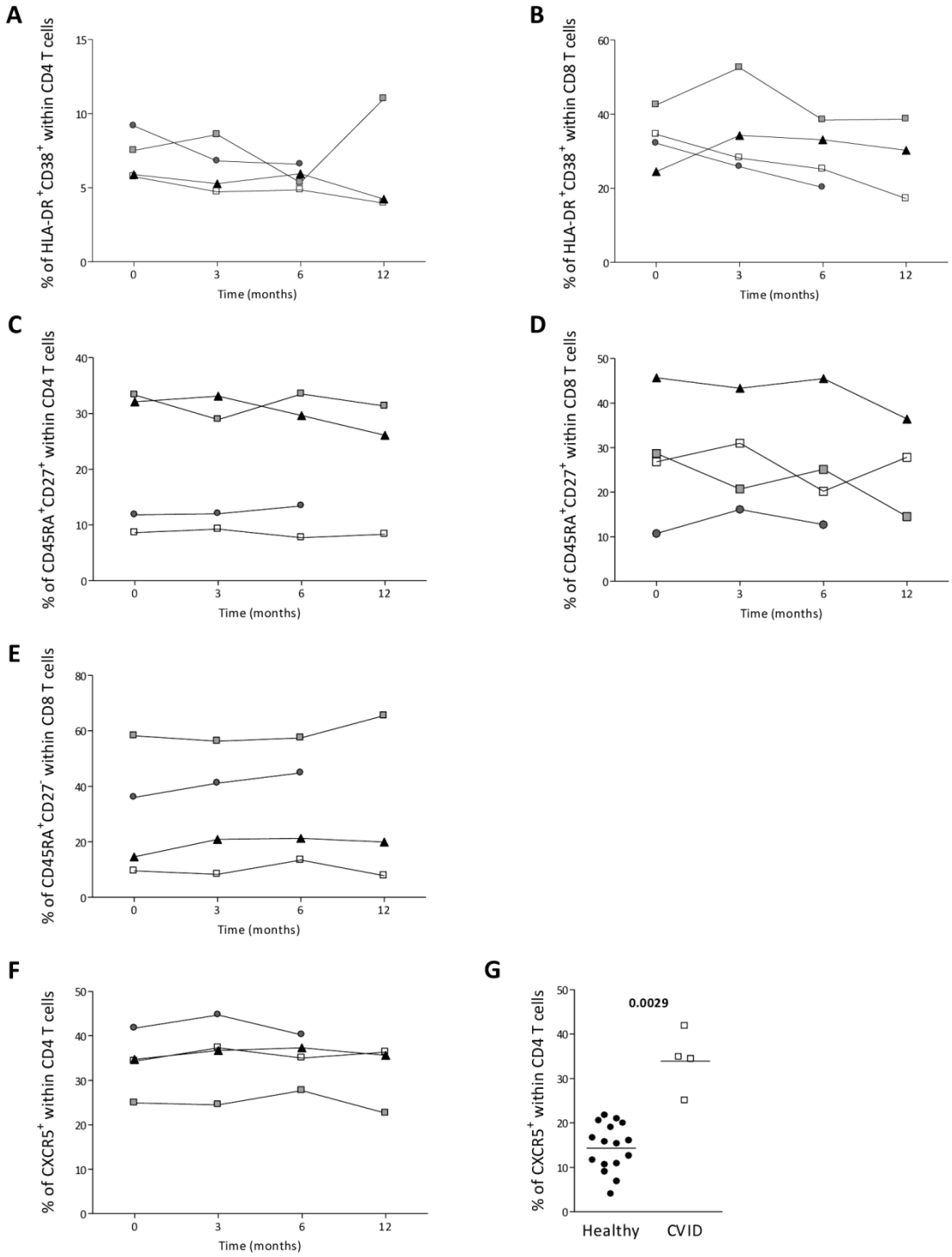

Figure 2. T-cell populations in CVID patients before and up to 12 months after starting IgG replacement therapy. Frequencies of activated (HLA-DR$^+$CD38$^+$) CD4 (A) and CD8 (B) T cells, naive (CD45RA$^+$CD27$^+$) CD4 (C) and CD8 (D) T cells, terminally-differentiated (CD45RA$^+$CD27$^-$) CD8 T cells (E), and CXCR5$^+$ CD4 T cells (F),), in four CVID patients before (time 0) and after starting IgG replacement therapy. (G) Circulating CXCR5$^+$ CD4 T cells in healthy individuals (filled circles) and CVID patients (open squares). Each symbol represents one individual. Bars represent the mean value. Data were compared using the Mann-Whitney test, and *p* values are shown.

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CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVES

The overall aim of this work was to study two clinical settings of primary B-cell immunodeficiencies, CVID and Congenital Agammaglobulinemia, in order to better understand the impact of B-cell dysfunction on the immune system. Ultimately, the study of these human clinical models was aimed not only to expand our knowledge on the function of the immune system as a whole, but also to help provide better tools to treat these patients.

In summary, we found that *ex vivo* BAFF-R expression was reduced in CVID patients, particularly in those that present very low frequencies of memory B cells. In contrast, TACI expression was significantly increased. Both the decrease in BAFF-R expression and the increase in TACI were directly associated with high serum levels of BAFF found in the patients. *In vitro* studies showed that BAFF induced BAFF-R down-regulation, both in healthy individuals and in CVID patients. However, the degree of this modulation was impaired in CVID patients, suggesting that these dynamics are affected, with a possible impact in B-cell homeostasis. We also observed that CVID was associated with monocyte activation unrelated to plasma LPS levels, but in direct association with T-cell activation and B-cell imbalances. The search to understand the mechanisms underlying the increase in inflammatory manifestations observed in CVID patients led us to study the contribution of IL-17, a major pro-inflammatory cytokine implicated in autoimmunity and inflammatory conditions. However, no increase in T_H17 cells was found in CVID and their frequency was inversely correlated with markers of GC dysfunction. T_H17 cells were severely reduced in Congenital Agammaglobulinemia and directly associated with switched-memory B cells in healthy subjects, which raises important questions regarding the impact of B-cell depleting therapies on this population.

In section 1 of the chapter of Results, we showed that BAFF-R and TACI expression were altered in CVID patients, likely contributing to disease pathogenesis. It will be extremely useful to understand whether altered receptor expression translates into altered ligand-binding and consequently to perturbed ligand responsiveness. This can be achieved by studying BAFF-binding capacity in B cells from CVID patients with different levels of BAFF-R expression. We have also hypothesized that BAFF can modulate the levels of BAFF-R in a physiological context. Our data showing that BAFF-R expression decreases following 5 minutes of stimulation with BAFF is suggestive that BAFF-R is being internalized, but further experiments are necessary in order to

better evaluate this point, namely through the use of inhibitors of endocytosis. It will also be important to understand, in case internalization occurs, what is the fate of internalized BAFF-R. Whether it is a target for degradation or it recycles back to the cell membrane may have different biological significances and outcomes. This is particularly relevant given the fact that we found high BAFF serum levels in CVID patients, together with low BAFF-R expression on B cells. A scenario where BAFF ligation is targeting BAFF-R to degradation could explain our observations and imply that B cells from CVID patients are consequently less responsive to BAFF stimulation. However, a previous report has suggested, although through a distinct experimental approach, that this may not be the case[1]. Another possibility that can be responsible for the down-regulation of BAFF-R upon BAFF binding is that BAFF-R is being cleaved and shed into the extracellular space. This is a rather interesting possibility, since it would prevent BAFF from acting on its target B cells. BAFF-R shedding could then act as a buffer in situations where excess BAFF is present. This mechanism has been described to occur for TNF receptors and to be critical for the regulation of TNF activity *in vivo*[2]. Our results have thus revealed a new possibility in which BAFF regulates the levels of its receptor, BAFF-R. Understanding the mechanisms involved in this modulation will be particularly relevant to discern whether BAFF-depleting therapies[3] may have a place in the treatment of CVID. In the case of applying such therapies in CVID patients, the main aim would not probably be the total depletion of circulating BAFF but rather the elimination of the excess BAFF, in order to allow B cells to regain a normal response to this homeostatic cytokine. In this sense, BAFF-depleting therapies would have to be tailored to each patient.

In section 2 of the chapter of Results, we reported that the frequencies of T_H17 cells and switched-memory B cells are directly associated in healthy subjects, and that Congenital Agammaglobulinemia patients, who lack peripheral B cells, have a severe reduction of this CD4 T-cell population. In addition, in CVID patients, who present primary defects in mature B-cell differentiation, the frequency of T_H17 cells was inversely correlated with markers of GC dysfunction, suggesting that B cells play a role in the homeostasis of the T_H17 subset. These results raise important questions in what concerns the use of B-cell depleting therapies in clinical contexts where T_H17 have a pathological role, and the beneficial impact that these therapies can by affecting this population. Concerning our findings of markedly reduced frequencies of IL-17-producing CD4 T cells in patients with Congenital Agammaglobulinemia, it will be of greater importance to understand whether this is due to intrinsic T-cell defects or to a lack of adequate interactions *in vivo*. Our preliminary data on the assessment of the ability of naïve CD4 T cells

from Congenital Agammaglobulinemia patients to differentiate under T_H17-polarizing conditions suggest that there is actually a cell-specific defect, but this point needs further evaluation. In the case this cell-specific defect is confirmed, it will be important to understand which phase of T_H17 differentiation is affected: induction, amplification or survival. Given that each of these phases has different molecular requirements, identifying which one is affected would shed light into the molecular mechanism underlying the differentiation defect. However, the recent data showing that B-cell depletion with rituximab in rheumatoid arthritis patients specifically inhibits the T_H17 response, without affecting other T-cell subsets[4], appear to be against the view of Congenital Agammaglobulinemia patients having a cell-intrinsic defect in T_H17 differentiation, and rather suggest that B cells themselves are involved in the development of T_H17 responses. Nevertheless, we can also hypothesize that the fact that CD4 T cells from Congenital Agammaglobulinemia have not been exposed to interaction with B cells makes them less prone to develop into the T_H17 population.

Regarding the data we have generated on T_{reg} cells, we show that the frequencies of T_{reg} cells and T_H17 cells are directly associated in CVID patients. These data support the view that the impairment of the GCs found in CVID may have an impact in both populations. In addition, we report dissociation between FOXP3 and CD25 expression in CVID patients, with severely impaired expression of CD25 in the presence of normal frequencies of $FOXP3^+$ CD4 T cells. It will be important to clarify the putative T_{reg} disturbances in CVID [5-8]. In what concerns our data, we can hypothesize that T_{reg} cells are reduced, in which case expression of FOXP3 is mainly related to activation. However, the fact that we found no difference in the MFI of FOXP3 expression would be against this view, since high levels of FOXP3 expression are regarded as a good estimate of T_{reg} function[9]. Nevertheless, the fact that CVID patients with autoimmunity, lymphoid proliferation or chronic diarrhoea present a significant decrease in FOXP3 MFI may be pointing to a true decrease of T_{reg} cells, at least in some groups of CVID patients. We can also put forward a different picture where T_{reg} cells are not reduced in CVID, but their suppressive function is affected by the loss of CD25 expression. The expansion of $FOXP3^+CD25^-$ CD4 T cells, which has also been described in SLE with conflicting data regarding their suppressive function[10-14], seems to be in favour of this hypothesis. A third situation can also be envisaged, in which T_{reg} cells are not reduced in CVID and there is also no loss of suppressive function. This would mean that the low levels of CD25 expression would not impact on T_{reg} function and would be likely related to either lack of appropriate stimulation *in vivo* or to an intrinsic defect to respond to, for instance, TCR stimulation or γc cytokines.

Finally, in section 3 of the chapter of Results, we showed that CVID, contrary to Congenital Agammaglobulinemia, was associated with monocyte imbalances that directly correlated with T-cell activation markers and with B-cell imbalances, without an association with plasma LPS levels. A very recent report has corroborated our data, also showing that CVID patients present increased serum levels of sCD14 in direct correlation with T-cell activation, in the absence of increased levels of LPS[15]. We thus describe monocyte activation as being a feature of CVID, and hypothesize that a possible modulation of microbial translocation may be taking place by the action of the IgG replacement therapy. To properly evaluate this possibility, it will be crucial to assess the levels of LPS, LBP, sCD14 and anti-LPS antibodies, together with other more suitable indicators of microbial translocation, such as bacterial 16S ribosomal DNA, in the group of CVID patients that are being followed longitudinally prior to and after commencing IgG replacement therapy. Only through the study of these patients we will be able to ascertain whether IgG replacement therapy has a role in these processes. However, this seems to be an unlikely possibility, given the fact that we found a limited impact of IgG replacement therapy in parameters related to monocyte and T-cell activation in our longitudinal follow-up of CVID patients starting therapy, together with little impact on B- and T-cell compartments. It does seem that the immune imbalances that CVID patients present are stable and likely related to the clinical course of the disease, with little influence from the IgG replacement therapy.

The schematic diagram shown in Figure 1 summarizes the immune system disturbances that we observed in primary B-cell immunodeficiencies. In Congenital Agammaglobulinemia patients, who lack B cells, we do not observe major alterations in the monocyte compartment, while the impairments in T cells appear to be restricted to a reduction in the levels of circulating T_H17 and T_{FH} cells. In contrast, in CVID patients, who have B cells that are dysfunctional, both lymphoid and myeloid compartments are affected. CVID patients present both monocyte and T-cell activation, in direct association with B-cell imbalances. In addition, our preliminary data also suggest that CVID patients have increased frequencies of circulating T_{FH} cells, underpinning the role of disturbed GC function in CVID pathogenesis.

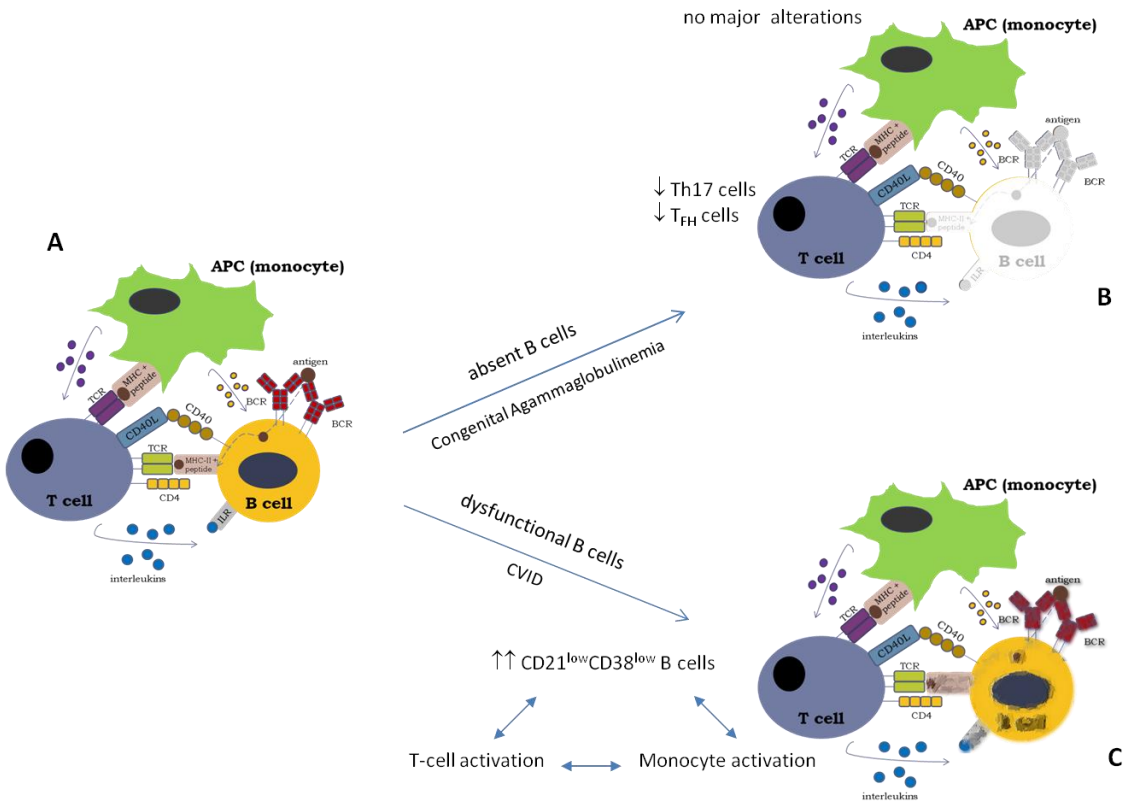

Figure 1. Simplified schematic representation of the disturbances of the immune system associated with primary B-cell immunodeficiencies. (A) Interaction between T cells, B cells and antigen-presenting cells (APC) in the normally-functioning immune system. (B) In the absence of B cells, as exemplified by Congenital Agammaglobulinemia patients, no major alterations are seen in the monocyte (APC) compartment, and imbalances at the level of T cells appear to be confined to a reduction in circulating T_H17 cells and T_{FH} cells. (C) When B cells are present but dysfunctional, as in the case of CVID patients, monocyte activation is observed, in direct association with T-cell activation and with the expansion of pathogenic $CD21^{low}CD38^{low}$ B cells.

Additionally, while patients with Congenital Agammaglobulinemia have clinical manifestations that are mostly related to the increased susceptibility to infections derived from the absence of B cells and antibody production, CVID patients have a much more complex clinical picture, with a high prevalence of autoimmunity and lymphoproliferation[16]. These clinical manifestations are sometimes difficult to treat and consequently there is a need for improved therapeutic strategies.

Given the plethora of clinical complications and immune alterations present in CVID patients[17], it can be argued that allogeneic haematopoietic stem cell transplantation (HSCT) might actually be considered a curative strategy. By wiping out the old compromised immune system and replacing it with a new one, normal immunity in CVID patients might be restored. HSCT has been reported as a treatment for CVID patients in the case of malignancy, for which patients are at a high risk[16]. Quite recently, the outcome of HSCT performed in four CVID patients, either for malignancy or for severe CVID with imminent organ failure, has been

reported, with promising results[18]. Even though only one of the four treated CVID patients has been clinically "cured", resolving all CVID-related complications, including Ig production, in addition to the lymphoma for which the patient received allogeneic HSCT, the proof of principle has been made[18]. HSCT can thus be envisioned as a curative approach, even if great care must be taken when selecting patients and donors for this procedure. HSCT implies the use of conditioning, even if at low doses. In addition, graft-versus-host disease is always a risk[19]. Consequently, only patients with severe clinical manifestations that are refractory to conventional treatments should be considered, but it should also be bore in mind that once serious organ damage has occurred, HSCT has very little potential to revert the situation. Given the great improvements that have been made in HSCT, both in terms of conditioning and the treatment of complications arising from transplantation, HSCT may be considered a true option in the treatment of CVID in the near future. Nevertheless, HSCT should only be regarded in a limited number of cases, and so the need for therapeutic strategies that may target the complexity of immune defects present in CVID still prevails.

In conclusion, we have shown that both B-cell intrinsic and extrinsic defects are underlying the pathogenesis of CVID. B-cell homeostasis is likely impaired, given that expression of BAFF-R is significantly reduced, while TACI expression is increased in CVID patients, both of which in direct correlation with high serum levels of BAFF. These data raise the possibility of BAFF-depleting therapies being envisaged in the treatment of CVID. In addition, we have reported that the homeostasis of the T_H17 population is related to B-cell maturation, illustrated by the severe reduction of T_H17 cells in patients with Congenital Agammaglobulinemia, with implications for the role of B-cell depleting therapies in this population. Finally, the finding of increased levels of monocyte activation in CVID patients that are directly associated with T-cell activation points out the need to design therapeutic strategies that target immune activation, in order to achieve better treatments for non-infectious complications.

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