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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica com especialização em Bioquímica Clínica, realizada sob a orientação científica do Doutor Artur Augusto Paiva, coordenador da Unidade de Gestão Operacional de Citometria/Serviço de Patologia Clínica dos CHUC equiparado a Professor Adjunto da ESTES Coimbra e do Doutor Pedro Miguel Dimas Neves Domingues, Professor Auxiliar do Departamento de Química da Universidade de Aveiro

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Palavras-chave: Gamapatias Monoclonais; Gamapatia Monoclonal de Significado Indeterminado; Mieloma Múltiplo; Células T Foliculares.

Resumo

As gamapatias monoclonais constituem um grupo de doenças, como a gamapatia monoclonal de significado indeterminado e o mieloma múltiplo (MM), que resultam da proliferação de células plasmáticas clonais. As células plasmáticas diferenciam-se a partir de linfócitos B que sobreviveram ao processo de maturação por afinidade no centro germinativo e que depois migraram para a medula óssea. Especula-se que o evento responsável pela transformação das células plasmáticas saudáveis em células patológicas ocorre no centro germinativo, provavelmente durante o processo de hipermutações somáticas. A manutenção dos centros germinativos, bem como a seleção de células B de elevada afinidade, depende das células T, especialmente do subtipo células T foliculares. Este estudo teve como objetivo a análise das diferentes populações de células T na medula óssea de doentes com gamapatias monoclonais, particularmente as células T foliculares. Recorrendo à técnica de citometria de fluxo, analisámos as subpopulações de células T $CD4^+$, $CD8^+$, $\gamma\delta^+$, $CD4^-CD8^-$ $\alpha\beta^+$ e $CD4^+CD8^+$. As células com fenótipo folicular foram identificadas com base na expressão de CXCR5 e as células T ativadas de acordo com a expressão de CD25 ou HLA-DR. Os nossos resultados mostraram um aumento na frequência das células T com fenótipo folicular nestes doentes, obtendo significado estatístico no subconjunto das células T reguladoras com fenótipo folicular, quando comparados os grupos com MM e controlo. Contudo, a frequência de células T $\gamma\delta^+$ com fenótipo folicular estava diminuída em todos os grupos com MM. Curiosamente, observou-se uma diminuição na frequência de células T com fenótipo folicular ativadas. Em suma, apesar de as células T com fenótipo folicular se apresentarem tendencialmente aumentadas em doentes com gamapatias monoclonais, estas parecem estar menos ativadas, o que sugere que estas células se encontram irresponsivas ao microambiente medular ou, por outro lado, estão a ser reguladas negativamente. Atendendo a que a nossa amostra de estudo era limitada, estudos adicionais são necessários para estabelecer o papel das células T foliculares na medula óssea e no microambiente tumoral de doentes com gamapatias monoclonais.

Keywords: Monoclonal Gammopathies; Monoclonal Gammopathy of Undetermined Significance; Multiple Myeloma; Follicular-like T Cells.

Abstract Monoclonal gammopathies result from the proliferation of a single clone of plasma cells (PCs) and include disorders such as monoclonal gammopathy of undetermined significance (MGUS) and Multiple Myeloma (MM). It is speculated that the triggering event that turns healthy PCs into pathological PCs happens in the germinal centre (GC), most likely during a process known as somatic hypermutation (SHM), which takes place during antigen affinity maturation. GC maintenance, as well as, GC B-cell selection depends on T cell help, particularly on follicular T cells. In this study we aimed to analyse different T cell populations in the BM microenvironment of patients with monoclonal gammopathies, focusing on follicular-like T cells. Through multiparameter flow-cytometry we analysed $CD4^+$, $CD8^+$, $\gamma\delta^+$, $CD4^-CD8^-$ $\alpha\beta^+$ and $CD4^+CD8^+$ T cells and identified follicular-like cells based on the expression of CXCR5, as well as activated T cells according to CD25 and HLA-DR expression, in all T cell subpopulations. We observed a general increase of follicular-like T cells in these patients, reaching statistical significance in the Th reg follicular-like cell subset when comparing the MM group with controls. The $\gamma\delta^+$ follicular-like T subset was, however decreased in all MM groups. Interestingly, a decrease in activated follicular-like T cells was observed. In conclusion, despite of follicular-like T cells being increased in monoclonal gammopathy patients, they tend to less activated, which suggests that these cells are either not responding to the BM microenvironment or being negatively regulated. Our study population was small so further studies concerning follicular-like T cells in the BM of patients with monoclonal gammopathies may help understand the role of these cells in the BM microenvironment.

Abbreviations and Acronyms

Ab – Antibody
Ag – Antigen
AICD – Activation-induced Cell Death
ADCC – Antibody-dependent Cellular Cytotoxicity
APC – Antigen Presenting Cell
BM – Bone Marrow
BMPC – Bone Marrow Plasma Cell
CLP – Common Lymphoid Progenitor
CRAB – Hypercalcemia, Renal Insufficiency, Anaemia and Bone Lesions
CSR – Class Switch Recombination
CT – Computed Tomography
DC – Dendritic Cell
DN – Double Negative
DNA – Deoxyribonucleic Acid
DP – Double Positive
EMA – European Medicines Agency
FDA – Food and Drugs Administration
FDC – Follicular Dendritic Cell
Fgl2 - Fibrinogen-like Protein 2
FLC – Free Light Chains
FOXP3 – Forkhead Box P3
FSC – Forward Scatter
GC – Germinal Centre
HDM – House Dust Mite
HIV – Human Immunodeficiency Virus
HSC – Hematopoietic Stem Cell
Hsp – Heat-shock Protein
ICOS – Inducible T-cell Co-stimulator
IFN – Interferon
Ig – Immunoglobulin
IL – Interleukin
IMGW – International Myeloma Working Group

MGUS – Monoclonal Gammopathy of Undetermined Significance

MHC – Major Histocompatibility Complex

MM – Multiple Myeloma

MRI – Magnetic Resonance Imaging

NK cells – Natural Killer cells

NO – Nitric Oxide

PAMP – Pathogen-associated Molecular Pattern

PB – Peripheral Blood

PBS – Phosphate-buffered Saline

PC – Plasma Cell

PET–CT – Positron Emission Tomography–Computed Tomography

Protein M – Monoclonal Protein

PRR – Pattern Recognition Receptor

ROS – Reactive Oxygen Species

RPM – Rotations *per* Minute

SHM – Somatic Hypermutation

SLAMF7 - Signaling Lymphocytic Activation Molecule Family 7

smoulMM – Smouldering Multiple Myeloma

SP – Single Positive

SSC – Side Scatter

symptMM – Symptomatic Multiple Myeloma

Tc cells – Cytotoxic T cells

TCR – T Cell Receptor

T_{FH} cells – Follicular Helper T cells

T_{FR} cells – Follicular Regulatory T cells

TGF – Transforming Growth Factor

Th cells – Helper T cells

Th_n cells – Helper T cells type *n*

TIGIT – T-cell Immunoreceptor with Ig and ITIM (immunoreceptor tyrosine-based inhibition motif) domains

TLR – Toll-like Receptor

T_{REG} cells – Regulatory T cells

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1. *State of the art*

1.1 Monoclonal Gammopathies

Monoclonal gammopathies, also called paraproteinaemias or immunoglobulinopathies, comprise a group of disorders including the monoclonal gammopathy of undetermined significance (MGUS), smouldering multiple myeloma (smoulMM) and multiple myeloma (symptMM), which result from the proliferation of a single clone of plasma cells (PCs) [1]. These pathological PCs secrete a homogeneous monoclonal (M) protein (paraprotein), characteristic of this group of disorders [1, 2], in the form of intact immunoglobulins, immunoglobulin fragments and/or free light chains (FLC) [3]. Each M protein consists of two heavy (H) polypeptide chains of the same class and two light (L) chains of the same type. The heavy polypeptide chains can be one of five and correspond to the major classes of immunoglobulins: μ (IgM), γ (IgG), α (IgA), δ (IgD) or ε (IgE). The two possible types of light chains are kappa (κ) and lambda (λ) [1, 4]. The preferred method to detect the presence of M protein in serum and urine samples is agarose gel electrophoresis, followed by immunofixation, in order to confirm the presence of the paraprotein and to ascertain its immunoglobulin heavy chain class and light chain type [5].

1.1.1 Monoclonal Gammopathy of Undetermined Significance

MGUS is defined by the presence of a monoclonal protein in the serum at a concentration lower than 3g/dL, 10% or fewer PCs in the bone marrow, as well as the absence of CRAB (i.e., hypercalcemia, renal insufficiency, anaemia and bone lesions) symptoms related to the proliferative process of PCs (Table 1) [2, 6, 7]. It is the most common monoclonal gammopathy and its incidence increases with age, with a prevalence of 3.2% among people 50 years or older, increasing to 5.3% among people over 70 years of age [7, 8]. According to a study conducted by Therneau et al., this increase in the prevalence of MGUS is a result of cumulative damage and is not related with an accumulation of new cases, because the longer the life-span the higher the probability of an individual to develop this disorder [8]. MGUS is more frequent in males than in females, with incidences of 4% and 2.75% at 50 years of age or older, respectively [7, 8]. Multiple studies found a 2- to 3-fold higher prevalence of

MGUS in the African and African-American populations than in the Caucasian population [9-11]. However, the reason for this race-related difference is yet to be determined.

MGUS can be classified in two main subtypes: IgM and non-IgM [12]. This distinction is of utter importance because the rate and nature of progression of IgM differs from that of non-IgM [13]. Moreover, the risk of progression is higher among patients with IgM MGUS than those with the non-IgM subtype of the disorder [14, 15]; IgM MGUS is associated with a risk of progression of 1.5% per year to malignant lymphoid disorders, such as Waldenström macroglobulinaemia, whereas non-IgM MGUS is associated with a risk of progression of 1% per year to symptMM [6, 12, 14]. Risk factors for progression of MGUS have been studied and include not only the type of serum M protein, but its size as well, i.e., its (initial) concentration in the serum, along with the free light-chain ratio and serum albumin concentration [6, 15-17].

According to the International Myeloma Working Group (IMWG) [18], management of MGUS should be based on: 1) the concentration, i.e., size of the serum monoclonal protein, 2) monoclonal protein type and 3) ratio of free light-chains. If the M protein is present at a concentration < 1.5 g/dL, the protein type is IgG and the free light-chain ratio is normal, a serum protein electrophoresis should be done 6 months after diagnosis and if the condition is stable the patient can be followed every 2-3 years, or when symptoms indicative of malignancy develop. In case the concentration of serum monoclonal protein is > 1.5 g/dL, the protein is IgM or IgA type, or the ratio of the free light-chains is abnormal, then an aspiration and biopsy of the bone marrow should be performed to verify whether an underlying PC malignancy is present on not. Levels of lactate dehydrogenase, β-2-microglobulin and C-reactive protein should be evaluated as well. If these tests' results are acceptable, the patient should have a complete blood count and a serum protein electrophoresis done in 6 months and then should be followed annually [18]. In both cases preventive strategies/trials are not advised because of the associated side-effects and elevated cost [16].

Table 1. Diagnostic criteria for monoclonal gammopathies

Disorder	Criteria
MGUS	✓ Serum M protein < 3 g/dL ✓ Bone marrow plasma cells < 10% ✓ Absence of end-organ damage (CRAB symptoms)
smoulMM	✓ Serum M protein (IgG or IgA) ≥ 3 g/dL or urinary M protein ≥ 500 mg/24h and/or clonal bone marrow plasma cells 10–60% ✓ Absence of end-organ damage (CRAB symptoms)
symptMM	✓ Percentage of clonal bone marrow plasma cells ≥ 10%, or presence of bony or extramedullary plasmacytoma and one or more of the following: • Tissue and/or organ damage attributable to the underlying plasma cell malignancy: ✓ Hypercalcemia: serum calcium > 11 mg/dL or > 1 mg/dL above the normal upper limit ✓ Renal Insufficiency: serum creatinine > 2 mg/dL or creatinine clearance < 40mL/min ✓ Anaemia: haemoglobin levels > 2 g/dL below the normal lower limit or a haemoglobin level < 10 g/dL ✓ Bone lesions: one or more site of osteolytic bone lesions on CT or PET-CT • Biomarkers of malignancy: ✓ Percentage of clonal bone marrow plasma cells ≥ 60% ✓ Serum free light chain ratio ≥ 100 ✓ > 1 focal lesion (involving bone or bone marrow) on MRI studies of at least 5mm in size

1.1.2 Smouldering (asymptomatic) Multiple Myeloma

smoulMM is a heterogeneous intermediate clinical stage between MGUS and symptMM, which encompasses patients whom, like MGUS, have no end-organ damage that can be attributed to the underlying PC proliferative disorder, but whose serum monoclonal protein and bone marrow plasma cell (BMPC) percentage values are higher than MGUS patients. This stage is characterized by a serum M protein (IgG or IgA) ≥ 3 g/dL and/or clonal BMPCs 10–60%, but no evidence of end-organ damage. A urinary

monoclonal protein ≥ 500 mg per 24 h may also be used as an inclusion factor for diagnosis (Table 1) [12, 18, 19].

Patients with smoulMM are at a higher risk for progression than MGUS patients due to the greater plasmacytosis extent and size of the monoclonal protein. The risk of progression is about 10% per year for smoulMM patients in the first 5 years after diagnosis [12]. Important risk factors for progression are increased numbers of PCs in the BM, elevated serum M protein level, abnormal FLC ratios and atypical MRI imaging, such as focal lesions and diffuse bone marrow abnormalities [12, 18, 20, 21]. In 2018, the IMWG proposed a risk stratification system based on BMPC percentage, serum M protein level and FLC ratio [21]. The cutoffs for these prognostic factors were: BMPC percentage > 20%, serum M protein level > 2g/dL, and FLC ratio > 20. smoulMM patients who didn't exhibit any of these risk factors were assigned to the "low-risk" category, those exhibiting one risk factor were assigned to the "intermediary-risk" category and those exhibiting two or more risk factors were considered at "high-risk" for progression. It was recommended that this latter group of patients be followed every 2-3 months and repeat blood and urine tests. Intermediate-risk patients should redo the tests 3 months after diagnosis and then repeat them every 4 months. Patients in the low-risk category should be re-tested 3 months after diagnosis as well. However, their evaluation can be carried out every 6 months for the first 5 years and then annually [21].

1.1.3 (Symptomatic) Multiple Myeloma

symptMM is a heterogeneous clonal PC proliferative disorder and is virtually always preceded by MGUS [12, 22]. In 2014, the International Myeloma Working Group (IMWG) updated the criteria for the diagnosis of symptMM. In contrast to the criteria released in 2003, there is now a minimum percentage of BMPCs that need to be met for the diagnosis of MM: clonal BMPCs must be ≥10% or, in those cases where patients present a number inferior to 10%, a biopsy must be performed to prove the presence of a bony or extramedullary plasmacytoma. All the criteria that should be met for the diagnosis of symptMM, according to the IMWG, is listed on Table 1 [12].

symptMM has a high symptom burden in all stages of disease, which impacts the patient's quality of life. Therefore, it is important to know the most common symptoms to provide better palliative care [23]. Furthermore, this knowledge may also be helpful for the diagnosis

because, clusters of specific symptoms when associated with signs and/or laboratory findings, can be indicative of symptMM [24]. Pain, primarily back pain, and fatigue are the most common symptoms among symptMM patients, followed by weight loss, breathlessness and malaise. Anaemia, bone fractures, nose bleeds and recurrent infections are some signs commonly observed in patients, as well as laboratory findings such as hypercalcemia, elevated creatinine levels, leukopenia and low haemoglobin values [24-26]. Table 2 displays the incidence of some these common signs and symptoms. According to Shephard et al., hypercalcemia alone has a low positive predicative value for symptMM, but when considered together with any one of the symptoms mentioned before, the probability of the patient suffering from symptMM increases and further investigation should be conducted [24]. Similar to what is observed in MGUS, symptMM is far more prevalent in the elderly population, with the median age at diagnosis being between 65 and 70 years old. MM is also more frequently diagnosed in men than women [26-28], and there is a 2-3 times higher incidence of symptMM in the black population. However, survival rates of the black population are higher than those of the white population, which may be suggestive of disease heterogeneity between races [26].

According to the 2021 European Society for Medical Oncology (ESMO) guidelines, treatment of newly diagnosed symptMM comprises induction therapy, followed by high-dose therapy (HDT) with autologous stem-cell transplantation (ASCT) and lenalidomide maintenance, in transplant-eligible patients. Induction therapy usually consists of either a three-drug combination, including bortezomib, lenalidomide and dexamethasone (VRd) or a four-drug combination, such as daratumumab, bortezomib, thalidomide, dexamethasone (DaraVTD). High-dose melphalan (HDM) is the standard regimen before ASCT. Daratumumab, bortezomib, melphalan and prednisone (DaraVMP), as well as daratumumab, lenalidomide and dexamethasone (DaraRd) are the standards of care for transplant-ineligible patients [29]. Recently, therapeutic monoclonal antibodies have been developed and added to the therapeutic arsenal for symptMM. The US Food and Drugs Administration (FDA), as well as the European Medicines Agency (EMA) approved, in 2015 and 2016, respectively, the use of two monoclonal antibodies for the treatment of symptMM patients, namely daratumumab, as mentioned above, and elotuzumab [30]. Daratumumab is a human monoclonal antibody whose target is CD38, a transmembrane glycoprotein expressed at low levels on normal hematopoietic cells and in non-hematopoietic tissues [31], but highly

expressed in MM cells [32]. It acts both as an ectoenzyme [33] and surface receptor which mediates cell adhesion and migration [34]. Daratumumab affects the immune microenvironment, depleting $CD38^+$ regulatory cells and promoting T-cell expansion, thereby enhancing an anti-tumour response [35]. Elotuzumab is a human monoclonal antibody as well and is directed against signalling lymphocytic activation molecule family (SLAMF)7, also known as CS1, which is a glycoprotein highly expressed on MM cells, as well as natural killer (NK) cells [36]. It exerts its anti-tumour activity through activation of NK cells, induction of NK-mediated antibody-dependent cellular cytotoxicity (ADCC) and inhibition of MM cell adhesion to BM stromal cells [37]. Therapeutic monoclonal antibodies differ from conventional anti-MM agents in their ability to recruit both innate and adaptive immune cells to eliminate the tumour and favourable toxicity profiles [36, 38], thus both commonly being used together in what is called combination therapy [32].

Table 2. Incidence of signs and symptoms present in patients with newly diagnosed multiple myeloma [26]

Signs and symptoms	Incidence, %
Anaemia (haemoglobin < 12 g per dL)	73
Bone pain	58
Elevated creatinine (> 1.3 mg/dL)	48
Fatigue	32
Hypercalcemia (calcium > 10.1 mg per dL)	28
Weight loss	24

1.2 The immune system and its role in monoclonal gammopathies

As mentioned above, monoclonal gammopathies result from the proliferation of a monoclonal population of PCs [1]. A PC is a type of cell of the immune system which synthesizes and secretes antibodies and results from the differentiation of activated B cells. Activation and differentiation of these cells into PCs requires the help of T cells and their secreted cytokines [39]. The immune system and monoclonal gammopathies are interconnected and, therefore, a good knowledge of the former is vital for a better understanding of the latter.

1.2.1 Immune System

When an individual is attacked by a pathogen the immune system initiates a response, which can be divided into two sequential stages, namely innate immunity and adaptive immunity. Innate immunity is the first line of defence against pathogens functioning in two steps: 1) prevention and 2) elimination. Prevention against entry of foreign pathogens into the host is achieved through physical and/or chemical barriers. Physical barriers comprise the epithelial layers of the skin and mucous membranes, while the chemical barriers consist of acidic pH, temperature and soluble substances that possess antimicrobial/antiviral properties, such as lysozymes and interferons [39, 40]. When pathogens manage to breach these barriers, the second step of the innate immune system – elimination – is activated. This step consists on a cellular response, carried out by phagocytic cells, such as macrophages, neutrophils and dendritic cells, which express surface receptors capable of recognizing specific patterns present on the surface of pathogens. These patterns, which can be anything from carbohydrates to lipoproteins and/or nucleic acids, are called pathogen-associated molecular patterns (PAMPs). Pattern recognition receptor (PRR) is the designation given to the cell surface receptors that bind PAMPs [41]. Regarding microbes, phagocytes are also able to recognize them through soluble proteins, called opsonins, that bind to the microbial surface, in a process called opsonization. This recognition process is possible because phagocytes carry specific receptors on their surface that bind opsonins, called membrane opsonin receptors [39]. Both direct or indirect recognition of pathogens, through PAMPs or opsonins, respectively, lead to phagocytosis, followed by intracellular killing. Upon activation of the phagocytic cell, it can secrete inflammation-promoting cytokines and chemokines, that recruit white blood cells to the infection site, promoting inflammation [39-41]. The innate immune system consists on a rapid, non-specific response to invading pathogens that, simultaneously, helps activating a response from the adaptive (acquired) immune system [39].

The adaptive immune response is slower but much more specific, relying on antigen receptors of a single specificity expressed by B and T lymphocytes. Lymphocytes that bind to an antigen are activated and undergo proliferation, leading to their clonal expansion in the lymph node. Activated B cells can develop into either PCs or memory B cells. PCs secrete soluble antigen-specific antibodies that can circulate and bind to antigen, marking it for elimination. Unlike B cells, which recognize free antigen, T cells usually only recognize

antigen that has been digested by antigen presenting cells (APC) into small peptides and are complexed to the Major Histocompatibility Complex (MHC) [41, 42]. Both B and T lymphocytes exhibit immunological memory, meaning that upon subsequent encounter with the same antigen, the immune system responds faster and with heightened efficiency.

Adaptive immunity and innate immunity are interconnected. The phagocytic cells that participate in the innate immune response are involved in the activation of the adaptive immune response. One example is that of dendritic cells which phagocyte and process antigens and then function as an APC, migrating to the lymph nodes and presenting the processed antigen to T cells, a process that initiates the adaptive immune response [39, 40].

1.2.1.1 B Lymphocytes

B lymphocytes arise from the differentiation of hematopoietic stem cells (HSC) in the BM. HSC, through multiple rounds of differentiation, originate a progenitor cell called common lymphoid progenitor (CLP), which, in turn, can give rise to either B cells or T cells [39]. CLPs that stay in the bone marrow differentiate into pro-B cells [43], which are irreversibly committed to the B-cell lineage. Once these cells express immunoglobulin M (IgM) on their surface, through recombination of the immunoglobulin genes, they migrate to the secondary lymphoid organs, such as the spleen and lymph nodes, to complete the maturation stage.

In the secondary lymphoid organs, mature B cells start expressing IgD on their surface in higher levels than IgM. Naïve B cells circulate in the blood and lymphoid organs, where they enter the B-cell follicles [39]. There, with the help of follicular dendritic cells (FDCs), B cells encounter antigen, becoming activated and start producing antibodies as a response, in a $CD4^+$ helper T cell (Th cells) dependent or independent manner. However, despite T-independent or -dependent activation, optimal proliferation of B-cells always requires involvement of cytokines produced by Th cells [40]. B-cells are professional antigen-presenting cells (APCs) that process antigen and present it as a peptide-MHC complex on their surface to Th cells. Upon binding of the complex to a Th cell, the B cell receives signals to become fully activated and initiate proliferation [39, 42].

Some of the activated B cells move to the primary foci, located at the borders of T-cell and B-cell zones, where they differentiate into short-lived antibody-secreting PCs, whereas others re-enter the follicle and form a GC under the influence of follicular helper T cells

(Tfh) [39, 44]. In the GC, B cells undergo clonal expansion, affinity maturation and differentiation (Fig.1). An antigen-specific B cell clone proliferates and experiences somatic hypermutation (SHM) of the variable regions of the Ig chains that code for its antigen-receptors, followed by a selection process in which only B cells capable of binding antigen with high affinity survive. In addition, it also undergoes class switch recombination (CSR), where Th cells influence antibody production of isotypes other than IgM [39, 41, 45]. Some surviving cells become long-term memory B-cells, responsible for the immune response in case of subsequent attack by the same antigen. However, the majority differentiate into plasmablasts, which migrate to peripheral blood and after to BM and become long-term PCs, secreting high-affinity antibodies and releasing them into circulation [40, 41]. These antibodies are soluble versions of the antigen receptors present on the surface of the B cell that originated them, hence making them highly specific for the antigen that induced the immune response [39, 40].

PCs are terminally-differentiated B cells that are no longer capable of division and antigen-presentation [40]. Furthermore, they also stop expressing immunoglobulins on their surface, releasing them instead as soluble antibody molecules [42]. The common antibody structure consists of two heavy and two light polypeptide chains arranged in a Y shape. The tips of the Y make up two identical antigen-binding regions with portions of both the heavy- and light-chain amino-terminal domains [39]. Despite this common structure, there are five classes of antibodies that differ in their heavy chains. The designation of the antibodies corresponds to that of their heavy chains: IgG corresponds to γ, IgM to μ, IgA to α, IgD to δ, and finally IgE to ε [41]. IgG, IgA and IgM are regarded as the major immunoglobulin classes because together they make up over 95% of the total immunoglobulins in a healthy human individual [42]. Each heavy chain is linked to its assigned light chain by a disulfide bond. The light-chains can be one of two types: κ (kappa) or λ (lambda). The antibody molecule can only have one type of light chain, never both. In normal individuals, production of heavy- and light-chains is balanced, so that assembly into an antibody molecule without surplus is possible [41, 42].

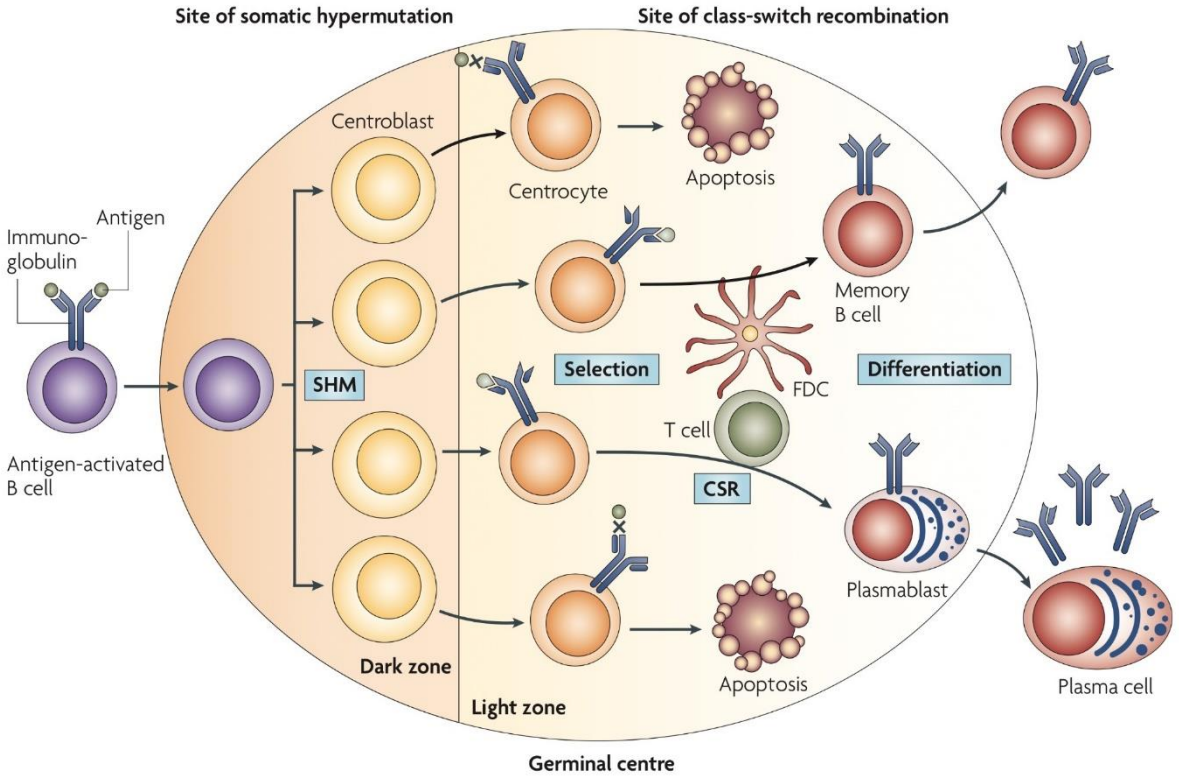


***Figure 1**. B cells undergo clonal expansion, affinity maturation and differentiation in germinal centres. Activated B cells enter the germinal centre and undergo clonal expansion and somatic hypermutations in the dark zone. They then migrate to the light zone where they are subjected to a selection process: those presenting disadvantageous mutations are induced to die by apoptosis whereas those positively selected undergo class switch recombination. B cells that bind antigen with high affinity differentiate into memory B cells or plasma cells and leave the germinal centre microenvironment. Abbreviation: CSR, class switch recombination; FDC, follicular dendritic cell; SHM, somatic hypermutations. Reprinted from "Germinal centres: Role in B-cell physiology and malignancy" by Klein U and Dalla-Favera R, 2008, Nature Reviews Immunology , 8, p.24 [(46)]. Copyright 2008 by Nature Publishing Group.*

B lymphocytes undergo somatic hypermutations, clonal selection and differentiation into either PCs or memory B-cells (Fig. 1), being responsible for the humoral immune response and long-term protection of the host against subsequent attacks by the same antigen, respectively.

1.2.1.2 T Lymphocytes

The maturation stage of T lymphocytes takes place in the thymus, hence their designation. There, immature T lymphocytes, also known as thymocytes, generate surface antigen-receptors and undergo selection events, in order to become mature T cells. In general, mature T lymphocytes are either $CD4^+$ or $CD8^+$ receiving the designations of T helper (Th) or T cytotoxic (Tc) cells, respectively [(39)].

T cell precursors migrate from the BM into the thymus through the blood stream, where they become committed to the T cell lineage. These cells lack a T-cell receptor (TCR) and expression of the co-receptors CD4 and CD8, and are therefore called double negative ($CD4^-$ $CD8^-$, DN). Through rearrangement events of TCR genes, specifically TCR α-chain and β-chain genes, T cell precursors become double positive (DP) thymocytes, expressing both CD4 and CD8 co-receptors [41, 42]. Then, DP cells are subjected to a selection process based on their affinity to bind self MHC-peptide complexes, present on the surface of thymic stromal cells. Cells that are moderately reactive to self-MHC undergo positive selection, receiving survival signals through their TCRs and then proliferating, whereas cells whose TCRs are highly reactive to self MHC-peptide complexes undergo negative selection and are eliminated. Positively selected DP cells must then commit to an effector T cell lineage and become either $CD4^+$ helper or $CD8^+$ cytotoxic single positive (SP) T cells [40, 42].

T cells are considered naïve until they make contact with an antigen. As mentioned above, T cells can only recognize antigen-processed peptides complexed to MHC molecules expressed on the surface of APCs. There are two main classes of MHC molecules: class I MHC molecules which are expressed by virtually all nucleated cells in vertebrates and are specialized in presenting intracellular antigens, and class II MHC molecules which, by contrast, are expressed mainly by APCs and specialize in presenting extracellular antigens. $CD4^+$ T cells recognize and bind to antigens complexed with MHC class II molecules, whereas $CD8^+$ T cells recognize and bind to peptide-MHC class I complexes [39]. Naïve T cells migrate from the thymus to the periphery in order to browse for peptide-MHC complexes. When they bind such complexes, they can become activated and receive signals to proliferate and differentiate into effector cells [41].

Stimulation of both the TCR and its CD4 or CD8 co-receptor by a peptide-MHC complex on the surface of an APC is not enough to activate a naïve T cell. Simultaneous co-stimulation of the TCR and its co-receptor CD28 is needed for efficient activation [47, 48]. Thus, two signals are required to successfully activate a T cell: signal 1, which is provided by the engagement of the TCR with a peptide-MHC complex (and enhanced by the co-receptors CD4 and CD8) and signal 2, which is provided by the interaction of the co-receptor CD28 on the T cell with its ligand (CD80 or CD86) on the APC. When the T cell receives both signals, it enters the cell cycle, moving from the G_0 to the G_1 phase, proliferates and becomes either an effector or a memory cell [39-42]. However, T-cell activation is also

influenced by the activity of local cytokines produced by APCs and T cells. These cytokines are, therefore, mentioned, by some, as signal 3 [39]. After T-cell stimulation by signal 1 and signal 2, soluble cytokines bind surface cytokine receptors, triggering a gene expression program that will promote T-cell proliferation and survival (Fig. 2) [41].

When a $CD8^+$ T cell is successfully activated it acquires cytotoxic properties, earning the designation of cytotoxic T cell. This newly activated Tc cell kills the target-cell via release of pro-apoptotic molecules which contain two different types of cytolytic granules: perforins, which are proteins capable of forming pores on the membrane of the target-cell, and granzymes, which are serine proteases that indirectly induce DNA fragmentation of the target-cell, activating an apoptotic pathway. Because Tc cells recognize antigen bound to MHC class I molecules, which, as mentioned above, is present in virtually all nucleated cells of the body, they are perfect for clearance of cells that have been infected by an intracellular pathogen, such as a virus, or have become cancerous [39, 41].

On the other hand, activated $CD4^+$ T cells acquires the ability of assisting in the activation and proliferation of other cells, like B cells and $CD8^+$ T cells, hence the designation of helper T cells. Following activation, some Th cells stay in the secondary lymphoid organs and regulate activation of B cells, antibody production and generation of lymphocyte memory, whereas others migrate to the site of infection, where they enhance the activity of macrophages and other cells involved in the immune response [39]. Th cells can be divided into a variety of subsets according to the functions they exert and cytokines they excrete: Th1 cells regulate the response to intracellular pathogens and secrete IL-2 and interferon (IFN)-γ; Th2 cells activate B-cell production of antibodies as a response to extracellular pathogens and secrete IL-4, IL-5, IL-10 and IL-13 [50]; Th17 cells promote elimination of extracellular bacteria and fungi by inducing a B-cell immune response and secrete IL-17, IL-17F and IL-21 [41, 51]; follicular helper T (T_{FH}) cells play a crucial role in GC maintenance, aid B-cell differentiation and secrete IL21 [52, 53]. The predominant helper cell subtype during an immune response depends mainly on the type of pathogen (e.g.: extracellular bacteria) that has infected an individual [39].

There is yet another subpopulation of $CD4^+$ T cells that doesn't fit in the category of Th cells, the regulatory T (T_{REG}) cells. They are characterized by the presence of CD25 on their surface, as well as the expression of the intracellular transcription factor forkhead box protein 3 (FoxP3). These cells regulate the magnitude of immune responses, assuring that

other immune cells attack the pathogen and not the host (self-tolerance) [39, 41]. T_{REG} cells maintain self-tolerance by supressing the roles of a variety of other cells assisting in the immune response, including proliferation and cytokine production of conventional $CD4^+$ T cells [54], as well as, B-cell antigen-specific antibody production and affinity maturation [55]. Depletion of T_{REG} cells can, therefore, lead to the development of autoimmune diseases and organ-specific autoimmunity [56, 57].

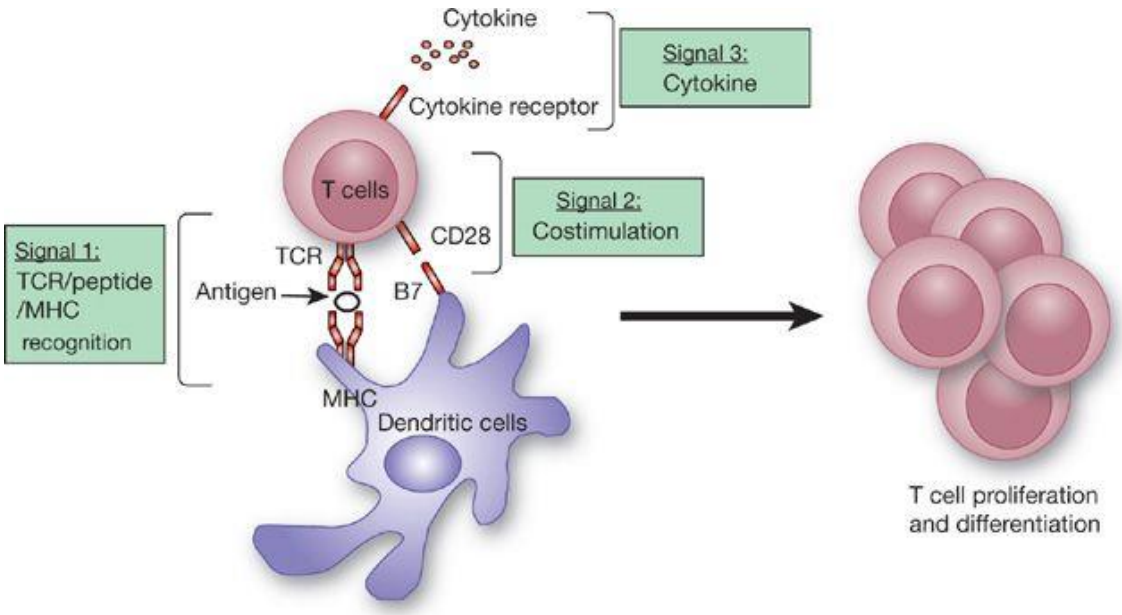


***Figure 2**. T cell activation requires 3 signals. Signal 1 consists on the recognition of the peptide-MHC complex, present on the surface of APCs, such as dendritic cells, by the TCR. Signal 2 is provided by binding of costimulatory molecule CD28 on the T cell to its ligand B7 (CD80 or CD86) on the APC. Signal 3 results from the influence of polarizing cytokine signals from local environment and nearby APCs and T cells. Reprinted from Fishman's Pulmonary Diseases and Disorders (p. 253), by Grippi MA et al., 2015, New York: McGraw-Hill Education [49]. Copyright 2015 by McGraw-Hill Education.*

T lymphocytes can be categorized based on their receptors. This categorization generates yet another subpopulation, the $\gamma\delta^+$ T cells. $CD4^+$ and $CD8^+$ T cells express α/β TCRs, which are the most common TCRs among recirculating T cells, and bind antigen that has been processed by the APC and then complexed to a type I or type II MHC molecule. In contrast, T cells bearing γδ TCRs are usually found in mucosal tissues and, despite some $\gamma\delta^+$ T cells bind the conventional antigenic peptide-MHC complex, others recognize components of the pathogen (e.g.: virus, bacteria), or molecules produced by the latter as a result of the interaction with the host. Furthermore, there is evidence that some $\gamma\delta^+$ T cells are capable of recognizing self-generated heat-shock proteins (Hsp) which are produced by a variety of cells during stress, either because of host-bacteria or host-tumour cells interactions [50, 58].

Most $\gamma\delta^+$ T cells are DN, not expressing either of the MHC co-receptors, CD4 or CD8, perhaps because, unlike T cells bearing α/β TCR, they recognize antigen that isn't complexed with a MHC molecule [51].

T lymphocytes are comprised by a complex group of subpopulations which can be further divided into more subpopulations, all being crucial for the correct functioning of the immune system. (α/β^+) T cells are responsible for the initiation of the adaptive immune response by binding with antigenic peptide-MHC complexes on APCs. This interaction activates T cells and initiates a cascade of events that eventually lead to the elimination of the pathogen and generation of immunologic memory against that same pathogen upon subsequent attack.

1.2.1.3 Regulatory and follicular T cells

Regulatory T cells are not restricted to the $CD4^+$ T cell population. $CD8^+$ and DP T cells have also been described to have regulatory properties [59-62], though these reports are few. Similarly, $CD4^+$ T cells are also the most well studied concerning follicular phenotype, but $CD8^+$ and $\gamma\delta^+$ T cells have been reported to express $CXCR5^+$ as well [63-66].

a) Regulatory T cells

T_{REG} cells consist of a heterogeneous population responsible for suppressing immune responses and maintaining peripheral tolerance, and reside mainly within the $CD4^+$ T cell compartment. This population is mainly made up of thymic-derived T_{REG} (tT_{REG}) cells, also called natural T_{REG} (tT_{REG}) cells, and induced T_{REG} (iT_{REG}) cells [67, 68]. The latter originate from $CD4^+CD25^-FoxP3^-$ T cells activated by an antigen in the presence of TGF-β and absence of the pro-inflammatory cytokine IL-6 [68]. T_{REG} cells are $CD4^+CD25^+$ T cells and the majority express FoxP3. FoxP3 is a transcription factor which expression on T_{REG} cells is required for the maintenance of tolerance [69].

TGF-β-mediated generation of iT_{REG} cells is inhibited by IL-6 and activation of T cells in the presence of this cytokine and TGF-β generates Th17 cells, which might be suggestive of both populations stemming from the same precursor T cell, depending on the cytokines present at the time of differentiation. Therefore, in the absence of inflammation and hence, in the absence of pro-inflammatory cytokines, production of effector cells is supressed and TGF-β induces the differentiation of T_{REG} cells, thereby enforcing peripheral tolerance [63]. Accordingly, conditions that induce generation of effector cells and suppress T_{REG} cells,

resulting in reduced numbers or even complete depletion of the latter, lead to chronic inflammation and autoimmune disease [56, 57, 70].

Expression of trafficking markers on FoxP3$^+$ T_{REG} cells depends on their stage of activation and differentiation. Naïve T_{REG} cells (CD45RA$^+$) mainly express CCR7, CD62L and CXCR4, which are receptors associated with lymphoid tissue homing phenotype [71]. Activated memory T_{REG} cells (CD25RO$^+$) express few lymphoid tissue homing receptors. However, they highly express receptors associated with effector cells trafficking, such as CCR4, CCR5, CCR6, CXCR3 and CLA, which represent the phenotype for nonlymphoid and inflamed tissue sites [71, 72]. This change in trafficking receptors is crucial for T_{REG} tissue-specific migration.

A subset of T_{REG} cells specialized in the regulation of GC reactions has been identified [73]. Cells in this subset are called follicular regulatory T (T_{FR}) cells given their resemblance to T_{FH} cells, including expression of the chemokine receptor CXCR5 and its ligand CXCL13, transcription factor Bcl-6, programmed cell death protein 1 (PD-1) and inducible T cell co-stimulator (ICOS), as well as their ability to access the B-cell follicle and GCs [74, 75]. T_{FR} cells also retain the suppressive function and expression of several markers characteristic of T_{REG} cells, such as CD25 and FoxP3 [76]. CXCR5$^+$ T_{REG} cells don't exist in the thymus but are rather induced in the periphery. There is some debate regarding their origin and specificity. Initial reports described T_{FR} cells as deriving from CXCR5$^-$FoxP3$^+$ thymic T_{REG} cells [74-77] and, therefore, being specific for self-antigen [77]. However, a recent study reported that these cells can originate from naïve conventional CD4$^+$ T cells, consequently being specific for the immunizing antigen, either self or foreign [78]. T_{FR} cells are suggested to undergo a multi-stage differentiation process similar to that of T_{FH} cells [74, 75], which is described below. However, unlike T_{FH} cells which only express Bcl-6, T_{FR} cells express, simultaneously, Bcl-6 and B lymphocyte-induced maturation protein-1 (Blimp-1), which are mutual antagonists and repressors. Linterman et al., suggested that while Bcl-6 is important for the acquisition of T_{FH}-like phenotype, Blimp-1 regulates the size of the T_{FR} population and may be necessary for their T_{REG}-like suppressive function [74]. T_{FR} cells have been shown to regulate GC reactions by inducing a suppressive state on T_{FH} and GC B cells, which translates in the inhibition of T_{FH} cell-mediated antibody production, IL-4 and IL-21 production by T_{FH} cells and class-switch recombination [79]. Other groups have also reported

the ability of T_{FR} cells to control the numbers of T_{FH}, antibody affinity maturation and differentiation of plasma cells [74, 75].

$CD8^+$ T cells can also regulate an immune response by downregulating lymphocyte activation and proliferation. These cells belong to a specialized subset of $CD8^+$ T with regulatory activity [80,81]. $CD8^+$ regulatory T (T_r) cells may be generated both in the thymus and in the periphery [82] and share some phenotypic markers with $CD4^+$ T_{REG} cells, such as expression of CD25 and FoxP3 [59]. One study reported that BM stromal cells could induce the generation of $CD8^+$ T_r cells from $CD8^+$ T cells, but these cells presented lower levels of FoxP3 mRNA [61]. DP T_r cells have been reported to acquire regulatory phenotype during the DP stage in the thymus and are thought to be the precursors of SP T_r cells [62, 83].

b) Follicular T cells

$CD4^+$ $CXCR5^+$ T cells, designated follicular helper T (T_{FH}) cells, are a population of cells which localize to the lymphoid follicles and are specialized in B cell help, inducing B cell differentiation and high affinity antibodies production [53, 84, 85]. They are considered a distinct subset of effector T cells with different gene expression and cytokine production, as well as, an independent developmental pathway [84, 86, 87]. T_{FH} cells don't express the typical markers for Th1 or Th2, such as IFN-γ and T-bet, as well as, IL-4 and GATA3, respectively [84, 86, 87]. Despite IL-21 expression being shared between T_{FH} cells and Th17, the former population doesn't express the distinctive Th17 markers, which include IL-17A, IL-17F and RORγt [86]. T_{FH} cells express PD-1 [65, 79, 84, 85, 87-89], ICOS [65, 84, 87], Bcl-6 [65, 87, 89] and CXCR5 [53, 84, 86, 87], among other markers, and secrete IL-21 [53, 84, 87, 89]. Furthermore, blockage of IL-4, IFN-γ and TGF-β, using antibodies, did not have any effect on the proportion of T_{FH} cells nor did it change the cytokines these cells secrete [86], which is suggestive of an independent developmental regulation. Both the distinct gene expression pattern and the developmental pathway indicate that T_{FH} cells are, in fact, a distinct subset of $CD4^+$ T cells.

$CD4^+$ T cells are activated in the T cell zone by dendritic cells (DCs). During this priming phase, induction of Bcl-6 is mediated by ICOS-ICOSL interactions and results in the upregulation of CXCR5 [90]. After this stage, almost all $CD4^+$ T cells transiently express CXCR5 [91], but only T_{FH} cells continue to express this chemokine receptor during the effector phase. Bcl-6 induction through intracellular ICOS signalling is probably enhanced by cytokines present in the environment, including IL-21 and IL-6 [78, 90]. DCs are sufficient

to initiate the differentiation process of T_{FH} cells, but B cells are required for their maintenance [90, 92, 93], most likely because antigen-presenting mature DCs die approximately four days after initiation of infection. Additionally, B cells share the same location with T_{FH} cells and undergo clonal expansion, which results in increased availability of antigen-specific B cells to function as APCs [90]. Upon DC priming, $CD4^+$ T cells also downregulate CCR7, allowing T_{FH} cells to migrate to the B-cell follicle [89]. There, they interact with their B-cell cognates and receive a second round of ICOS signals needed for T_{FH} maintenance [90].

T_{FH} cells express the costimulatory molecule CD40L [84, 94], which is required for B cell maturation and survival [84, 95], as well as maintenance of GCs in vivo [95, 96]. These cells also secrete IL-21, which has been implicated as one of the mechanisms through which T_{FH} cells exert their B cell helper function. IL-21 stimulates B cells to undergo proliferation and differentiation into antibody secreting cells [52, 53, 88, 97], being a potent inducer of antibody production both by naïve and memory B cells [53]. This cytokine induces production of the three major Ig classes, IgG, IgA and IgM [53]. Furthermore, a study performed with IL-21 deficient mice outlined the importance of this T_{FH} cell-derived cytokine in promoting normal GC formation and survival, optimal affinity maturation and maximal expression of Bcl-6 [88]. However, T_{FH} cells can also promote negative selection of GC B cells through CD95 (Fas) signalling [98]. Fas/FasL interactions control the number of memory B cells and prevent the accumulation of mutated B cells in the memory B cell pool.

$CXCR5^+$ $CD8^+$ T cells are predominantly located in B cell follicles [99, 100], where they regulate autoantibody production [63]. These cells develop in response to prolonged Ag exposure and are capable of eradicating infected T_{FH} and B cells [64]. $CXCR5^+$ $\gamma\delta^+$ T cells have been reported to originate from $\gamma\delta^+$ T cells through $\gamma\delta$ TCR activation [65]. They can act as APCs, presenting Ag to naïve $CD4^+$ T cells via MHC-II [65], as well as aid B cell activation and Ab production [66].

1.2.2 Immune System in Monoclonal Gammopathies

Centroblasts, which are activated B cells that have undergone proliferation, are submitted to multiple rounds of SHMs in GCs, and differentiate into centrocytes. These cells undergo a selection process known as affinity maturation; those that bind the antigen with high affinity survive, whereas those that secrete low affinity antibodies or are autoreactive are

eliminated by apoptosis. Positively selected centrocytes can further differentiate into memory B cells or PCs [45].

However, during the SHM or CSR processes, aberrant genetic events can occur which originate malignant centrocytes that proliferate uncontrollably. Such events, which frequently are chromosomal translocations, can cause B-cell lymphoma or MGUS, depending on their intensity. If malignant centrocytes replicate at a fast-enough rate, they accumulate at the exit of the GC and give rise to a B-cell lymphoma. Translocations that do not cause B-cell lymphoma but still originate malignant centrocytes will cause either indolent or more aggressive forms of MGUS. Although these translocations are required for onset of MGUS, a second step is necessary for progression of disease in the BM. Further genetic events take place in the BM which allow cells to survive and proliferate in hypoxia and to replicate independently of inflammatory cytokines, eventually leading progression of disease to symptMM [101].

T cells play an important role in B cell activation and differentiation into memory B cells or PCs. Both T-cell count and function are altered in monoclonal gammopathies [102]. A decrease in the PB $CD4^+/CD8^+$ T-cell ratio has been reported in MGUS and symptMM patients, either due to an increase in $CD8^+$ T cells, a decrease in the $CD4^+$ T cell population, or a combination of both [103-108]. In contrast, Perez-Andres et al., found that the proportion of $CD4^+$ T cells was elevated in the BM of patients with monoclonal gammopathies but observed no significant changes in the $CD4^+/CD8^+$ T-cell ratio, when compared to healthy subjects [109].

There are conflicting reports regarding the frequency and function of T_{REG} cells in MGUS and symptMM patients, most likely due to differences in the methods and monoclonal antibodies used. Feng et al., described a decrease in the T_{REG} population and its secreted cytokines, IL-10 and TGF-β, in the PB of symptMM patients [110]. Another study by Prabhala et al., also found reduced T_{REG} cell numbers, as well as decreased suppressive function of these cells in the PB of MGUS and symptMM patients [111]. However, other groups reported increased T_{REG} frequencies in PB of patients with monoclonal gammopathies [112, 113]. The exact role of T_{REG} cells in MGUS and symptMM remains unclear, but the abnormal activity of these cells might be a contributing factor for MM-related immune dysfunction [114].

Despite their important role in the generation of long-term PCs, T_{FH} cells have hardly been studied in the context of monoclonal gammopathies. A study by Zhou et al., found increased ratios of peripheral T_{FH} cells in symptMM patients, as well as elevated expression of the T_{FH} markers ICOS and PD-1 [115]. They also observed a decreased T_{FH} ratio in those patients with effective treatment and an even lower ratio in those patients with complete remission, which suggests a possible involvement of this cell subset in the progression of symptMM. Similar results were observed regarding ICOS and PD-1 expression. Additionally, IL-21 levels were reported to be increased in the plasma of symptMM patients, compared to healthy subjects, however, significant differences were only found between pre-treatment and second treatment course, with IL-21 levels being decreased after two rounds of treatment. A negative correlation was established between prognosis and IL-21 [115], which is consistent with the finding of IL-21 growth-promoting and anti-apoptotic effects on myeloma cells [116]. A group studying T_{FH} cells in the context of follicular lymphoma found that tumour cells produce the chemokines CCL17 and CCL22 in response to cross talk with T_{FH} cells. Overexpression of these chemokines induces preferential recruitment of T_{REG} and IL-4-producing $CD4^+$ T cells to the tumour microenvironment, which will stimulate further chemokine production, leading to the generation of an autocrine loop. Such an immunosuppressive microenvironment stimulates tumour growth and survival and promotes immunosurveillance evasion. This group also reported that the IL-4-producing T cells were T_{FH} rather than Th2 cells [117]. Considering that follicular lymphomas are B-cell lymphomas and that both B-cell lymphomas and symptMM originate from B cells that have undergone SHM in the GCs [101], it is fair to speculate that T_{FH} cells might behave similarly in both diseases.

Given the role of the immune system in symptMM, immunomodulatory drugs (IMiDs) are used in the treatment of symptMM. IMiDs are thalidomide analogues, which display a variety of anti-myeloma effects, including T-cell co-stimulatory, anti-proliferative and anti-angiogenic effects [118]. These compounds can function as a secondary co-stimulatory signal for partially activated T cells (either by anti-CD3 or DC), thereby enhancing the T-cell response [119]. IMiDs are also capable of inducing production of Th1-type cytokines, IFN-γ and IL-2, while displaying inhibitory properties towards Th2-type cytokines, IL4 and IL-10 [118]. In addition, they have been shown to supress T_{REG} function and FOXP3 expression [120].

All this suggests that IMiDs may enhance tumour cell elimination by promoting tumour-specific immunity.

1.3 Flow Cytometry in the study of Monoclonal Gammopathies

Flow cytometry is a technique used to analyse the phenotype and other characteristics of cells, including cell size and granularity [9, 38]. In flow cytometric immunophenotyping, cells in suspension are labelled using fluorochrome-conjugated monoclonal antibodies [121]. These cells then pass through a laser beam and scatter light in all directions. The light scattered in the forward direction (forward scatter, FSC) is proportional to cell size whereas side scattered (SCC) light is proportional to the intracellular complexity of the cell [9, 38].

Multiparameter flow cytometry applied to immunophenotyping is regularly used in both clinical and research settings. It allows for the identification and qualification of a specific group of cells based on the detection of molecules expressed on the surface of these cells, known as cell surface markers, by antibodies [122]. This can be achieved by staining cells with fluorochrome-conjugated antibodies, all of which with different fluorescence excitation and emission patterns [123], commonly referred to as immunophenotyping panels.

Flow cytometry-based immunophenotyping is important for the diagnosis and monitoring of monoclonal gammopathies [124] and has many advantages, including the possibility of analysing the proportion of PCs in the BM that are normal, clonal and not reactive, which is relevant information for the differential diagnosis, as well as for predicting the risk of progression in MGUS and smoulMM patients. Moreover, it allows the assessment of treatment efficacy [125] and can often detect recurrence of cancer before changes in morphology, for instance, become noticeable [126].

2. Objectives

T cells play a crucial role in B-cell activation and further differentiation into either memory B cells or PCs. Particularly, T cells with follicular phenotype, such as T_{FH} cells, are required for GC maintenance and GC B-cell selection. Recently, a T cell subset with both follicular and regulatory properties has been identified, which is capable of regulating GC reactions. Given the importance of the follicular phenotype in GC reactions and B cell help and the fact that the triggering event that leads to monoclonal gammopathies is thought to occur in the GC during or after SHM, we propose to study different T cell populations, including $CD4^+$, $CD8^+$, $\gamma\delta^+$, DN $\alpha\beta^+$ and DP T cells and its subpopulations with follicular-like phenotype.

Therefore, the aims of this thesis are as follows:

- Evaluate the frequency of follicular-like and regulatory T cells in monoclonal gammopathy patients and in a control group
- Evaluate the frequency of activated T cells in monoclonal gammopathy patients and in a control group
- Analyse the implication of follicular-like and regulatory T cells in the physiopathology of monoclonal gammopathies and disease progression.

3. Material and Methods

3.1 Patients and controls

EDTA-anticoagulated BM samples from 5 individuals with normal BM, 14 MGUS patients, 7 smoulMM patients and 20 symptMM patients were used in this study. These BM samples were obtained from BM aspirates sent to the Unidade de Gestão Operacional de Citometria (UGOC) of the Centro Hospitalar e Universitário de Coimbra (CHUC) for diagnosis. Normal BM samples were acquired from normal BM aspirates which arrived at UGOC with suspicion of gammopathy which was not confirmed by immunophenotyping nor serology, and without inflammatory markers and cytopenias nor any other haematological disease. The characteristics of the studied population is summarized on table 3.

Table 3. Demographic characteristics of the study participants

	Controls		MGUS		smoulMM		symptMM	
Number	5		14		7		20	
Sex, n	F, 2	M, 3	F, 7	M, 7	F, 2	M, 5	F, 9	M, 11
Age (mean ± standard deviation)	54 ± 16	71 ± 8	76 ± 8	68 ± 12	65 ± 28	71 ± 12	77 ± 6	76 ± 12
	64 ± 14		72 ± 11		68 ± 19		77 ± 10	

F, female; M, male; MGUS, patients with monoclonal gammopathy of undetermined significance; n, number; smoulMM, patients with smouldering multiple myeloma; symptMM, patients with symptomatic multiple myeloma.

3.2 Multiparameter flow-cytometry immunophenotypic study of bone marrow T cell subsets

One antibody (Ab) panel was used to identify and characterize T cell subsets present in the bone marrow samples, as described in table 4. This panel aimed to quantify T follicular-like cells and analyse T_{FH}/T_{REG} plasticity. The volume of Ab used is in accordance with the laboratory recommendations.

Table 4. Antibody panel used for T cell identification and characterization

Ab	CD3	CD4	CD8	CXCR5	CD25	CD127	TCRγ/δ	HLA-DR
Fluorochrome	PerCP-Cy™5.5	PB	APC-H7	APC	PE	BV510	PE-Cy™7	FITC
Clone	SK7	RPA-T4	SK1	51505	2A3	HIL-7R-M21	11F2	L243
Volume (µL)	10	2,5	2,5	5	10	2,5	1	10
Brand	BDB	BDBP	BDB	R&D Systems	BDB	BDH	BDB	BDB

PerCP-Cy™5.5, peridinin-chlorophyll-protein Complex-cyanine 5.5; PB, pacific blue; APC-H7, allophycocyanin-Hilite® 7; APC, allophycocyanin; PE, phycoeritrin; BV510, brilliant violet™ 510; PE-Cy™7, phycoerythrin-cyanine 7; FITC, fluorescein isothiocyanate; BDB, Becton Dickinson Bioscience; BDBP, Becton Dickinson Bioscience Pharmingen™; BDH, Becton Dickinson Horizon™.

Direct immunofluorescence staining of the sample was performed. 100 µL of original sample or 150-200 µL of sample washed with PBS, (Corning) was aliquoted to the test tube already containing the monoclonal antibodies, followed by an incubation period of 15 minutes at room temperature in darkness. Then, a lyse and wash protocol was followed: incubation with 2mL of FACS Lysing Solution (BD Bioscience) for 15 minutes, centrifugation at 3500 rotations per minute (rpm) for 4 minutes and removal of supernatant, followed by a washing step with 2mL of PBS. Cells were subsequently resuspended in approximately 0,5mL of PBS before acquisition on the flow cytometer.

3.3 Flow cytometry data acquisition and analysis

Data acquisition was performed in a FacsCanto II flow cytometer (BD Bioscience) using the FacsDiva software (BD Bioscience). For data analysis the Infinicyt™ software, V.1.7 (Cytognos SL, Salamanca, Spain) was used.

T cells were identified according to their CD3 positivity and typical side scatter. Among these, $CD4^+$, $CD8^+$, $\gamma\delta^+$, DN $\alpha\beta^+$ and DP T cells were analysed: $CD4^+$ T cells were identified by the expression of CD4 and absence of CD8, whereas $CD8^+$ T cells were identified by the expression of CD8 and absence of CD4; $\gamma\delta^+$ T cells were identified according to their positivity to γδ and typical side scatter, as well as, by the absence of both CD4 and CD8, while DN $\alpha\beta^+$ T cells were identified by the lack of expression of CD4 and CD8, in addition to their negativity to γδ and typical side scatter; and, finally, DP T cells were identified by

the expression of both CD4 and CD8. Two subsets of these T cell populations were also analysed: Treg cells were identified based on their expression of CD25 and lower to no expression of CD127, and T follicular-like cells were identified by their positivity to CXCR5 and typical side scatter. Furthermore, activation of these subsets was also analysed according to their positivity to HLA-DR and CD25, and typical side scatter.

3.4 Statistical analyses

Data were statistically analysed using the non-parametric Kruskal-Wallis (KW) test. The pairwise Mann-Whitney test was used as a post hoc to identify the groups with statistical differences. Furthermore, a two-sample Mann-Whitney (MW) test was performed to determine the existence, or lack thereof, of significant statistical differences between two groups, specifically, Controls and MM, as well as, MGUS and MM, the MM group consisting of an agglomeration of both smoulMM and symptMM groups. Differences were considered to be statistically significant when the p-value was less than 0.05. All statistical analyses were performed using Paleontological Statistics software package for education and data analysis PAST 4.04 (Natural History Museum – University of Oslo, Norway) and GraphPad Prism 8.0.0 (San Diego, California USA).

4. Results

4.1 Frequency of regulatory and follicular-like cells in different T cell populations in the BM

To assess the BM T cell repertoire in patients with monoclonal gammopathies we performed a detailed FACS analysis of controls' and patients' BM samples, in which we analysed $CD4^+$, $CD8^+$, $\gamma\delta^+$, $CD4^-CD8^-$ $\alpha\beta^+$ and $CD4^+CD8^+$ T cells and identified follicular-like cells based on the expression of CXCR5, as well as activated T cells according to CD25 and HLA-DR expression, in all T cell subpopulations.

No significant differences were found between controls and patients with monoclonal gammopathies regarding the frequency of regulatory cells in the different T cell studied populations (Th, Tc and DP) (Table 5).

Concerning follicular like T cells we observed an increase in the frequency of Th reg cells with a follicular like phenotype in all monoclonal gammopathies, reaching statistical significance in the MM group, when compared with controls. The same seemed to occur in DP T reg cells, although without statistical significance. On contrary, a significant decrease in the frequency of $\gamma\delta^+$ T follicular like cells was observed in all MM groups, when compared with controls and MGUS group. No significant differences were observed in the $CD8^+$, DN $\alpha\beta^+$ or DP T cell compartments (Table 5).

4.2 Frequency of activated ($CD25^+$ or $HLA\text{-}DR^+$) T cell subsets

As shown in figures 3A and 3B, respectively, activated Th reg cells with follicular phenotype were found at a lower frequency in all MM groups than in controls, reaching a statistically significant decrease in MM group when compared to MGUS, whereas activated Th reg cells without follicular phenotype were increased in symptMM patients in comparison to controls and MGUS patients. The MM group also displayed an increase in this cell subset comparing to controls and, despite not reaching statistical significance there is a clear tendency for these cells to be increased in the MM patient group in comparison to MGUS patients (p=0,0518). No significant differences were observed in activated $CD8^+$ and DP Treg cell frequencies between patients and controls (Figure 3C-F).

The frequencies of activated $CD4^+$, $CD8^+$ and DP follicular-like T cells were similar across patient and control groups (Figure 4A-F and 4M-O). A significant decrease of CD25

activated $\gamma\delta^+$ T follicular cells was observed in symptMM patients when compared to controls and smoulMM patients (Figure 4I). Furthermore, the frequency of CD25 activated DN $\alpha\beta^+$ T follicular cells was also found to be lower in symptMM and MM patients in comparison to controls and MGUS patients (Figure 4L).

Table 5. Mean and standard deviation of the main T cell populations and subpopulations in the BM

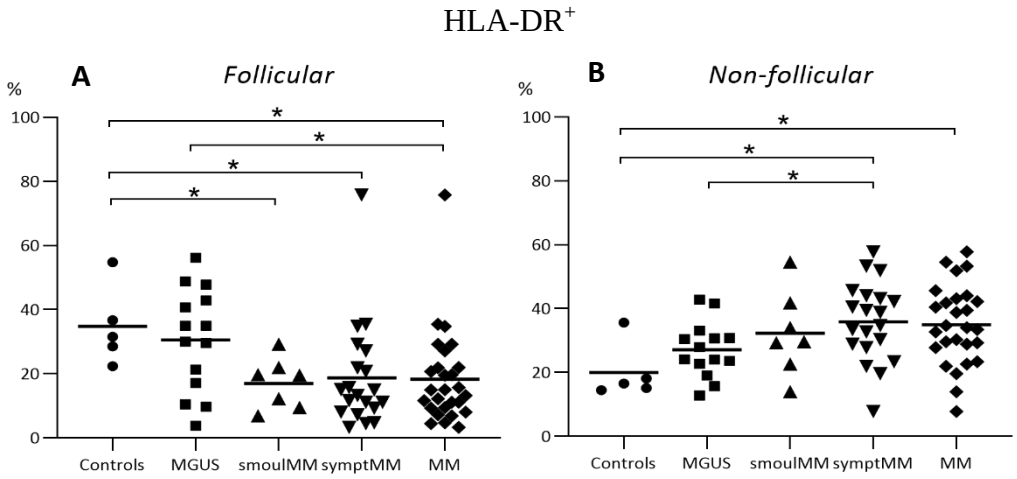
	Controls	**MGUS**	**smoulMM**	**symptMM**	**MM**
CD3$^+$	12,39 ± 6,25	11,23 ± 5,68	9,18 ± 7,59	7,70 ± 3,53	8,09 ± 4,78
CD4$^+$	48,77 ± 13,56	54,04 ± 9,13	44,13 ± 13,64	44,71 ± 14,17	44,56 ± 13,77
Reg	8,99 ± 2,17	11,14 ± 5,05	10,81 ± 4,02	10,85 ± 4,50	10,84 ± 4,30
Follicular like	5,75 ± 3,28	18,27 ± 16,79	16,62 ± 18,73	21,85 ± 13,90	20,49 ± 15,09 [a]
Follicular like	4,46 ± 1,55	7,79 ± 4,88	8,82 ± 7,51	8,94 ± 4,87	8,91 ± 5,51
CD8$^+$	38,16 ± 13,75	35,85 ± 9,60	43,52 ± 10,51	45,84 ± 14,04	45,24 ± 13,06
Reg	0,07 ± 0,16	0,70 ± 0,88	0,66 ± 0,64	0,24 ± 0,39	0,35 ± 0,49
Follicular like	0,00 ± 0,00	4,01 ± 7,44	4,57 ± 4,10	2,27 ± 4,69	2,86 ± 4,58
Follicular like	5,64 ± 1,85	8,61 ± 9,99	4,56 ± 2,78	6,26 ± 5,09	5,82 ± 4,61
γδ+	6,46 ± 5,71	4,86 ± 4,41	6,41 ± 4,62	4,85 ± 2,68	5,25 ± 3,26
Follicular like	13,46 ± 10,52	11,76 ± 13,58	2,79 ± 2,48 [a b]	5,80 ± 7,42 [b]	5,02 ± 6,59 [a b]
DN αβ+	1,72 ± 1,62	1,34 ± 1,08	1,62 ± 1,71	1,47 ± 1,27	1,51 ± 1,36
Follicular like	6,43 ± 4,40	10,91 ± 14,48	9,22 ± 13,96	9,19 ± 10,14	9,20 ± 10,96
DP	2,15 ± 2,61	2,24 ± 1,78	2,62 ± 3,11	1,74 ± 1.73	1,97 ± 2,14
Reg	0,99 ± 2,20	2,91 ± 5,82	5,59 ± 6,90	2,53 ± 3,52	3,32 ± 4,68
Follicular like	0,00 ± 0,00	12,09 ± 21,50	42,00 ± 29,99	23,83 ± 35,18	28,54 ± 34,32
Follicular like	13,18 ± 13,81	9,54 ± 11,97	17,51 ± 13,79	17,93 ± 19,74	17,82 ± 18,13

[a] : $p < 0,05$ vs controls, [b] : $p < 0,05$ vs MGUS. DN, double negative; DP, double positive; Reg, regulatory cells; MGUS, patients with monoclonal gammopathy of undetermined significance; MM, patients with either smouldering or symptomatic multiple myeloma; smoulMM, patients with smouldering multiple myeloma; symptMM, patients with symptomatic multiple myeloma.

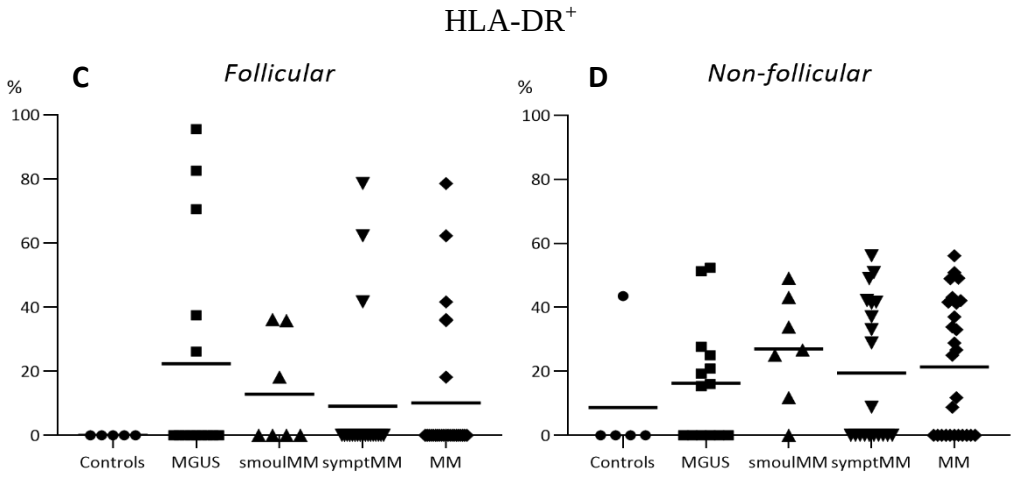
Activated CD4$^+$, CD8$^+$ and DP T cells were observed at similar frequencies in patients with monoclonal gammopathies and controls (Figure 5A-D, 5I and 5J). Both HLA-DR and CD25 activated DN $\alpha\beta^+$ T cell frequencies were altered in monoclonal gammopathy patients, as represented in Figures 5G and 5H, respectively. An increase in HLA-DR

activated cells was observed in symptMM patients when compared to controls, MGUS and smoulMM patients, as well as in the MM patient group in comparison to MGUS and controls, despite not reaching statistical significance with the latter (p=0,0528). Contrarily, CD25 activated DN $\alpha\beta^+$ T cells were decreased in symptMM patients in comparison to MGUS and smoulMM patients. We also observed a tendency for HLA-DR activated $\gamma\delta^+$ T cells to be increased in patients with monoclonal gammopathies, especially in symptMM patients despite not reaching statistical significance (p=0,0834), as depicted in figure 5E.

Regulatory T helper cells



Regulatory T cytotoxic cells



Regulatory DP T cells

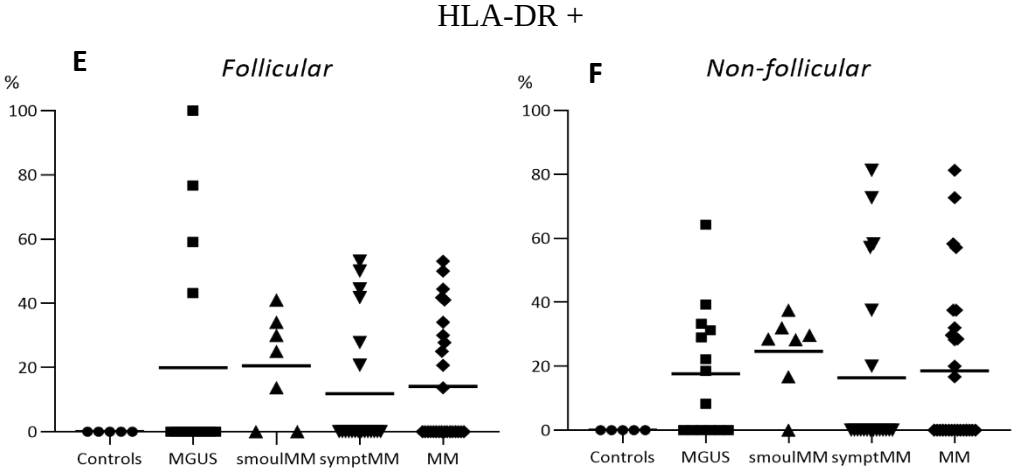
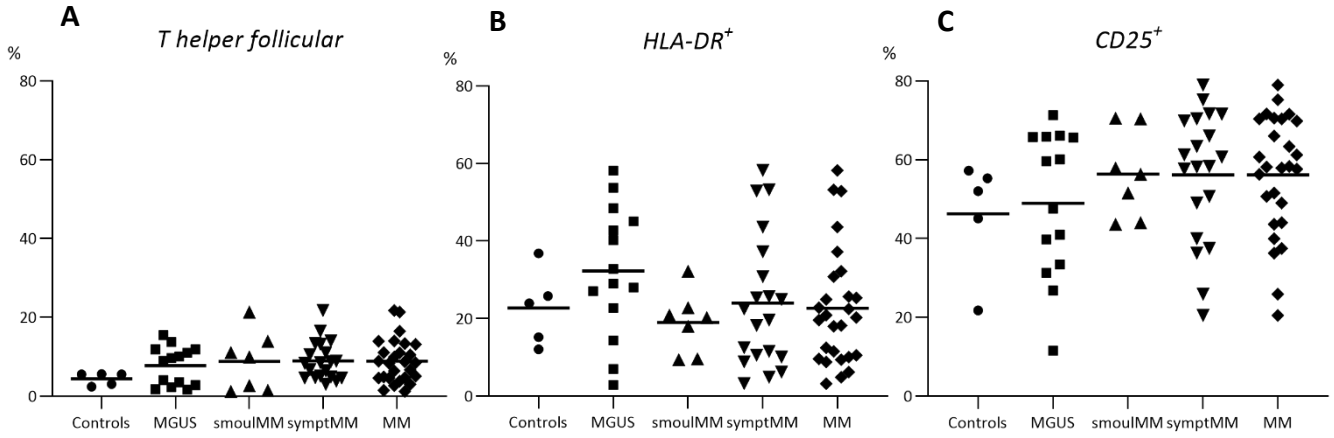
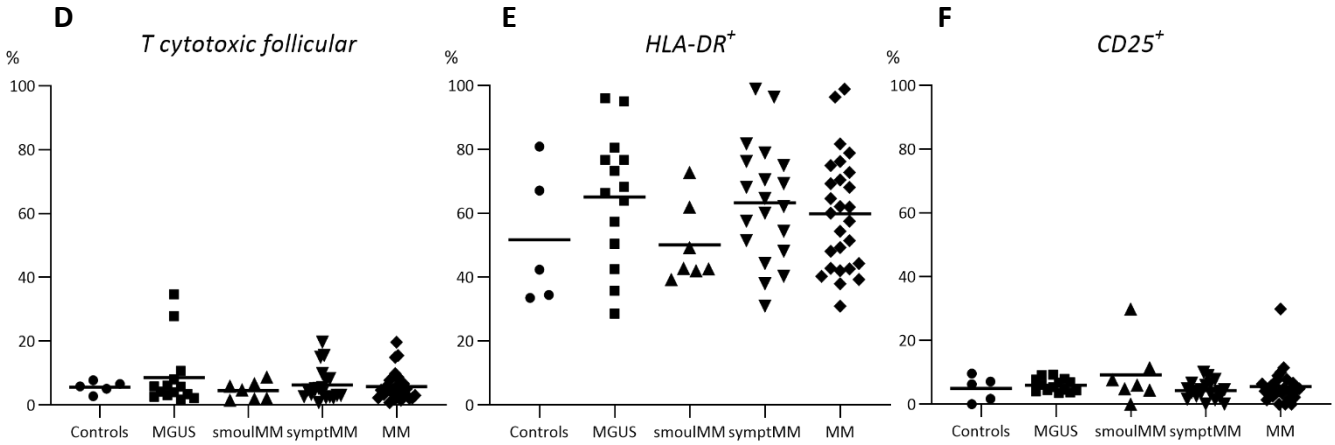


Figure 3. Percentage of HLA-DR activated cells among T regulatory follicular and non-follicular cells, T helper (A and B), T cytotoxic (C and D) and DP T (E and F) cells. DP, double positive; MGUS, patients with monoclonal gammopathy of undetermined significance; MM, patients with either smouldering or symptomatic multiple myeloma; smoulMM, patients with smouldering multiple myeloma; symptMM, patients with symptomatic multiple myeloma. *$p < 0.05$.

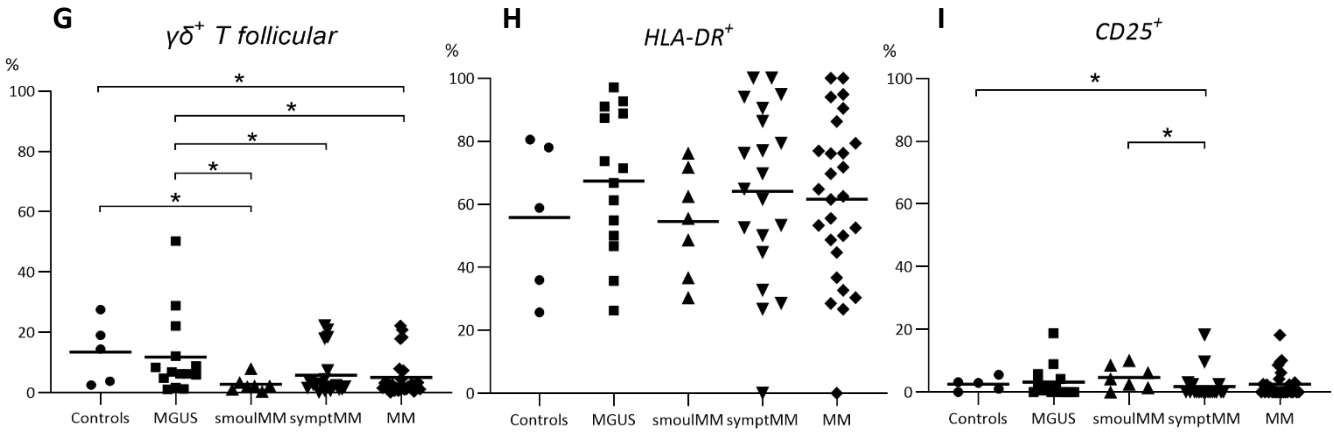
T Follicular like helper cells



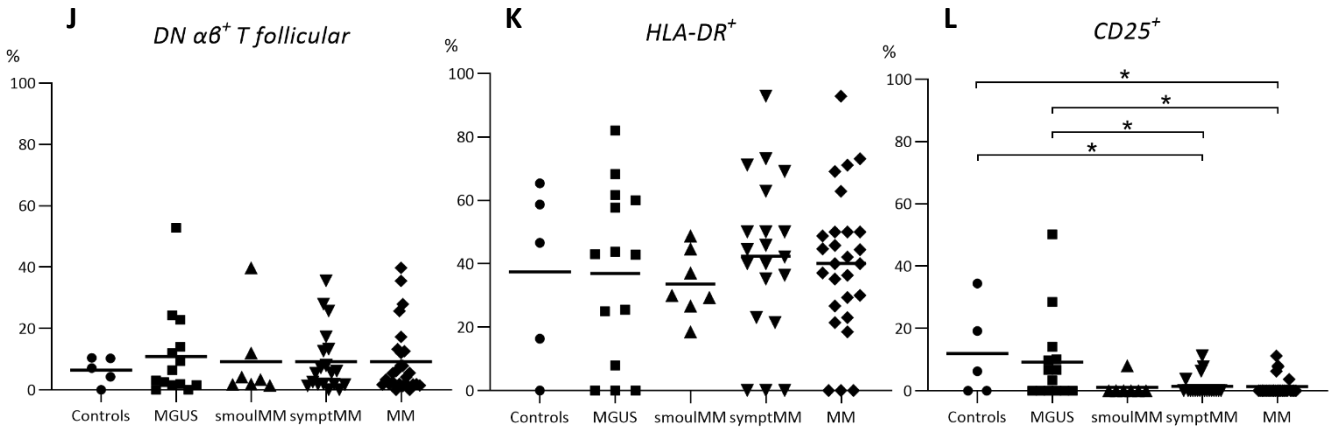
T Follicular like cytotoxic cells



γδ+ T Follicular like cells



CD4^{-}CD8^{-} (DN) αβ^{+} T Follicular like cells



CD4^{+}CD8^{+}(DP) T Follicular like cells

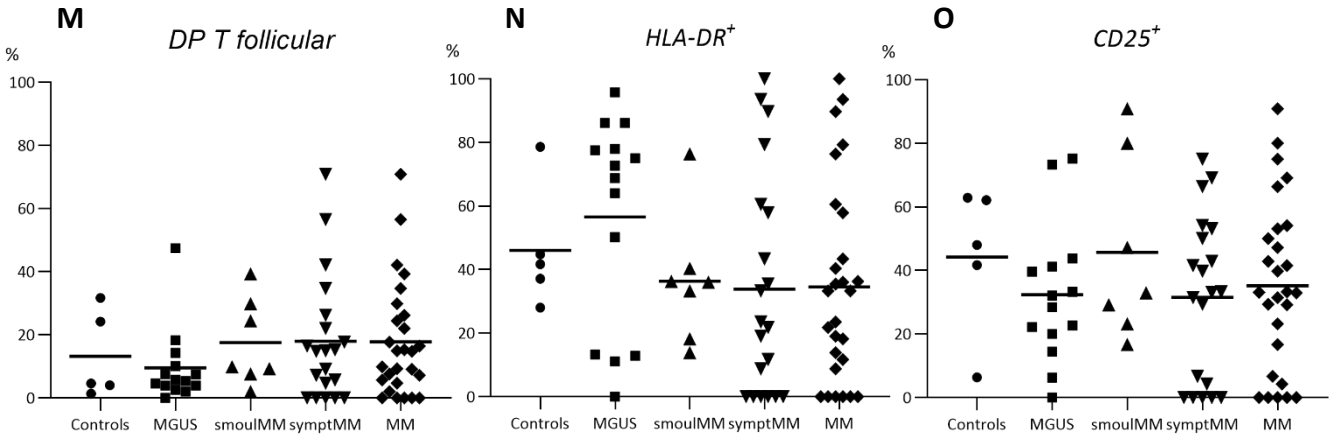
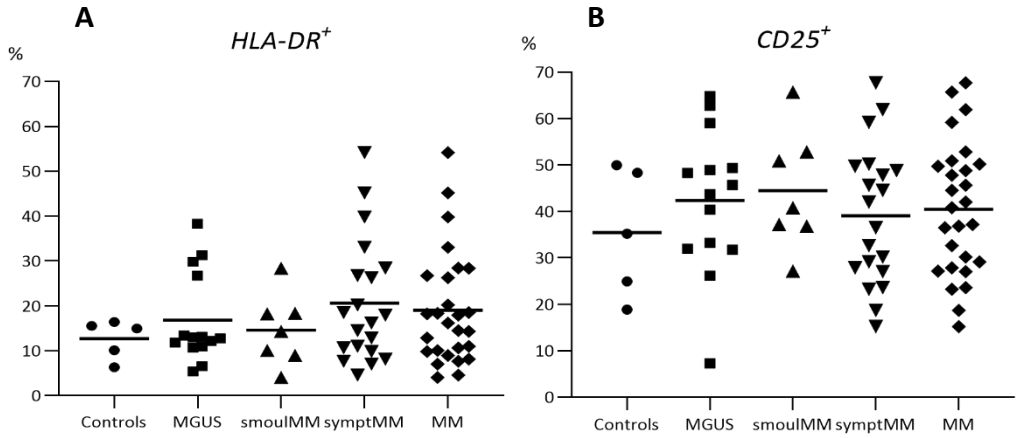
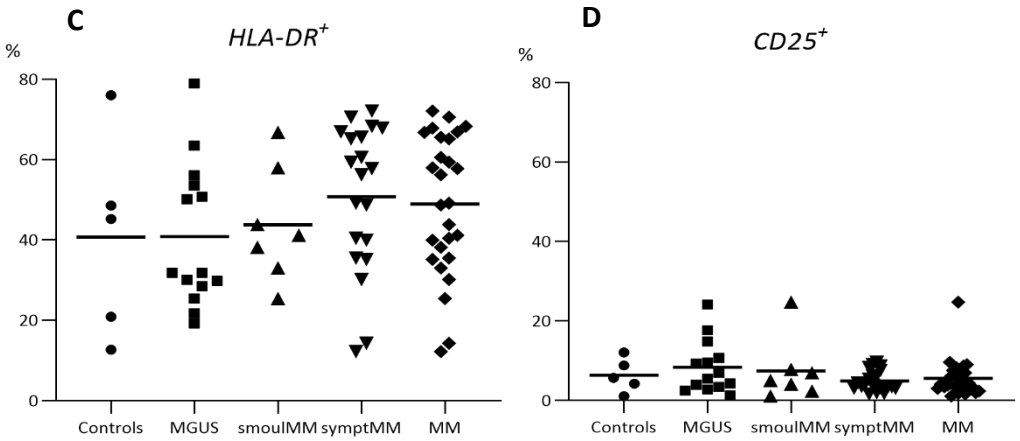


Figure 4. Frequency of T follicular like cells among each T cell subpopulation (Thelper-A, Tcitotoxic -D, γδ: G, DN: J, DP: M). Percentage of HLA-DR (B) and CD25 (C) activated T helper follicular like cells ; HLA-DR (E) and CD25(F) activated T cytotoxic follicular like cells HLA-DR (H) and CD25 (I) activated γδ+ T follicular like cells ,HLA-DR (K) and CD25 (L) activated DN αβ^{+} T follicular like cells ; HLA-DR (N) and CD25 (O) activated DP T follicular like cells. DN, double negative; DP, double positive; MGUS, patients with monoclonal gammopathy of undetermined significance; MM, patients with either smouldering or symptomatic multiple myeloma; smoulMM, patients with smouldering multiple myeloma; symptMM, patients with symptomatic multiple myeloma. *p< 0.05.

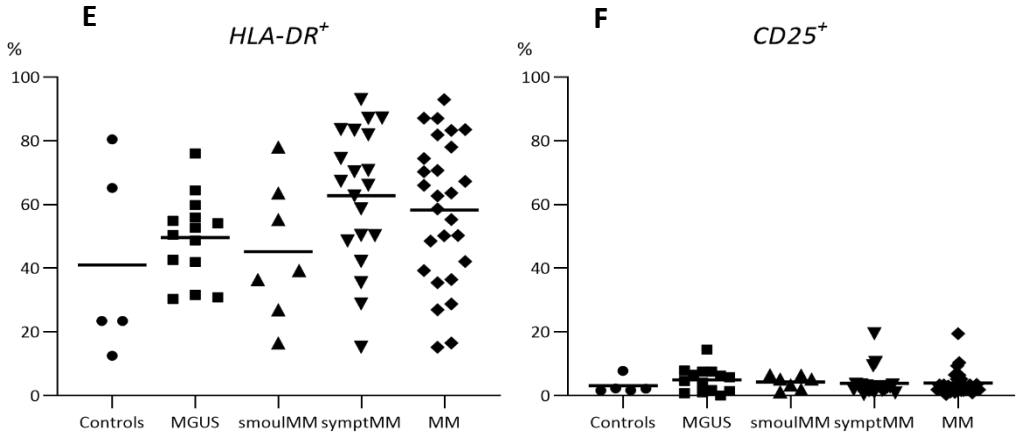
T helper cells (non-follicular; non-regulatory cells)



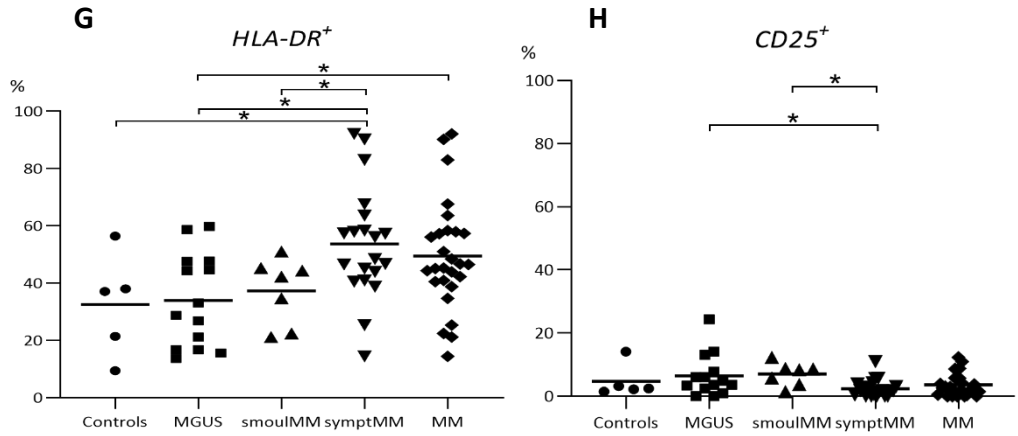
T cytotoxic cells (non-follicular; non-regulatory cells)



γδ+ T cells (non-follicular; non-regulatory cells)



$CD4^{-}CD8^{-}$ (DN) $\alpha\beta^{+}$ T cells (non-follicular; non-regulatory cells)



$CD4^{+}CD8^{+}$ (DP) T cells (non-follicular; non-regulatory cells)

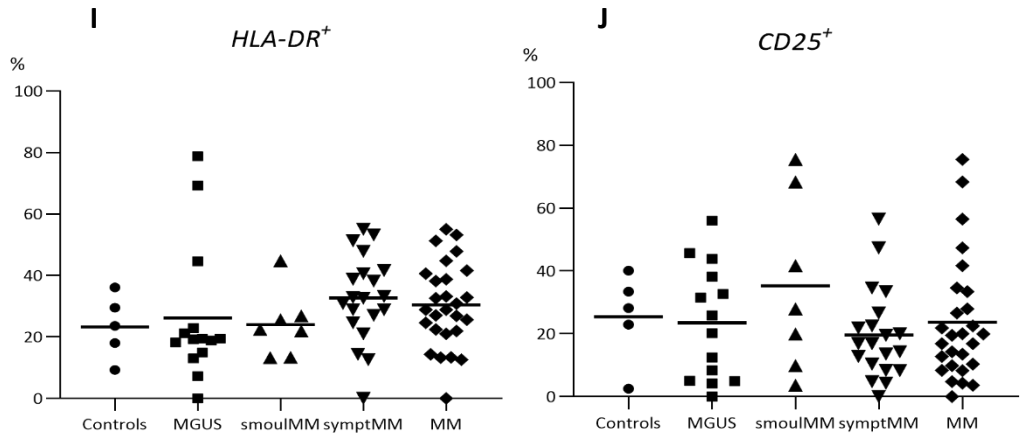


Figure 5. Frequency of HLA-DR and CD25 activated non-follicular non-regulatory T cells: T helper (A and B), T cytotoxic (C and D), $\gamma\delta^{+}$ (E and F), DN $\alpha\beta^{+}$ (G and H) and DP T (I and J) cells. DN, double negative; DP, double positive; MGUS, patients with monoclonal gammopathy of undetermined significance; MM, patients with either smouldering or symptomatic multiple myeloma; smoulMM, patients with smouldering multiple myeloma; symptMM, patients with symptomatic multiple myeloma. *$p< 0.05$.

5. Discussion

The T cell compartment is crucial for the correct functioning of the immune system and therefore, if the T cell equilibrium is altered, then the immune system will be compromised. Changes in the T cell population can lead to and/or be caused by disease. In this study we demonstrate that some T cell subpopulations are altered in patients with monoclonal gammopathies.

A skewed PB $CD4^+/CD8^+$ T cell ratio has been reported in MGUS and sympMM patients [94-99], as well as an increase in $CD4^+$ T cells in the BM of patients with monoclonal gammopathies [100]. In this study, however, no alterations were observed in the frequency of these major T cell populations in the BM. This may be due to the fact that our study population is relatively small and therefore, further studies with a larger number of participants should be performed.

Levels of T cells with a regulatory phenotype were found to be similar in controls and all patient groups. Regarding Th reg cells, several studies have reported conflicting results about the frequency of these cells and the possible correlation between them and tumour burden or disease status [111, 127, 128]. Our study is in agreement with that of Foglietta et al., in which no differences in the frequency of these cells were found between controls and MGUS and MM patients [129]. These results indicate that the Th reg cell pool seems not to be influenced by disease status. Although the frequency of Th reg cells was similar across all groups, HLA-DR activated Th reg cells without follicular phenotype were found at a significantly higher frequency in symptMM patients in comparison to controls and MGUS patients. This finding is in agreement with a study by Raja et al., which observed a significant increase in the frequency of activated Th reg cells in the PB of symptMM patients compared to healthy individuals [128]. Wang et al., also observed a higher proportion of activated Th reg cells in the PB and BM of MGUS and symptMM patients than controls [130]. These cells have been demonstrated to inhibit T cell proliferation and cytokine production [128, 131] in a contact-dependent manner and can act as APCs to other Treg cells *in vivo* by presenting self Ag [131]. In addition, TIGIT, an inhibitory immune receptor, is highly expressed and upregulated on activated Th reg cells [132, 133]. TIGIT inhibits T cell responses by binding to its ligand CD155 on DCs which inhibits IL-12 but induces IL-10 production [134]. TIGIT-CD155 interactions induces expression of the effector molecule Fgl2 on Th reg cells, which shifts the immune response toward a Th2 cell response by inhibiting Th1 and Th17 cell

cytokine production [133]. High expression of TIGIT and its ligands has been reported in the BM of symptMM patients, which may indicate a role for this inhibitory immune receptor in regulating immune activation [134]. All these findings indicate that the increase in activated Th reg cells in symptMM patients observed in this study is suggestive of an immunosuppressive state and may be a useful marker of disease progression.

In general, we observed that the frequency of follicular-like T cells is increased in monoclonal gammopathy patients, except for the $\gamma\delta^+$ T cell compartment (Table 5). The exact reason behind this increase is unknown, but we speculate it may be due to a possible upregulation of Bcl-6 in MM cells in the BM microenvironment. Myeloma and BM stromal cells, as well as DN T cells produce IL-6 and IFN-γ, respectively, which upregulate Bcl-6 [136-138]. Furthermore, ICOSL has been reported to be expressed by MM cells [139]. ICOS-ICOSL interactions induce Bcl-6 [90]. Bcl-6 in turn induces a higher expression of CXCR5 [90], the hallmark of follicular phenotype. The increase in the frequency of follicular-like T cells reached statistical significance in the Th reg follicular-like cell subset when comparing the MM group with controls. T follicular regulatory cells have been mainly reported on blood and lymph nodes in humans [140], and to our knowledge, our work is the first identifying regulatory T cells with a follicular-like phenotype in the BM. Several studies have reported a suppressive function for Th reg follicular (T_{FR}) cells [141, 142]. T_{FR} cells suppress B cells at different steps during the B-cell differentiation process [143]. Sage and Sharp hypothesize that the T_{FR} suppression exerted on PCs may restrict antibody production by these cells [143]. The observed increase in Th reg follicular-like cells may therefore indicate an attempt of the immune system to control the level of monoclonal protein produced by malignant PCs. However, despite Th reg follicular-like cells being increased, they were less activated. HLA-DR activated Th reg follicular-like cells were decreased in all MM groups in comparison to controls. This finding may point towards the opposite direction of the previous one as these cells seem to be quiescent and, therefore, cannot regulate antibody production by tumour cells. In line with this observation, CD25 activated DN $\alpha\beta^+$ T follicular-like cells were also reduced in symptMM and MM patients in comparison to controls and MGUS patients.

We observed a significant decrease in the frequency of $\gamma\delta^+$ T follicular like cells in all MM groups, when compared with controls and MGUS group, as well as in the frequency of CD25 activated $\gamma\delta^+$ T follicular-like cells in symptMM patients when compared to controls and smoulMM patients. This reduction may be due to a possible less propension of $\gamma\delta^+$ T

cells to migrate to the follicles. Although they are reduced in number, we observed a tendency for these cells to be (HLA-DR) activated in patients with monoclonal gammopathies, especially in symptMM patients, despite not reaching statistical significance. Activated $\gamma\delta^+$ T cells can act as APCs for $\alpha\beta^+$ T cells [144] and secrete IFN-γ [145], which may further help explain the increase of the frequency of follicular-like T cells observed in the BM microenvironment. However, Resende et al., reported that CXCR5-expressing $\gamma\delta^+$ T cells do not express Bcl-6, have limited expression of CD40-L and fail to produce IL-21, which are critical factors for B-cell help [65]. These results suggest that CXCR5-expressing $\gamma\delta^+$ T cells may not be follicular-like cells. Nonetheless, these cells are important for the initiation of the Tfh cell program [65]. Further studies concerning cytokine expression and production would have to be performed on BM CXCR5-expressing $\gamma\delta^+$ T cells to ascertain whether or not they are follicular-like T cells.

A decrease in CD25 activated (non-follicular non-regulatory cells) DN $\alpha\beta^+$ T cells was observed in symptMM patients when compared to MGUS and smoulMM patients. On contrary, the frequency of those cells expressing HLA-DR (late activated cells) was higher in the symptMM patient group than in controls, MGUS and smoulMM patients. DN $\alpha\beta^+$ T cells are cytotoxic cells that are able to eliminate tumour cells in a TCR-independent manner [146, 147]. FasL expressed on DN T cells connects to the Fas receptor present on target cells thereby inducing apoptosis [148, 149]. Chen et al., demonstrated that DN T cells inhibits pancreatic cancer growth through the Fas/FasL signalling pathway [149]. DN T cells have also been reported to induce cytotoxicity in melanoma target cells in a perforin/granzyme B dependent manner [150]. In fact, Merim et al., observed that DN T cells from acute myeloid leukaemia patients expressed high levels of perforin and granzyme B, and that perforin is critical for DN T cell-mediated cytotoxicity against leukaemia cells. Furthermore, they also observed increased expression of IFN-γ and TNF-α on DN T cells [151]. As already mentioned, IFN- γ may be responsible for the increase of follicular-like T cells observed in this study. Furthermore, this cytokine can exert an antiproliferative and pro-apoptotic effect on tumour cells [152, 153] and has been shown to block myeloma cell proliferation *in vitro* by both inhibiting IL-6-dependent growth of MM cells and downregulating the expression of the IL-6 receptor [154]. Contrarily, TNF-α acts as a proliferation and survival factor for myeloma cells, promoting their entry into the cell cycle as well as their long-term growth [155]. DN T cells have also been reported to secrete IL-10 and IL-17 [156], which are

upregulated in patients with multiple myeloma and promote MM cell proliferation [157, 158]. DN T cells have, therefore, a dual role in cancer immunity, acting both as inhibitors and stimulators. Their exact role in MM development is still not clear so further research is necessary. However, if further studies corroborate our findings of increased activated DN T cells in MM patients, this could perhaps be used as a marker of disease progression.

6. Conclusion

Monoclonal gammopathies arise from the proliferation of clonal PCs in the BM. It has been hypothesized that the triggering event that turns healthy PCs into pathological PCs happens in the GC, most likely during the SHM process, which takes place during antigen affinity maturation [101]. GC maintenance, as well as, GC B-cell selection depends on T-cell help, particularly those with follicular phenotype. Therefore, we aimed to study different T cell populations, focusing on follicular-like T cells, in the BM microenvironment of patients with monoclonal gammopathies.

In summary, our study observed a general tendency for follicular-like T cells to be increased in patients with monoclonal gammopathies, except for the $\gamma\delta^+$ T cell compartment, which was significantly decreased in all MM groups. Despite follicular-like T cells being increased they appear to be less activated, which may indicate these cells are impaired, or just overall less efficient in these patients compared to controls. Furthermore, significant increases in the frequency of HLA-DR activated Th reg and DN $\alpha\beta^+$ T cells in symptMM patients were also observed, which could possibly be used as markers of prediction of disease progression. However, the fact that our study population is relatively small must be taken into consideration.

The exact role of follicular T cells in the BM is unknown. Future studies aimed at studying the function of these cells in the BM microenvironment in both healthy individuals and patients with monoclonal gammopathies might further help understand the onset and progression of these disorders.

7. References

1. Kyle RA, Child JA, Anderson K, Barlogie B, Bataille R, Bensinger W, et al. Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: A report of the International Myeloma Working Group. Br J Haematol. 2003;121(5):749–57.
2. Parreira J, Lúcio P, João C, Macedo A, Sarmento AB, Geraldes C, et al. Gamapatias monoclonais de significado indeterminado: Critérios de diagnóstico e acompanhamento clínico. Acta Med Port. 2014;27(5):661–4.
3. Attaelmannan M, Levinson SS. Understanding and identifying monoclonal gammopathies. Clin Chem. 2000;46(8B):1230–8.
4. Wang H, Gao C, Xu L, Yang Z, Zhao W, Kong X. Laboratory characterizations on 2007 cases of monoclonal gammopathies in East China. Cell Mol Immunol. 2008;5(4):293–8.
5. Kyle RA, Rajkumar SV. Monoclonal gammopathy of undetermined significance and smouldering multiple myeloma: Emphasis on risk factors for progression. Br J Haematol. 2007;139(5):730–43.
6. Kyle RA, Therneau TM, Rajkumar SV, Offord JR, Larson DR, Plevak MF, et al. A Long-Term Study of Prognosis in Monoclonal Gammopathy of Undetermined Significance. N Engl J Med. 2002;346(8):564–9.
7. Kyle RA, Therneau TM, Rajkumar SV, Larson DR, Plevak MF, Offord JR, et al. Prevalence of monoclonal gammopathy of undetermined significance. N Engl J Med. 2006;354(13):1362–9.
8. Therneau TM, Kyle RA, Melton LJ, Larson DR, Benson JT, Colby CL, et al. Incidence of monoclonal gammopathy of undetermined significance and estimation of duration before first clinical recognition. Mayo Clin Proc. 2012;87(11):1071–9.
9. Landgren O, Gridley G, Turesson I, Caporaso NE, Goldin LR, Baris D, et al. Risk of monoclonal gammopathy of undetermined significance (MGUS) and subsequent multiple myeloma among African American and white veterans in the United States. Blood. 2006; 107(3):904–6.
10. Landgren O, Katzmann JA, Hsing AW, Pfeiffer RM, Kyle RA, Yeboah ED, et al. Prevalence of Monoclonal Gammopathy of Undetermined Significance Among Men in Ghana. Mayo Clin Proc. 2007;82(12):1468-73.

11. Singh J, Dudley AW Jr, Kulig KA. Increased incidence of monoclonal gammopathy of undetermined significance in blacks and its age-related differences with whites on the basis of a study of 397 men and one woman in a hospital setting. J Lab Clin Med. 1990;116(6):785-9.
12. Rajkumar SV, Dimopoulos MA, Palumbo A, Blade J, Merlini G, Mateos MV, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Vol. 15, The Lancet Oncology. Elsevier Ltd; 2014. p. e538–48.
13. Fonseca R, Bailey RJ, Ahmann GJ, Rajkumar SV, Hoyer JD, Lust JA, et al. Genomic abnormalities in monoclonal gammopathy of undetermined significance. Blood. 2002;100(4):1417-24.
14. Kyle RA, Therneau TM, Rajkumar SV, Remstein ED, Offord JR, Larson DR, et al. Long-term follow-up of IgM monoclonal gammopathy of undetermined significance. Blood. 2003;102(10):3759–64.
15. Kyle RA, Larson DR, Therneau TM, Dispenzieri A, Kumar S, Cerhan JR, et al. Long-term follow-up of monoclonal gammopathy of undetermined significance. N Engl J Med. 2018;378(3):241–9.
16. Rajkumar SV, Kyle RA, Therneau TM, Melton LJ, Bradwell AR, Clark RJ, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. *Blood* 2005; 106 (3): 812–817.
17. Kyle RA, Rajkumar SV, Therneau TM, Larson DR, Plevak MF, Melton LJ. Prognostic factors and predictors of outcome of immunoglobulin M monoclonal gammopathy of undetermined significance. Clin Lymphoma. 2005;5(4):257–60.
18. Kyle RA, Durie BGM, Rajkumar SV, Landgren O, Blade J, Merlini G, et al. Monoclonal gammopathy of undetermined significance (MGUS) and smouldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. Leukemia. 2010;24(6):1121–7.
19. Kyle RA, Rajkumar S V. Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. Leukemia. 2009;23(1):3–9.
20. Kyle RA, Remstein ED, Therneau TM, Dispenzieri A, Kurtin PJ, Hodnefield JM, et al. Clinical course and prognosis of smouldering (asymptomatic) multiple myeloma. N Engl J Med. 2007;356:2582–90.

21. Lakshman A, Vincent Rajkumar S, Buadi FK, Binder M, Gertz MA, Lacy MQ, et al. Risk stratification of smouldering multiple myeloma incorporating revised IMWG diagnostic criteria. Blood Cancer J. 2018;8(6).
22. Landgren O, Kyle RA, Pfeiffer RM, Katzmann JA, Caporaso NE, Hayes RB, et al. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: A prospective study. Blood. 2009;113(22):5412–7.
23. Ramsenthaler C, Osborne TR, Gao W, Siegert RJ, Edmonds PM, Schey SA, et al. The impact of disease-related symptoms and palliative care concerns on health-related quality of life in multiple myeloma: A multi-centre study. BMC Cancer. 2016;16,427.
24. Shephard EA, Neal RD, Rose P, Walter FM, Litt EJ, Hamilton WT. Quantifying the risk of multiple myeloma from symptoms reported in primary care patients: A large case-control study using electronic records. Br J Gen Pract. 2015;65(631):e106–13.
25. Ong F, Hermans J, Noordijk EM, Wijermans PW, Kluin-Nelemans JC. Presenting signs and symptoms in multiple myeloma: High percentages of stage III among patients without apparent myeloma-associated symptoms. Ann Hematol. 1995;70(3):149–52.
26. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, et al. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc. 2003;78(1):21–33.
27. Waxman AJ, Mink PJ, Devesa SS, Anderson WF, Weiss BM, Kristinsson SY, et al. Racial disparities in incidence and outcome in multiple myeloma: A population-based study. Blood. 2010;116(25):5501–6.
28. Castañeda-Avila MA, Ortiz-Ortiz KJ, Torres-Cintrón CR, Birmann BM, Epstein MM. Trends in cause of death among patients with multiple myeloma in Puerto Rico and the United States SEER population, 1987-2013. Int J cancer. 2020;146(1):35–43.
29. Dimopoulos MA, Moreau P, Terpos E, Mateos MV, Zweegman S, Cook G, et al. Multiple myeloma: EHA-ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2021;32(3):309–22.
30. ACTIP. Monoclonal Antibodies Approved by the EMA and FDA for Therapeutic Use (status 2017) [Internet]. La Nucia (Alicante): Animal Cell Technology Industrial Platform; 2020 [cited 2020 Jan 25]. Available from: https://www.actip.org/products/monoclonal-antibodies-approved-by-the-ema-and-fda-for-therapeutic-use/

31. Laubach JP, Richardson PG. CD38-Targeted immunochemotherapy in refractory multiple myeloma: A new horizon. Clin Cancer Res. 2015;21(12):2660–2.
32. Iftikhar A, Hassan H, Iftikhar N, Mushtaq A, Sohail A, Rosko N, et al. Investigational Monoclonal Antibodies in the Treatment of Multiple Myeloma: A Systematic Review of Agents under Clinical Development. Antibodies. 2019;8(2):34.
33. Hogan KA, Chini CCS, Chini EN. The Multi-faceted Ecto-enzyme CD38: Roles in Immunomodulation, Cancer, Aging, and Metabolic Diseases. Front Immunol. 2019;10:1–12.
34. Deaglio S, Aydin S, Grand MM, Vaisitti T, Bergui L, D'Arena G, et al. CD38/CD31 interactions activate genetic pathways leading to proliferation and migration in chronic lymphocytic leukemia cells. Mol Med. 2010;16(3–4):87–91.
35. Krejcik J, Casneuf T, Nijhof IS, Verbist B, Bald J, Plesner T, et al. Daratumumab depletes CD38+ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. Blood. 2016;128(3):384–94.
36. Touzeau C, Moreau P, Dumontet C. Monoclonal antibody therapy in multiple myeloma. Leukemia. 2017;31(5):1039–47.
37. van Rhee F, Liu Y-C, Szmania S. Profile of elotuzumab and its potential in the treatment of multiple myeloma. Blood Lymphat Cancer Targets Ther. 2014;2014(4):15.
38. Donk NWCJ, Moreau P, Plesner T, Palumbo A, Gay F, Laubach JP, et al. Clinical efficacy and management of monoclonal antibodies targeting CD38 and SLAMF7 in multiple myeloma. Blood. 2016;127(6):681–95.
39. Owen JA, Punt J, Stranford SA, Jones PP. Kuby - Immunology. 7th ed. New York: W.H. Freeman and Company; 2013.
40. Mohanty SK, Leela KS. Textbook of Immunology. 2nd ed. New Delhi: Jaypee Brothers Medical Publisher (P) Ltd; 2014.
41. Delves PJ, Martin SJ, Burton DR, Roitt IM. Roitt's Essential Immunology. 13th ed. Hoboken, NJ: John Wiley & Sons, Inc.; 2017.
42. Virella G. Medical Immunology. 5th ed. New York: Marcel Dekker, Inc.; 2001.
43. Barwick BG, Gupta VA, Vertino PM, Boise LH. Cell of origin and genetic alterations in the pathogenesis of multiple myeloma. Front Immunol. 2019;10:1121.

44. Tarte K, Zhan F, De Vos J, Klein B, Shaughnessy J. Gene expression profiling of plasma cells and plasmablasts: Toward a better understanding of the late stages of B-cell differentiation. Blood. 2003;102(2):592–600.
45. Meyer-Hermann ME, Maini PK, Iber D. An analysis of B cell selection mechanisms in germinal centers. Math Med Biol. 2006;23(3):255–77.
46. Klein U, Dalla-Favera R. Germinal centres: Role in B-cell physiology and malignancy. Nat Rev Immunol. 2008;8(1):22–33. Figure 1, The germinal centre microenvironment; p.24.
47. Noel PJ, Boise LH, Green JM, Thompson CB. CD28 co-stimulation prevents cell death during primary T cell activation. J Immunol. 1996;157(2):636–42.
48. Morin SO, Giroux V, Favre C, Bechah Y, Auphan-Anezin N, Roncagalli R, et al. In the absence of its cytosolic domain, the CD28 molecule still contributes to T cell activation. Cell Mol Life Sci. 2015;72(14):2739–48.
49. Grippi MA, Elias JA, Fishman J, Kotloff RM, Pack A, Senior RM et al. Fishman's pulmonary diseases and disorders. 5th ed. New York: McGraw-Hill Education; 2015. Figure 20-3, Generation of an immune response; p.253.
50. Kutukculer N, Azarsiz E, Aksu G, Karaca NE. CD4+CD25+Foxp3+ T regulatory cells, Th1 (CCR5, IL-2, IFN-γ) and Th2 (CCR4, IL-4, Il-13) type chemokine receptors and intracellular cytokines in children with common variable immunodeficiency. Int J Immunopathol Pharmacol. 2016;29(2):241–51.
51. Mitsdoerffer M, Lee Y, Jäger A, Kim HJ, Korn T, Kolls JK, et al. Pro-inflammatory T helper type 17 cells are effective B-cell helpers. PNAS. 2010;107(32):14292–7.
52. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J Exp Med. 2010;207(2):365–78.
53. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. J Immunol. 2007;179(12):8180–90.
54. Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk AH. Infectious tolerance: Human CD25+ regulatory T cells convey suppressor activity to conventional CD4+ T helper cells. J Exp Med. 2002;196(2):255–60.

55. Eddahri F, Oldenhove G, Denanglaire S, Urbain J, Leo O, Andris F. CD4+CD25+ regulatory T cells control the magnitude of T-dependent humoral immune responses to exogenous antigens. Eur J Immunol. 2006;36(4):855–63.
56. Stephens LA, Gray D, Anderton SM. CD4+CD25+ regulatory T cells limit the risk of autoimmune disease arising from T cell receptor cross-reactivity. PNAS. 2005;102(48):17418–23.
57. Nyström SN, Bourges D, Garry S, Ross EM, van Driel IR, Gleeson PA. Transient Treg-cell depletion in adult mice results in persistent self-reactive CD4+ T-cell responses. Eur J Immunol. 2014;44(12):3621–31.
58. Heng MK, Heng MCY. Antigen Recognition by γδ T-Cells. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013 [cited 2019 Oct 10]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK6165/
59. Mahic M, Henjum K, Yaqub S, Bjørnbeth BA, Torgersen KM, Taskén K, et al. Generation of highly suppressive adaptive CD8+CD25+FOXP3+ regulatory T cells by continuous antigen stimulation. Eur J Immunol. 2008;38(3):640–6.
60. Hu Z, Zhang W, Usherwood EJ. Regulatory CD8 + T Cells Associated with Erosion of Immune Surveillance in Persistent Virus Infection Suppress In Vitro and Have a Reversible Proliferative Defect. J Immunol. 2013;191(1):312–22.
61. Poggi A, Zocchi MR. Role of bone marrow stromal cells in the generation of human CD8+ regulatory T cells. Hum Immunol. 2008;69(11):755–9.
62. Nunes-Cabaço H, Caramalho Í, Sepúlveda N, Sousa AE. Differentiation of human thymic regulatory T cells at the double positive stage. Eur J Immunol. 2011;41(12):3604–14.
63. Chen Y, Yu M, Zheng Y, Fu G, Xin G, Zhu W, et al. CXCR5+PD-1+ follicular helper CD8 T cells control B cell tolerance. Nat Commun. 2019;10(1):1–15.
64. Leong Y, Chen Y, Ong H, Wu D, Man K, Deleage C, et al. CXCR5+ follicular cytotoxic T cells control viral infection in B cell follicles. Nat Immunol. 2016(17):1187-96.
65. Rezende RM, Lanser AJ, Rubino S, Kuhn C, Skillin N, Moreira TG, et al. γδ T cells control humoral immune response by inducing T follicular helper cell differentiation. Nat Commun. 2018;9(3151):1-13.

66. Caccamo N, Battistini L, Bonneville M, Poccia F, Fournié JJ, Meraviglia S, et al. CXCR5 Identifies a Subset of Vγ9Vδ2 T Cells which Secrete IL-4 and IL-10 and Help B Cells for Antibody Production. J Immunol. 2006;177(8):5290–5.
67. Caza T, Landas S. Functional and Phenotypic Plasticity of CD4 + T Cell Subsets. Biomed Res Int. 2015;2015:1–13.
68. Hall BM, Tran GT, Robinson CM, Hodgkinson SJ. Induction of antigen specific CD4+CD25+Foxp3+T regulatory cells from naïve natural thymic derived T regulatory cells. Int Immunopharmacol. 2015;28(2):875–86.
69. Kendal AR, Chen Y, Regateiro FS, Ma J, Adams E, Cobbold SP, et al. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. J Exp Med. 2011;208(10):2043–53.
70. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, et al. TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell–mediated pathology. Nat Immunol. 2007;8(12):1390–7.
71. Lim HW, Broxmeyer HE, Kim CH. Regulation of Trafficking Receptor Expression in Human Forkhead Box P3 + Regulatory T Cells. J Immunol. 2006;177(2):840–51.
72. Lim HW, Lee J, Hillsamer P, Kim CH. Human Th17 Cells Share Major Trafficking Receptors with Both Polarized Effector T Cells and FOXP3 + Regulatory T Cells. J Immunol. 2008;180(1):122–9.
73. Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. J Clin Invest. 2004;114(11):1640–9.
74. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal centre response. Nat Med. 2011;17(8):975–82.
75. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal centre reactions. Nat Med. 2011;17(8):983–8.
76. Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the Germinal Center Reaction by Foxp3+ Follicular Regulatory T Cells. J Immunol. 2011;187(9):4553–60.

77. Maceiras AR, Almeida SCP, Mariotti-Ferrandiz E, Chaara W, Jebbawi F, Six A, et al. T follicular helper and T follicular regulatory cells have different TCR specificity. Nat Commun. 2017;8(15067):1–12.
78. Aloulou M, Carr EJ, Gador M, Bignon A, Liblau RS, Fazilleau N, et al. Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. Nat Commun. 2016;7(10579):1–10.
79. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sungnak W, et al. Suppression by T_{FR} cells leads to durable and selective inhibition of B cell effector functions. Nat Immunol. 2016;17(12):1436–46.
80. Xystrakis E, Dejean AS, Bernard I, Druet P, Liblau R, Gonzalez-Dunia D, et al. Identification of a novel natural regulatory CD8 T-cell subset and analysis of its mechanism of regulation. Blood. 2004;104(10):3294–301.
81. James EA, Kwok WW. CD8+ suppressor-mediated regulation of human CD4+ T cell responses to glutamic acid decarboxylase 65. Eur J Immunol. 2007;37(1):78–86.
82. Aandahl EM, Torgersen KM, Taskén K. CD8+ regulatory T cells-A distinct T-cell lineage or a transient T-cell phenotype? Hum Immunol. 2008;69(11):696–9.
83. Tuovinen H, Pekkarinen PT, Rossi LH, Mattila I, Arstila TP. The FOXP3+ subset of human CD4+CD8+ thymocytes is immature and subject to intrathymic selection. Immunol Cell Biol. 2008;86(6):523–9.
84. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med. 2000;192(11):1545–51.
85. Rodríguez-Bayona B, Ramos-Amaya A, Bernal J, Campos-Caro A, Brieva JA. Cutting Edge: IL-21 Derived from Human Follicular Helper T Cells Acts as a Survival Factor for Secondary Lymphoid Organ, but Not for Bone Marrow, Plasma Cells. J Immunol. 2012;188(4):1578–81.
86. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages. Immunity. 2008;29(1):138–49.
87. Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T Follicular Helper Cells Express a Distinctive Transcriptional Profile, Reflecting Their Role as Non-Th1/Th2 Effector Cells That Provide Help for B Cells. J Immunol. 2004;173(1):68–78.

88. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. J Exp Med. 2010;207(2):353–63.
89. Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, et al. Follicular Helper T Cell Differentiation Requires Continuous Antigen Presentation that Is Independent of Unique B Cell Signaling. Immunity. 2010;33(2):241–53.
90. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. Immunity. 2011;34(6):932–46.
91. Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J Exp Med. 1999;190(8):1123–34.
92. Lu KT, Kanno Y, Cannons JL, Handon R, Bible P, Elkahloun AG, et al. Functional and Epigenetic Studies Reveal Multistep Differentiation and Plasticity of In Vitro-Generated and In Vivo-Derived Follicular T Helper Cells. Immunity. 2011;35(4):622–32.
93. Ballesteros-Tato A, Randall TD, Lund FE, Spolski R, Leonard WJ, León B. T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite. Immunity. 2016;44(2):259–73.
94. Iwasaki K, Kitahata N, Hiramitsu T, Yamamoto T, Noda T, Okada M, et al. Increased CD40L+PD-1+ follicular helper T cells (Tfh) as a biomarker for predicting calcineurin inhibitor sensitivity against Tfh-mediated B-cell activation/antibody production after kidney transplantation. Int Immunol. 2018;30(8):345–55.
95. Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan ICM. Mechanism of antigen-driven selection in germinal centres. Nature. 1989;342(6252):929–31.
96. Han S, Hathcock K, Zheng B, Kepler TB, Hodes R, Kelsoe G. Cellular interaction in germinal centres. Roles of CD40 ligand and B7-2 in established germinal centres. J Immunol. 1995;155(2):556–67.
97. Ozaki K, Spolski R, Ettinger R, Kim H-P, Wang G, Qi C-F, et al. Regulation of B Cell Differentiation and Plasma Cell Generation by IL-21, a Novel Inducer of Blimp-1 and Bcl-6. J Immunol. 2004;173(9):5361–71.

98. Takahashi Y, Ohta H, Takemori T. Fas is required for clonal selection in germinal centres and the subsequent establishment of the memory B cell repertoire. Immunity. 2001;14(2):181–92.
99. Yu D, Ye L. A Portrait of CXCR5+ Follicular Cytotoxic CD8+ T cells. Trends Immunol. 2018;39(12):965–79.
100. Perdomo-Celis F, Taborda NA, Rugeles MT. Follicular CD8+ T Cells: Origin, function and importance during HIV infection. Front Immunol. 2017;8(SEP):1–13.
101. Silva AS, Gatenby RA. Adaptation to survival in germinal centre is the initial step in onset of indolent stage of multiple myeloma. Mol Pharm. 2011;8(6):2012–20.
102. Pratt G, Goodyear O, Moss P. Immunodeficiency and immunotherapy in multiple myeloma. Br J Haematol. 2007;138(5):563–79.
103. Ogawara H, Handa H, Yamazaki T, Toda T, Yoshida K, Nishimoto N, et al. High Th1/Th2 ratio in patients with multiple myeloma. Leuk Res. 2005;29(2):135–40.
104. Magalhães RJP, Vidriales MB, Paiva B, Fernandez-Gimenez C, García-Sanz R, Mateos MV, et al. Analysis of the immune system of multiple myeloma patients achieving long-term disease control by multidimensional flow cytometry. Haematologica. 2013;98(1):79–86.
105. Kay NE, Leong TL, Bone N, Vesole DH, Greipp PR, Van Ness B, et al. Blood levels of immune cells predict survival in myeloma patients: Results of an Eastern Cooperative Oncology Group phase 3 trial for newly diagnosed multiple myeloma patients. Blood. 2001;98(1):23–8.
106. Koike M, Sekigawa I, Okada M, Matsumoto M, Iida N, Hashimoto H, et al. Relationship between CD4+/CD8+ T cell ratio and T cell activation in multiple myeloma: Reference to IL-16. Leuk Res. 2002;26(8):705–11.
107. Mills KHG, Cawley JC. Abnormal monoclonal antibody-defined helper/suppressor T-cell subpopulations in multiple myeloma: relationship to treatment and clinical stage. Br J Haematol. 1983;53(2):271–5.
108. Miguel JFS, González M, Gascón A, Moro J, Hernández JM, Ortega F, et al. Lymphoid subsets and prognostic factors in multiple myeloma. Br J Haematol. 1992;80(3):305–9.
109. Péres-Andres M, Almeida J, Martin-Ayuso M, Moro MJ, Martin-Nuñez G, Galende J, et al. Characterization of bone marrow T cells in monoclonal gammopathy of

undetermined significance, multiple myeloma, and plasma cell leukemia demonstrates increased infiltration by cytotoxic/Th1 T cells demonstrating a squed TCR-Vβ repertoire. Cancer. 2006;106(6):1296–305.

110. Feng P, Yan R, Dai X, Xie X, Wen H, Yang S. The Alteration and Clinical Significance of Th1/Th2/Th17/Treg Cells in Patients with Multiple Myeloma. Inflammation. 2015;38(2):705–9.

111. Prabhala RH, Neri P, Bae JE, Tassone P, Shammas MA, Allam CK. Dysfunctional T regulatory cells in multiple myeloma. Blood. 2006;107(1):301–4.

112. Beyer M, Kochanek M, Giese T, Endl E, Weihrauch MR, Knolle PA, et al. In vivo peripheral expansion of naive $CD4^+$ $CD25^{high}$ $FoxP3^+$ regulatory T cells in patients with multiple myeloma. 2006;107(10):3940–9.

113. Lad D, Huang Q, Hoeppli R, Garcia R, Xu L, Song K, et al. Evaluating the role of Tregs in the progression of multiple myeloma. Leuk Lymphoma. 2019;60(9):2134–42.

114. Allegra A, Innao V, Allegra AG, Pugliese M, Di Salvo E, Ventura-Spagnolo E, et al. Lymphocyte Subsets and Inflammatory Cytokines of Monoclonal Gammopathy of Undetermined Significance and Multiple Myeloma. Int J Mol Sci. 2019;20(11):1–25.

115. Zhou DM, Xu YX, Zhang LY, Sun Y, Wang ZY, Yuan YQ, et al. The role of follicular T helper cells in patients with malignant lymphoid disease. Hematology. 2017;22(7):412–8.

116. Brenne AT, Ro TB, Waage A, Sundan A, Borset M, Hjorth-Hansen H. Interleukin-21 is a growth and survival factor for human myeloma cells. Blood. 2002;99(10):3756–62.

117. Rawal S, Chu F, Zhang M, Park HJ, Nattamai D, Kannan S, et al. Cross Talk between Follicular Th Cells and Tumor Cells in Human Follicular Lymphoma Promotes Immune Evasion in the Tumor Microenvironment. J Immunol. 2013;190(12):6681–93.

118. Quach H, Ritchie D, Stewart AK, Neeson P, Harrison S, Smyth MJ, et al. Mechanism of action of immunomodulatory drugs (IMiDS) in multiple myeloma. Leukemia. 2010;24(1):22–32.

119. LeBlanc R, Hideshima T, Catley LP, Shringarpure R, Burger R, Mitsiades N, et al. Immunomodulatory drug costimulates T cells via the B7-CD28 pathway. Blood. 2004;103(5):1787–90.

120. Galustian C, Meyer B, Labarthe MC, Dredge K, Klaschka D, Henry J, et al. The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. Cancer Immunol Immunother. 2009;58(7):1033–45.
121. Büscher M. Flow Cytometry Instrumentation – An Overview. Curr Protoc Cytom. 2019;87(1):1–16.
122. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Crit Rev Biotechnol. 2017;37(2):163–76.
123. O'Donnell EA, Ernst DN, Hingorani R. Multiparameter Flow Cytometry: Advances in High Resolution Analysis. Immune Netw. 2013;13(2):43.
124. Raja KRM, Kovarova L, Hajek R. Review of phenotypic markers used in flow cytometric analysis of MGUS and MM, and applicability of flow cytometry in other plasma cell disorders. Br J Haematol. 2010;149(3):334–51.
125. Rawstron AC, Orfao A, Beksac M, Bezdickova L, Brooimans RA, Bumbea H, et al. Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders. Haematologica. 2008;93(3):431–8.
126. Betters DM. Use of Flow Cytometry in Clinical Practice. J Adv Pract Oncol. 2015;6(5):435–40.
127. Feyler S, Von Lilienfeld-Toal M, Jarmin S, Marles L, Rawstron A, Ashcroft AJ, et al. CD4+CD25+FoxP3+ regulatory T cells are increased whilst CD3+CD4-CD8 - αβTCR+ Double Negative T cells are decreased in the peripheral blood of patients with multiple myeloma which correlates with disease burden. Br J Haematol. 2009;144(5):686–95.
128. Muthu Raja KR, Rihova L, Zahradova L, Klincova M, Penka M, Hajek R. Increased T Regulatory Cells Are Associated with Adverse Clinical Features and Predict Progression in Multiple Myeloma. PLoS One. 2012;7(10).
129. Foglietta M, Castella B, Mariani S, Coscia M, Godio L, Ferracini R, et al. The bone marrow of myeloma patients is steadily inhabited by a normal-sized pool of functional regulatory T cells irrespective of the disease status. Haematologica. 2014;99(10):1605–10.
130. Wang JN, Cao XX, Zhao AL, Cai H, Wang X, Li J. Increased activated regulatory T cell subsets and aging Treg-like cells in multiple myeloma and monoclonal gammopathy of undetermined significance: A case control study. Cancer Cell Int. 2018;18(1):1–8.

131. Baecher-Allan C, Wolf E, Hafler DA. MHC Class II Expression Identifies Functionally Distinct Human Regulatory T Cells. J Immunol. 2006;176(8):4622–31.
132. Fuhrman CA, Yeh W-I, Seay HR, Saikumar Lakshmi P, Chopra G, Zhang L, et al. Divergent Phenotypes of Human Regulatory T Cells Expressing the Receptors TIGIT and CD226. J Immunol. 2015;195(1):145–55.
133. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. Immunity. 2014;40(4):569–81.
134. Lozano AE, Mena M, Díaz T, Martin-Antonio B, León S. Nectin-2 expression on malignant plasma cells is associated with better response to TIGIT blockade in multiple myeloma. Clin Cancer Res. 2020;26(17):4688–98.
135. Yu X, Harden K, C Gonzalez L, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol. 2009;10(1):48–57.
136. Lauta VM. A review of the cytokine network in multiple myeloma: Diagnostic, prognostic, and therapeutic implications. Cancer. 2003;97(10):2440–52.
137. Brandt D, Sergon M, Abraham S, Mäbert K, Hedrich CM. TCR+ CD3+ CD4− CD8− effector T cells in psoriasis. Clin Immunol. 2017;181:51–9.
138. Crispín JC, Tsokos GC. Human TCR-αβ + CD4 − CD8 − T Cells Can Derive from CD8 + T Cells and Display an Inflammatory Effector Phenotype. J Immunol. 2009;183(7):4675–81.
139. Wang B, Ma N, Cheng H, Zhou H, Qiu H, Yang J, et al. Effects of ICOSLG expressed in mouse hematological neoplasm cell lines in the GVL reaction. Bone Marrow Transplant. 2013;48(1):124–8.
140. Maceiras AR, Fonseca VR, Agua-Doce A, Graca L. T follicular regulatory cells in mice and men. Immunology. 2017;152(1):25–35.
141. Sage PT, Alvarez D, Godec J, Von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. J Clin Invest. 2014;124(12):5191–204.
142. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal centre reactions. Nat Med. 2011;17(8):983–8.

143. Sage PT, Sharpe AH. T follicular regulatory cells. Immunol Rev. 2016;271(1):246–59.

144. Brandes M, Willimann K, Moser B. Professional Antigen-Presentation Function by Human γδ T Cells. Science. 2005;309(5732):264–8

145. Kunzmann V, Bauer E, Feurle J, Weißinger F, Tony H, Wilhelm M. Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Hematology. 2000;96(2):384–92.

146. Li Y, Dong K, Fan X, Xie J, Wang M, Fu S, et al. DNT Cell-based Immunotherapy: Progress and applications. J Cancer. 2020;11(13):3717–24.

147. Lee JB, Minden MD, Chen WC, Streck E, Chen B, Kang H, et al. Allogeneic human double negative t cells as a novel immunotherapy for acute myeloid leukemia and its underlying mechanisms. Clin Cancer Res. 2018;24(2):370–82.

148. Young KJ, Kay LS, Phillips MJ, Zhang L. Antitumor Activity Mediated by Double-Negative T Cells. Cancer Res. 2003;63(22):8014–21.

149. Chen J, Hu P, Wu G, Zhou H. Antipancreatic cancer effect of DNT cells and the underlying mechanism. Pancreatology. 2019;19(1):105–13.

150. Voelkl S, Moore T V., Rehli M, Nishimura MI, Mackensen A, Fischer K. Characterization of MHC class-I restricted TCRαβ+ CD4− CD8− double negative T cells recognizing the gp100 antigen from a melanoma patient after gp100 vaccination. Cancer Immunol Immunother. 2009;58(5):709–18.

151. Merims S, Li X, Joe B, Dokouhaki P, Han M, Childs RW, et al. Anti-leukemia effect of ex vivo expanded DNT cells from AML patients: A potential novel autologous T-cell adoptive immunotherapy. Leukemia. 2011;25(9):1415–22.

152. Castro F, Cardoso AP, Gonçalves RM, Serre K, Oliveira MJ. Interferon-gamma at the crossroads of tumor immune surveillance or evasion. Front Immunol. 2018;9(MAY):1–19.

153. Detjen KM, Farwig K, Welzel M, Wiedenmann B, Rosewicz S. Interferon γ inhibits growth of human pancreatic carcinoma cells via caspase-1 dependent induction of apoptosis. Gut. 2001;49(2):251–62.

154. Portier M, Zhang XG, Caron E, Lu ZY, Bataille R, Klein B. γ-Interferon in multiple myeloma: Inhibition of interleukin-6 (IL-6)- dependent myeloma cell growth and downregulation of IL-6-receptor expression in vitro. Blood. 1993;81(11):3076–82.

155. Jourdan M, Tarte K, Legouffe É, Brochier J, Rossi JF, Klein B. Tumor necrosis factor is a survival and proliferation factor for human myeloma cells. Eur Cytokine Netw. 1999;10(1):65–70.

156. Brandt D, Hedrich CM. TCRαβ+CD3+CD4−CD8− (double negative) T cells in autoimmunity. Autoimmun Rev. 2018;17(4):422–30.

157. Alexandrakis MG, Goulidaki N, Pappa CA, Boula A, Psarakis F, Neonakis I, et al. Interleukin-10 Induces Both Plasma Cell Proliferation and Angiogenesis in Multiple Myeloma. Pathol Oncol Res. 2015;21(4):929–34.

158. Wang S, Ma Y, Wang X, Jiang J, Zhang C, Wang X, et al. IL-17A Increases Multiple Myeloma Cell Viability by Positively Regulating Syk Expression. Transl Oncol. 2019;12(8):1086–91.