UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di Ricerca in Oncologia e Chirurgia Sperimentali
Dipartimento di Discipline Chirurgiche, Oncologiche e Stomatologiche
Settore Scientifico Disciplinare MED 15

EVALUATION AND POTENTIAL ROLE OF BONE MARROW TISSUE-RESIDENT MEMORY T-CELLS IN PATIENTS WITH PLASMA CELL DYSCRASIAS

IL DOTTORE
Dott. Melania Carlisi

IL COORDINATORE
Prof. Antonio Russo

IL TUTOR
Prof. Sergio Siragusa

CICLO XXXII
ANNO CONSEGUIMENTO TITOLO 2020

Sommario

INTRODUCTION

One of the main characteristics of the immune system is the "immunological memory" that is the possibility, following exposure to a specific antigen, to enhance the future ability to respond to that same antigen with a faster and more intense secondary immune response. This immune memory is guaranteed as both B and T memory cells are more efficient than virgin cells.

In the T lymphocyte group, the naïve T-cells, once activated by a specific antigen, undergo a huge clonal expansion and at the same time differentiate into a short-lived effector, which generally dies at the end of the immune response, and in long-lived memory T-cells, ready to provide protection in case of new contact with the same antigen.

These memory T-cells can be localized in any part of the body, with a particular preference for the bone marrow, in which they remain for a long time with the ability to recirculate. In the group of memory T-cells, two subgroups are distinguished, in relation to the phenotype and functional potential: the "central memory cells (T_{CM})", which express the CCR7 chemokine receptor at high intensity, and the " effector memory T cells (T_{EM}) "which do not express CCR7. In the panorama of lymphocyte T memory we can also find a group of non-circulating resident memory T cells (T_{RM}), which live permanently in some peripheral tissues and where they survive in a state of quiescence.

It appears that circulating memory T cells are responsible for systemic immunity, while T_{RM} cells represent immediate protection in the main pathogen entry sites, mostly participating in local defence.

Several studies have shown that bone marrow (BM) is included among the organs in which TRM cells can be observed.

In fact, BM is considered to be the largest T-cell memory maintenance site, since it has a high frequency of antigen-specific T memory lymphocytes against vaccines, pathogens and several tumor antigens. The BM in this case provides survival signals, making the interaction with stromal cells expressing homeostatic cytokines, such as IL-7 or IL-15. BM T_{RM} cells are phenotypically characterized by the expression of CD69 and by the integrins CD103 and VLA1, molecules that contribute to the localization of these cells in the tissues.

In recent years, we have also learned that T_{RM} can contribute to the defence of the host in the neoplastic conditions, representing potentially reactive cells against hematological tumors infiltrating the bone marrow. For example, this has been proven in some studies of "adoptive BM T-cell therapy" in multiple myeloma cases.

In relation to the latter topic and to the possibility that T_{RM} cells may have a role in the immunological surveillance in the course of neoplasms, including plasma cell dyscrasias, we conducted a study aimed at the description and functional analysis of bone marrow T_{RM} in patients with new diagnosis of multiple myeloma.

The aim of this study is to evaluate the frequency and phenotype of CD8 + memory T lymphocytes resident in the BM of patients with multiple myeloma. In fact, the CD8+ T_{RM} could represent a source of tumor-specific effector cells capable of controlling tumor growth. In this case, they can provide an immunological marker of prognosis of the pathology or, even more, they can become a specific target in the context of the most modern immunotherapy.

1. MULTIPLE MYELOMA AND IMMUNOTHERAPY

1.1 Multiple Myeloma

Multiple myeloma (MM) is a malignant neoplasm characterized by the clonal expansion of plasma cells that can release monoclonal immunoglobulins (protein M, or paraprotein or monoclonal component), or their fractions (free light chains, FLC) [1].

MM is part of a broader spectrum of diseases called "plasma cell dyscrasias" or "plasma cell disorders", which include other conditions such as monoclonal gammopathy of undetermined significance, smoldering multiple myeloma, solitary plasmacytoma, Waldenström's macroglobulinemia, amyloidosis AL and POEMS syndrome.

All plasma cell dyscrasias are characterized by the accumulation and expansion of monoclonal plasma cells. In most cases, these pathological plasma cells maintain the ability to produce and release immunoglobulins or parts of them with the production therefore of identical Ig.

1.1.1 Epidemiology

MM accounts for 1.7% of all cancers and 10% of all hematological malignancies in the United States. However, globally the incidence varies and is higher in more developed countries such as the United States and Europe; moreover, the incidence is 2-3 times higher in black than in white people, although it is lower in Asians and Hispanics [2].

Most patients are between 65 and 74 years old at the time of diagnosis, with an average age of 69. Only 4% of cases concern subjects under the age of 45. Men are more affected than women (1.6: 1).

The prevalence of MM has increased compared to the past, due to the improvement of diagnostic techniques and the increase in survival (an ever increasing use of autologous transplantation and development of new therapeutic agents) [3].

Several epidemiological studies have shown that there is a greater predisposition to MM development (up to 4 times) in the first-degree relatives of individuals suffering from plasma cell dyscrasias [4]. Furthermore, there is also a correlation with other hematological malignancies; a recent study has shown the existence of common biological pathways that determine the development of MM and chronic lymphatic leukemia (LLC) [5].

Among the environmental factors, some studies have shown an association between MM and body mass index: in obese people, adipocytes secrete greater quantities of IL-6 and IGF-1, that are useful for the growth and survival of myeloma cells [6].

Another hypothesis suggests a possible role of chronic immune stimulation such as occurs during allergies, infections (HIV, HCV) or other autoimmune disorders [7].

1.1.2 Pathogenesis, genetic alterations and bone marrow microenvironment

It is believed that the development of MM is a multistep process, characterized by the initial establishment of specific genetic lesions in neoplastic cells, the formation of a privileged relationship with the marrow microenvironment (that contributes to the growth, survival, migration and drug resistance of myeloma cells) and the subsequent appearance of further genetic aberrations that make the neoplastic clone independent of external factors for its own expansion.

In fact, it seems possible to postulate that all patients diagnosed with MM have had a previous phase corresponding to a condition of monoclonal gammopathy of uncertain significance (MGUS) [8, 9]. This can remain stable for years or it can progress through the phases of asymptomatic or smoldering MM (SMM), in which

there are still no signs of organ damage, as far as the symptomatic MM. The final evolution may be the loss of dependence on the marrow microenvironment, with the dissemination of myeloma cells in the blood circulation (plasma cell leukemia) or the invasion of other tissues (extramedullary myeloma).

MM is a heterogeneous disease with a complex genetic background characterized by anomalies in the karyotype, chromosomal translocations and gene duplications or deletions.

The first genetic "event" of the aforementioned multistep process could occur during the isotype switch process with translocations involving the IgH locus (the locus containing the heavy chain gene) on chromosome 14q32. These translocations are considered primary events since they are found with the same frequency in both MGUS and MM.

The MM clone, for its instability, can undergo further modifications (secondary genetic anomalies) over time.

On the basis of the chromosomal kit it is possible to divide patients with MM into two groups: hyperdiploid MM and non-hyperdiploid MM.

Hyperdiploid patients show trisomies in chromosomes 3, 5, 7, 9, 11, 15, 19 and 21 and generally have a more favourable prognosis. In non-hyperdiploid MM, translocations commonly involving the loci of heavy chains or, less frequently, light chains.

The most frequent translocations involving the 14q32 locus are 11q13 (15%), 4p16 (15%), 16q23 (5%), 20q11 (2%) and 6p21 (5%), while the genes involved are those of cyclin D1, cyclin D3, the MMSET / FGFR3 genes and the c-MAF gene [8].

The deletion of chromosome 13, deletion of chromosome 17p13 and amplification of chromosome 1q21 are the most frequent genetic aberrations.

The presence of the 1p23 deletion is important, especially for the prognosis [10].

In conclusion, other genetic alterations could be present in the MM pathogenesis such as N- and K-Ras mutations, involved in the activation of the NF-kB pathway [11] and the activity of small non-coding RNAs, called microRNAs (miRNAs) with an important role in the regulation of oncogenes and therefore in neoplastic transformation [12].

The BM microenvironment certainly plays a fundamental role in the development and maintenance of the plasma cell clone. It consists of an extracellular compartment (matrix) consisting of proteins, such as collagen, laminin, fibronectin, and numerous hematopoietic and non-haemopoietic cells (medullary stromal cells), such as osteoblasts, osteoclasts, fibroblasts and immune effector cells. Plasma cells are recalled to the BM through a homing process driven by the CXCL12 / SDF-1 chemokine produced by stromal cells [13]. Once in the BM, the plasma cells, through cell-cell reciprocal interactions (homotypic or heterotypic) and cell-matrix, survive, proliferate and become chemoresistant.

A crucial role is played by IL-6, produced by stromal cells and, partly, MM cells [14], but there are many cytokines involved and one of the most activated molecular pathways is the NF-κB pathway (*nuclear factor κB*) [15].

The formation of new vessels induced by VEGF satisfies both the nutritional intake and the transmission of further cytokines; an increased microvascular density (microvessel density, MVD) in BM appears to correlate with disease progression and with a poorer prognosis [16].

1.1.2 Diagnosis and Staging

The International Myeloma Working Group (IMWG) updated the MM diagnostic criteria in 2014 [17]. The diagnosis requires a quantity of plasma cells in the BM ≥

10% or the presence of a bone or extramedullary plasmocytoma associated with one of the following *myeloma-defining events* (MDE):

- Evidence of organ damage. The main clinical manifestations are summarized in the English acronym CRAB: hyperCalcemia, Renal failure, Anemia and Bone lesions;
- One of the following biomarkers of malignancy (SLiM criteria):
    - Quantity of BM plasma cells ≥ 60%;
    - Serum free light chains ratio (rFLC) (involved/not involved) ≥ 100 (the chain involved must be present in an amount greater than 100 mg / L);) ≥ 100
    - ≥ 1 focal lesion in MRI (each of which must be ≥ 5mm).

In most cases, the key element supporting the suspected diagnosis is the finding of a monoclonal component (MC) in serum and/or urine. The monoclonal peak is shown at the serum proteins electrophoresis as a net band in the γ region, more rarely in the β or 2 regions (Fig. 1).

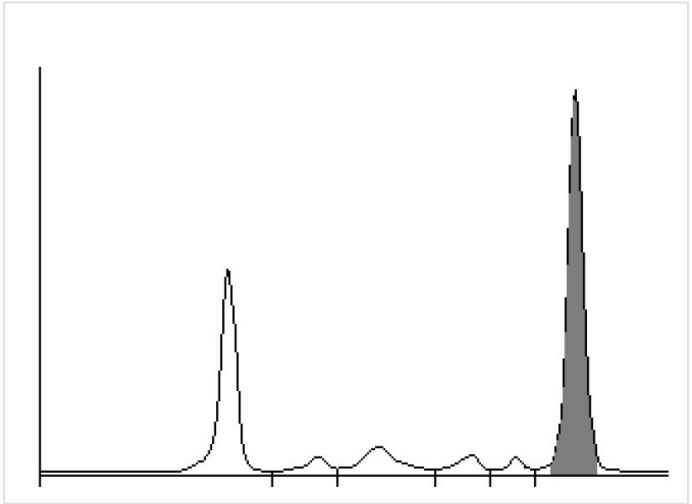


Fig. 1. Serum protein electrophoresis of a patient with MM. The monoclonal component, located in the γ area, is equal to 5.04 g dL.

Once the presence of the monoclonal peak at the electrophoresis is underlined, the nature of the secreted immunoglobulin (typing or immunological characterization) is confirmed with the electrophoretic immunofixation (IFE) or with the immunosottration (ISE), more specific and sensitive technique of the electrophoresis. Not all MM isotypes have the same incidence: 52% is IgG, 21% is IgA, 16% is a micromolecular or light chain MM (LCMM), 2% is bi-clonal (the light chain or heavy chain may be different) and 0.5% is IgM; IgD and IgE MM are rare [18]. Furthermore, a percentage of patients may present an oligosecreting MM or a non-secretory MM, in which the MC is null. In these last two groups of patients it may be useful to evaluate the serum rFLC; in fact, an unbalanced ratio indicates the presence of a clonal disorder [19].

Furthermore, a reduction in γ-globulin not included in the monoclonal peak may be evident; this phenomenon can be evaluated more precisely by measuring serum immunoglobulins (IgM, IgG, IgA) with a reduction in the non-clonal type (phenomenon of retroinhibition or immunoparesis) [20].

The 24-hour urine collection and its study can reveal the presence of a Bence-Jones proteinuria (PBJ) that is the presence of significant urinary FLC. For the PBJ research, urine immunofixation is used and this is the only one with which it is possible to ascertain the two characteristics of the PBJ: the monoclonality and the absence of the heavy chain.

The blood count can show normochromic anemia, leukopenia and/or thrombocytopenia. Blood tests show an increase in serum calcium, urea, uraemia, creatinine, LDH and β2-microglobulin (B_2M). The latter is important in terms of prognostic stratification; its levels related to the stage of myeloma and survival [21, 22]. The MM diagnosis also requires the bone marrow assessment.

MM cells express CD38 and CD138 on their surface and, unlike normal ones, present a weak or absent expression of CD19, CD27, CD45, and CD81 and elevated levels of CD28, CD33 and CD56. Sometimes they can express CD20 and CD117 aberrantly [23, 24]. From a histological point of view, the neoplastic infiltrate can develop with a widespread growth pattern, or it can be distributed in the interstice or organized into nodules (*patchy*).

The bone damage present in MM patients can be evidenced by several radiological investigations. Traditional radiography allows the detection of lytic lesions or diffuse osteopenia when the loss of trabecular bone exceeds 50% of the basal values. For this reason, it has been supplanted by the much more sensitive skeleton CT at low doses. The lesions are variable, from osteoporosis to the presence of crushed and deformed vertebral bodies due to vertebral collapses, but the typical alteration is that of an osteolytic lesion lacking the usual peripheral radiopaque osteo-thickening oracle. It is also useful to combine CT/PET with 18F-FDG, in particular to evaluate the presence of extramedullary disease. The risk of pathological fractures or neurological compression should be assessed in patients with osteolytic lesions. In this sense, MRI is preferred for revealing medullary compressions.

Obviously, monoclonal plasma cellular disorders must be distinguished from the polyclonal forms of marrow plasmacytosis that can occur in autoimmune disorders, chronic liver diseases, chronic infections and AIDS.

The variable outcome of patients with MM depends on biological differences, tumor load and patient characteristics. In MM staging, the use of the Durie and Salmon classification, which allowed the estimate of the burden of disease based on the magnitude of the neoplastic mass and the extension of the terminal organ

damage of the host, was adopted for years [25]. This allowed defining disease stages with distinct expectations of survival.

More recently, a new staging system (ISS) has been introduced into clinical practice. It is based on the concentrations of β_2-microglobulin and albumin at the time of diagnosis. The patients are then divided into three prognostic groups related to progressively lower overall survival [26]. The ISS was amended in 2015 by adding the genetic risk assessment by FISH and the LDH values [27]. The ISS and R-ISS provide prognostic information at diagnosis but have not been validated in relapsed or refractory MM.

STAGE	DS	ISS	R-ISS
I	All of the following: - Hb > 10 mg/dl - Serum calcium normal or ≤ 12 mg/dl - Bone x-ray: normal bone structure (scale 0) or solitary bone plasmacytoma only - Low M-component production rate: IgG <5 g/dl, IgA < 3 g/dl and Bence-Jones protein < 4 g/24 hours	- Serum B_2M ≤ 3,5 g/dl - Serum albumin ≥ 3,5 g/dl	- ISS I - Standard-risk chromosomal abnormalities* - Normal LDH
II	Neither stage I nor stage III	Neither stage I nor stage III according to the following subcategories: • Serum B_2M < 3,5 g/d and serum albumin < 3,5 g/dl; • Serum B_2M 3,5 - 5,5 g/dl irrespective of serum albumin level	
III	One or more of the following: - Hb < 8,5 g/dl - Serum calcium > 12 mg/dl - Advanced lytic bone lesions (scale 3)	- Serum B_2M ≥ 5,5 g/dl	- ISS III - High-risk chromosomal abnormalities ** - High LDH

	- High M-component production rate: IgG > 7 g/dl, IgA > 5 g/dl and Bence-Jones protein > 2 g/24 hours
	A: creatinine > 2mg/dl **B**: creatinine ≥2mg/dl

Table 1. Staging systems DS, ISS e R-ISS
*Standard-risk chromosomal abnormalities= absence of high-risk chromosomal abnormalities
** High-risk chromosomal abnormalities = del(17p) e/o t(4;14) e/o t(14;16)

An adequate therapeutic choice also depends on the patient characteristics, and for this reason, a correct evaluation of the latter should always be considered. In 2016, the IMWG published the result of a study conducted on 869 patients with a new diagnosis of MM, all undergoing a pre-treatment geriatric assessment. This study demonstrated not only age but also the comorbidity burden and the performance status of patients in everyday life are variables able to stratify three distinct groups of patients, each of which characterized by a different tolerance to the treatment and a different survival outcome [28]. The comorbidity burden was assessed by the Charlson comorbidity index (CCI), while the ADL and IADL scales defined the performance status. A score was then defined (later nicknamed "Frailty score") consisting of four variables: age, CCI, ADL and IADL. This score stratified the patients into three groups: fit, intermediate fit and frail. Compared to fit patients, those defined as “frail” have a higher, statistically significant, probability of facing non-haematological adverse events and death and/or disease progression.

1.1.3 General information on treatment

Despite the considerable progresses made in recent years in the therapeutic field, MM remains today an incurable disease, characterized by an alternation of latency and recurrence phases, with a chronic course of the pathology.

At the moment, only the symptomatic MM or associated with one of the biological markers (MDE) must be treated.

Once the need for an anti-myeloma treatment has been ascertained, the patient is subjected to a first line therapy; it is followed by remission periods of varying lengths, based on the effectiveness of the treatment itself. Almost inevitably, however, the plasma cell clone returns to proliferate, thus configuring a recurrent disease.

Subsequent to a possible second line therapy there is a new period of remission, the length of which is widely variable and dependent on several factors.

In the early 1980s, the combination of vincristine, doxorubicin, and dexamethasone (VAD) was used as standard induction therapy. Today MM therapy has significantly changed through the use of new drugs, which work differently from traditional drugs. Among these there are proteasome inhibitors (Bortezomib, Carfilzomib, Ixazomib), immunomodulating drugs (Thalidomide, Lenalidomide, Pomalidomide), inhibitors of histone deacetylases (Panobinostat) and monoclonal antibodies directed against molecules expressed on the surface of plasma cells (Daratumumab, Elotuzumab).

The central issue in choosing the correct therapy is the eligibility of patients for autologous stem cell transplantation (ASCT). The choice depends on the patient's age, comorbidities and performance status.

Patients under 65 years old and fit patients under 70 years old (normal cardiac, pulmonary and hepatic function) are considered suitable for autologous transplantation.

Maintenance therapy, on the other hand, is aimed at maintaining the response obtained with a treatment at low dosages for longer.

Patients older than 65-70, not eligible for transplantation, undergo chemotherapy regimens, with the dosage modified according to the patient's frailty.

A refractory MM is a disease that has not presented at least a partial response to 3 or more cycles of anti-myeloma therapy or that has progressed within 60 days of the last treatment.

On the other hand, we define a relapsed MM as a disease that requires a new rescue therapy after a partial or complete remission interval of at least 60 days. The latter is defined as "biochemical relapse" in the presence of plasma cell proliferation and the consequent increase in the monoclonal blood and urine component; or it is defined "clinical relapse" when it follows the appearance of new symptoms. The IMWG guidelines recommend starting a new therapy line in cases of clinical recurrence or "aggressive" biochemical recurrence, i.e. characterized by a rapid increase in the monoclonal component.

It is clear that a correct evaluation of the treatment response is essential in MM management. In 2016, the IMWG criteria for response and definition of the minimum residual disease (MRD) were updated (Table 2).

A fundamental part of the patient's therapy with MM is the so-called supportive therapy. In fact, as highlighted, the manifestations occur at systemic level and determine a multi-organ involvement.

Bisphosphonates (BSF) are the primary therapeutic aid in the treatment of bone disease. Denosumab, an antibody against RANK-L, can also be used in the prevention of SRE in patients with MM [29]. Orthopedic surgery must be considered in the event of fractures to long bones, instability or vertebral collapses with spinal or radicular compression. Both vertebroplasty and kyphoplasty give good analgesic and biomechanical results [30]. Radiation therapy, on the other hand, is useful for analgesic purposes and for skeletal localizations of disease at risk of fracture [31].

Table 2. International Myeloma Working Group consensus criteria for response and minimal disease assessment in multiple myeloma

Response category	Response criteria
Complete Response (CR)	Negative immunofixation on the serum and urine and disappearance of any soft tissue plasmacytomas and < 5% plasma cells in bone marrow
Complete Response stringent (sCR)	CR as defined below plus normal FLC ratio and absence of clonal cells in bone marrow3 by immunohistochemistry or immunofluorescence
Very Good Partial Response (VGPR)	Serum and urine M-protein detectable by immunofixation but not on electrophoresis or > 90% reduction in serum M-protein plus urine M-protein level < 100 mg/24 h
Partial Response (PR)	> 50% reduction of serum M-protein and reduction in 24 hours urinary M-protein by >90% or to < 200 mg/24 h. If the serum and urine M-protein are unmeasurable,5 a > 50% decrease in the difference between involved and uninvolved FLC levels is required in place of the M-protein criteria If serum and urine M-protein are not measurable, and serum free light assay is also not measureable, > 50% reduction in plasma cells is required in place of M-protein, provided baseline bone marrow plasma cell percentage was > 30% In addition to the above listed criteria, if present at baseline, a > 50% reduction in the size of soft tissue plasmacytomas is also required
Stable Disease (SD)	Not meeting criteria for CR, VGPR, PR, or progressive disease
Progressive Disease (PD)	Increase of > 25% from lowest response value in any one or more of the following: • Serum M-component and/or (the absolute increase must be > 0.5 g/dL) • Urine M-component and/or (the absolute increase must be > 200 mg/24 h) • Only in patients without measurable serum and urine M-protein levels; the difference between involved and uninvolved FLC levels. The absolute increase must be > 10 mg/dL • Bone marrow plasma cell percentage; the absolute percentage must be > 10% • Definite development of new bone lesions or soft tissue plasmacytomas or definite increase in the size of existing bone lesions or soft tissue plasmacytomas;

	• Development of hypercalcemia (corrected serum calcium > 11.5 mg/dL or 2.65 mmol/L) that can be attributed solely to the plasma cell proliferative disorder
"Sustained MRD-negative"	MRD negativity in the marrow (NGF or NGS, or both) and by imaging as defined below, confirmed minimum of 1 year apart. Subsequent evaluations can be used to further specify the duration of negativity (e.g., MRD-negative at 5 years)
"Flow MRD-negative"	Absence of phenotypically aberrant clonal plasma cells by NGF on bone marrow aspirates using the EuroFlow standard operation procedure for MRD detection in multiple myeloma (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
"Sequencing MRD-negative"	Absence of clonal plasma cells by NGS on bone marrow aspirate in which presence of a clone is defined as less than two identical sequencing reads obtained after DNA sequencing of bone marrow aspirates using the LymphoSIGHT platform (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
"Imaging-positive MRD-negative"	MRD negativity as defined by NGF or NGS plus disappearance of every area of increased tracer uptake found at baseline or a preceding PET/CT or decrease to less mediastinal blood pool SUV or decrease to less than that of surrounding normal tissue

1.2. Immunotherapy in Multiple Myeloma

In the last 20 years, the introduction of autologous stem cell transplantation, followed by proteasome inhibitors and immunomodulatory agents increased the survival of MM patients by 50%. However, a high proportion of patients relapse and become refractory, especially patients with adverse cytogenetic.

Therefore, novel strategies, such as immunotherapy, have been developed in the last few years to improve the survival of these patients. Immunotherapy treatments include a high number of different strategies used to attack the tumor cells by using the immune system.

Interestingly, immunotherapy has arisen as a new modality treatment with very promising results and less toxic effects. Recent drugs include the next generation of PIs (carfilzomib, ixazomib, marizomib, and oprozomib), small and targeted molecules such as histone deacetylase inhibitors, venetoclax, selinexor, Hsp90 inhibitors and PI3K/AKT/mTOR inhibitors, which are currently under development. Immunotherapy also includes other treatments, such as monoclonal antibodies, bi-specific T cell engaging antibodies (BiTEs), bi-specific antibodies, antibody-drug conjugates (ADC), immune checkpoint inhibitors and adoptive cell immunotherapy. A specific strategy includes the immunotherapy treatment based on antibodies that target antigens expressed on tumor cells. Whereas normal plasma cells express CD38, CD138, CD19 and CD45, malignant PCs loose CD45 and CD19 and usually acquire high expression of CD56 and CD117 [32].

The panel of surface plasma cell markers includes CD150 (SLAMF1), CD48 (SLAMF2), CD229 (SLAMF3), CD352 (SLAMF6), CD319 (SLAMF7 or CS1), CD272, CD86, CD200, and CD184. Therefore, they could be possible targets for immunotherapy.

Bi-Specific T-Cell Engagers Antibodies (BiTEs) and Bi-Specific Antibodies have shown promising results in the treatment of R/R MM in pre-clinical studies. BiTEs are designed to bind a tumor cell and an immune cell by engaging usually CD3 with an antigen expressed in the tumor cell. Consequently, the T cell becomes activated and attacks the target cell. There are different BiTEs targeting BCMA and CD3, which have shown potent anti-MM activity in vitro and in vivo murine and monkey models of MM [33].

Also bi-specific antibodies are being tested for the treatment of MM. NKG2D is an activating receptor expressed on NK cells, CD8+ T cells, γδ T cells and NKT cells. Simultaneous targeting of NKG2D and CS1 should engage both innate and

adaptive immune cells to target MM cells and to show in vitro and in vivo anti-MM activity.

Immune checkpoints are negative signals established between immune and tumor cells that hamper immune cells and prevent them from eliminating malignant cells. Antibodies such as ipilimumab, which blocks CTLA-4, pembrolizumab and nivolumab, which block PD-1 in T cells, and atezolizumab, durvalumab and avelumab, which block PD-L1 on tumor cells, have been developed and approved by the FDA for solid tumors. However, targeting immune checkpoints has not been very successful in MM as of yet.

Genetically modifying autologous T cells to express chimeric antigen receptors (CARs) thus redirecting them to eliminate tumor cells or other harmful cells is a new and revolutionary therapeutic modality for cancer treatment [34].

The success of new CAR T cell therapies relies on selecting the right antigen to target. The antigen should be expressed broadly in tumors of a given type, but should not be present in healthy tissues, as to avoid off-target effects and associated toxicity. There are currently 32, completed or on-going, clinical trials involving CAR T cell therapy in MM and the vast majority of them are evaluating the efficacy of anti-BCMA CAR and a few assessing anti-CD19 or anti-CD138 CAR.

To summarize, the revolution of immunotherapy in cancer treatment in the last years is a field that is being incorporated to the treatment of MM. Among the different types of immunotherapy, the introduction of monoclonal antibodies targeting CD38 (daratumumab) and CS1 (elotuzumab) in combination with IMiDs or bortezomib has significantly improved the outcome of patients with R/R MM. Other monoclonal antibodies targeting IL6, CD56, CD138 and CD40 were tested but, unfortunately, did not show any benefit or just limited responses were observed. Moreover, immunocheckpoint inhibitors have not shown a beneficial

effect in MM patients, and additionally, high toxicities were detected. In the field of CAR-modified T cells, BCMA has proven to be the star among other CARs tested in MM and very promising clinical results are expected. It is expected that in the next decade, as new alternatives appear, novel combination of treatments will be tested and hopefully will lead to higher complete remission rates and prolonged survival in patients with MM along with the lowest possible toxicity.

2. MEMORY T CELLS

2.1 General informations about memory T cells

When naïve T-cells meet cognate antigen in the draining lymph node (LN), the cells undergo activation and proliferation program, and differentiate into a heterogeneous population of effector T-cells. These effector T cells then migrate to the site of infection and eliminate pathogen-infected cells. Most effector cells die after clearance of the pathogens but some cells, subsequently, can differentiate into memory T-cells. During the immunological activity, T-cells receive specific signals that impact their ultimate fate; either death or differentiation into different types of memory cells with distinct functional and migratory properties [35, 36]. T-cells with weak stimulatory potential preferentially remain in the LN and differentiate into central memory T-cells (T_{CM} cells) where they survey lymph and blood [37]. Instead, T-cells with high stimulatory potential differentiate into potent effector cells that migrate to inflamed tissues and subsequently die.

The effectors that do not receive these tissue-instructive signals may differentiate into effector memory T-cells (T_{EM} cells) that circulate between blood and certain peripheral tissues. Instead, some effector cells receive tissue-specific instructive signals to differentiate into tissue-resident memory T-cells (T_{RM} cells) and establish permanent residency within the tissues [38].

T_{EM} cells play a supportive role to T_{RM} due to an immediate effector functions and their ability to rapidly move to infection sites.

The external or internal surfaces of the body such as the skin and the mucosal linings of the gastrointestinal, respiratory, and urogenital tracts are a major gateway for infectious pathogens to access to the body.

Inside these tissues reside different types of immune system cells, such as macrophages, dendritic cells (DC), γδ T-cells, and innate lymphoid cells (ILC), and they play important roles in maintaining the integrity of these epithelial barriers.

Accumulating evidence has revealed that there are dynamic and complex interactions between T_{RM} cells in peripheral tissues and the original resident cell populations. For example, some tissue-resident immune provide to T_{RM} cells factors for their maintenance [39, 40].

Furthermore, it is now known that T_{RM} cells are also established in non-barrier tissues (such as the brain, liver, and kidney) as well as the primary lymphoid organs and secondary lymphoid organs (SLOs) and protect tissues from infectious pathogens disseminated by haematogenous or cellular pathways [41].

In all these locations we can found CD4+ and CD8+ memory T-cells but T_{RM} cells are best characterized for the CD8+ subset and, therefore, it is to these that reference will mostly be made.

2.2 Tissue-Resident Memory CD8+ T Cells Phenotype

Tissue-resident memory CD8+ T-cells was formally described in 2009. CD8+ T_{RM} cells have been shown to stably reside in different tissues, where they provide rapid and potent protective immunity against re-infecting pathogens. Phenotypically, T_{RM} cells constitutively express CD69, integrin αE(CD103)β7 (commonly referred to as CD103), CD45RA, CD8, CD3 and lake of CD62L and CCR7 [42, 43].

Interestingly, CD69 expression is not limited to CD8+ T-cells, and it is also present on other immune subsets including natural killer cells. While CD69 is expressed on the majority of T_{RM} subsets, absence of this lectin on CD8+ T-cells only limits the size of the population and does not result in complete ablation [44]. This suggests that CD69 is not an absolute requirement, and while its expression, although

advantageous, is not mandatory [45].

CD103 is the ligand for E-cadherin, which is expressed in epithelial cells. For this reason, is conceivable that CD103 is responsible for residency in epithelial tissues. Though physical retention by ligand binding is the best-known role for CD103, engagement of CD103 may have a number of other functional ramifications outside of adhesion. For example, the effects of CD103 binding have been also studied in tumor models. CD103+ tumor-infiltrating CD8+ T-cells are more capable of killing tumor cells [46]. This is likely attributed to the fact that CD103+ T-cells form more stable synapses with target cells than their CD103-negative counterparts [47]. Moreover, engagement of CD103 also positions cytolytic granules to organize in a polarized fashion, and the addition of signaling through the TCR results in lytic granule exocytosis [48, 49].

2.3 Tissue-Resident Memory CD8+ T Cells in Non-Lymphoid Organs

2.3.1 Barrier Tissues

The skin is composed of three main layers, the epidermis, dermis, and subcutaneous fatty region and it represents one of the tissue sites in which T_{RM} can reside. The epidermis and dermis are separated by a basement membrane and they harbor numerous populations of innate and adaptive immune cells. In detail, the hair follicles provide unique niches for immune cells including T_{RM} cells [50].

Skin CD8+ T_{RM} cells express canonical T_{RM} makers such as the activation marker CD69, the E-cadherin- binding integrin CD103, and the collagen-binding integrin CD49a and, although are widely found throughout the body, their number is generally elevated at sites of infection and/or inflammation [51].

In addition to the skin, T_{RM} cells can also homing in the intestinal mucosa; this latter consists of a single layer of intestinal epithelial cells that overlies the lamina propria (LP), a thin layer of loose connective tissue. The epithelium and LP are separated by a basement membrane and each provides a distinct immunological niche for the maintenance of T_{RM} cells. Significant numbers of antigen-specific T_{RM} cells are established in the intraepithelial compartment following intestinal infections and most of them are CD8+ T cells. However, the accumulation of T_{RM} cells is also evident even in the absence of intestinal infection [52], suggesting the presence of additional niches that sustain T_{RM} cells in the infection/inflammation-inexperienced LP.

The migration of effector and memory T-cells into the mucosa of the Female Reproductive Tract (FRT) is significantly restricted in the absence of local infection and/or inflammation [53]. Once recruited, however, T_{RM} cells are formed and maintained in both compartments under the control of local environmental cues. Distinct from the skin CD8+ T_{RM} cells, however, the development and maintenance of CD8+ T_{RM} cells in the FRT is IL-15-independent [54] and, currently, the factors that regulate the maintenance of T_{RM} cells in the FRT are largely unknown.

Upper Respiratory Tract (URT) and Lower Respiratory Tract (LRT) are two other specific areas of T_{RM} cell localization. Most studies have largely focused on T_{RM} cells in the LRT, but the most common airborne pathogens primarily infect the URT. Thus, understanding the T_{RM} niches in both compartments is critical for the development of specific vaccines. The instructions required for the differentiation of CD8+ T_{RM} cells in the nasal mucosa are different from those in the LRT. Furthermore, the number of CD8+ T_{RM} cells in the nasal tissues is relatively stable (it was the same 3 months post-infection), whereas there is a significant decline in

number of these cells in the LRT (lung). This suggests the existence of different niches of CD8+ T_{RM} cells maintenance between URT and LRT [55].

Finally, among barriers tissues, it is also possible to observe the presence of T_{RM} cells in the Salivary Glands (SGs), exocrine epithelial tissues that secrete saliva into the oral cavity. It is known that the SGs can be a target of several bacterial and viral infections, for example cytomegalovirus (CMV). In latently infected individuals, resident populations of antigen-specific CD8+ and CD4+ T_{RM} cells are located in the SGs. CD4+ T_{RM} cells are observed predominantly in the stroma of the SGs [56], while CD8+ T_{RM} cells, that express CD103, are located in the epithelium of the acini and ducts [57]. These T_{RM} cells can confer protection upon recall by eliminating CMV infected non-epithelial cells, where CMV fails to achieve complete downregulation of MHC class I molecule.

2.3.2 Non-Barrier Tissues

The presence of T_{RM}, as cells responsible for immunological surveillance, it was also observed in Non-Barrier Tissue, including the brain. The presence of the blood–brain barrier (BBB) makes the central nervous system (CNS) an immune privileged site with severely limited ingress of blood-borne T lymphocytes and few, if any, T-cells are present in the healthy brain parenchyma under non inflammatory conditions. For this reason, the accumulation of T-cells in the brain parenchyma has generally been considered a pathological condition [58]. However, it is now known that few peripheral T cells can be present in the brain in the absence of inflammation and they play there a key role in surveying the CNS [59], as the lack of these cells can result in opportunistic infections in the CNS [60]. Among these T-cells, it is possible to observe also in the brain la presence of T_{RM} cells. These last

are primarily established at brain surface structures, such as meninges and confer protection against reinfection [61].

In addition to the brain, the presence of T_{RM} cells was also observed in the liver and kidney.

Recent studies have demonstrated that liver-resident memory CD8+ T-cells are established in the sinusoid following systemic infection or vaccination [62] and are present in both, healthy and hepatitis B virus infected individuals.

Lymphocytes are relatively rare in healthy kidneys but a small numbers of resident immune cells such as macrophages and T-cells can be found in the interstitium in normal condition. CD8+ T_{RM} cells can be observed in extravascular renal compartments following direct [63] or regional infections with pathogens, where they seem to play an important role in immunological surveillance against possible reinfections

Finally, the presence of T_{RM} cells also in White Adipose Tissue (WAT) and in some solid tumors tissue has been described. In this last case, it appears that T_{RM} cells may also have a protective role for the tumor, but this protection is limited to primary tumors, and not metastases, since CD8+ T_{RM} cells are segregated from the circulation [64].

2.4 Tissue-Resident Memory CD8+ T Cells in Lymphoid Organs

In addition to the anatomical sites mentioned above, T_{RM} cells can be also observed in Lymphoid Organ (LO), both in Secondary Lymphoid Organs (SLOs), like lymphnodes and spleen, and Primary Lymphoid Organs, such as thymus and bone marrow.

2.4.1 Secondary Lymphoid Organs (SLOs)

Lymphnodes, Spleen

The SLOs have generally been considered a transit place for T_{CM} and T_{EM} cells. However, recent studies have demonstrated that there are also small numbers of memory CD4+ and CD8+T-cells that are resident in the LN, spleen and tonsils without recirculation [65]. Unlike circulating memory T cells, T_{RM} cells in the SLO share phenotypic characteristics and gene expression profiles with those in the non-lymphoid tissues, including the expression of CD69. Since surface expression of CD69 is generally transient, however, it is likely that repetitive antigen stimulation is required for the maintenance of CD69 expression and the retention of T_{RM} cells in the SLO [66]. In this regard, there is considerable evidence that residual antigen persists in the draining LN for several months after vaccination or the resolution of an acute infection and presumably facilitates the accumulation of memory T-cells [67]. The distribution of T_{RM} cells in the SLO depends on an antigen niche and the T_{RM} cells are preferentially localized at the common antigen entry sites: the marginal zone and red pulp of the spleen and the subcapsular sinuses of the LN [68]. T_{CM} cells in the SLO have a critical role in the pathogen clearance by generating massively increased numbers of secondary effector T cells during a recall response. For this reason, it will be important to determine the functional contribution of T_{RM} cells in the SLOs during the recall responses. It is possible that T_{RM} cells in the SLO do not actively contribute to the recall response to avoid unnecessary competition with T_{CM} cells, but are strategically positioned to protect the SLO from direct infection with pathogens.

2.4.2. Primary Lymphoid Organs

Thymus

CD8+T_{RM} cells have also been found in the thymus, a primary lymphoid organ [69]. Thymic CD8+ T_{RM} cells are observed after a specific infection with either thymus-tropic or non-tropic pathogens. As with T_{RM} cells in the peripheral tissues, thymic CD8+ T_{RM} cells exhibit a canonical T_{RM} phenotype (CD69+, CD103+). These cells are located predominantly in the medulla although a few cells lodge also in the cortex. The immune activation process strongly inhibits the migration of peripheral dendritic cells populations to the thymus to avoid unfavourable induction of acquired tolerance to the invading pathogens [70] and for this reason, it is reasonable to think that thymic CD8+ T_{RM} cells mainly function to protect the thymus, rather than contribute to the recall responses against systemic infections.

Bone marrow

As mentioned above, when a pathogen attacks a barrier tissue, primary immune responses are initiated in the draining lymph nodes (LN) and sometimes in the spleen. In the secondary lymphoid organs, mature antigen-presenting dendritic cells (DC) prime T-cells to undergo huge clonal expansion and differentiation into short-lived effector and long-lived memory T-cells. Both types of antigen experienced T cell display an increased capacity to migrate to inflamed tissues as well as to the BM as compared with naive T-cells. Memory T-cells can be found all over the body, with a peculiar enrichment also in the BM. The BM consists of islets of hematopoietic BM interspersed with fatty areas, all contained within spongy bone and inside central cavities of long bones. In healthy individuals BM contains mature T-cells that represent about 3–8% of total nucleated BM cells, and have a typically reduced CD4/CD8 T cell ratio, as compared with blood [71, 72]. The BM

contains several memory-phenotype T-cells, a cellular subset defined by the expression of activation/memory markers, including memory T-cells specific for previously encountered antigens. BM memory T-cells contain both central memory and effector memory T-cells [73, 74]. Thus, the BM is often described as a "reservoir" for long-lived memory T-cells [75, 76]. Some pivotal experiments suggest that the BM represents a temporary stopping point for recirculating memory T-cells but it also possible to observe T_{RM} [77]. About the homing molecules into the BM of memory T-cells, memory CD8 T-cells slow down and roll in BM microvessels via L-, P-, and E-selectin-mediated interactions; CD4 T-cells lodge into the BM via molecular mechanisms similar to those of CD8 T-cells but, for their retention in the BM the expression of β1-integrin by CD4 T-cells is required. Furthermore, is now also known that CD69 regulates local T-cell retention in the BM by different mechanisms.

Memory T-cells specific for previously encountered antigens are commonly found in the BM of healthy subjects as well as of individuals affected by infectious, immune-mediated, and neoplastic diseases. For example, specific CD4 and CD8 T-cells were present in the BM of immune individuals after resolution of acute infections but also in the BM of subjects infected by persistent viruses, including Cytomegalovirus (CMV), Epstein–Barr Virus (EBV), and Human Hepatitis C Virus (HCV) [78, 79]. Also BM from neoplastic patients can contain tumor antigen-specific T-cells.

BM memory T-cells present some phenotypic differences respect to corresponding cells from lymphoid periphery and blood. For example, a high proportion of CD4 and CD8 BM memory T-cells express CD69, but no critical differences were found by gene-expression analysis of memory T-cell paired samples obtained from either BM or blood/spleen.

In respect to the activity status, the majority of BM memory T-cells are quiescent, as demonstrated by staining with the cell cycle marker Ki67, but a small percentage of them divides under steady state. For example, CD8 T-cell proliferation in the BM is supported by local stimuli, including a dominant role for IL-15 [80].

About the function, as demonstrated by adoptive transfers in immunodeficient mice [81] and in vitro studies [82], BM memory T-cells perform a potent antigen-specific effector function. As evidenced in several papers available in the literature [83, 84], memory T-cells from BM of patients with different solid and hematological cancers were able to kill autologous tumor cells. Moreover, T cell-derived cytokines can modulate hematopoiesis, implying that BM T-cells can contribute to shaping hematopoiesis during both acute and chronic infections [85].

Finally, T-cells can regulate physiological processes occurring in the BM, like normal hematopoiesis and bone tissue homeostasis. Surprisingly, in physiological conditions, T-cells promote the maintenance of normal bone mass and bone mineral density stimulating the production of the RANK-L decoy receptor osteoprotegerin by B cells, through CD40L/CD40 interaction [86]. Therefore, a cross-talk between T-cells and hematopoietic precursors occurs in the BM also in normal healthy conditions [87, 88].

2.5 Concluding remarks

The identification of recirculating memory T-cells and T_{RM} cells offers a novel view of both memory maintenance and response to antigenic re-challenge, with a broader view respect to the previous perspective based on T_{CM}/T_{EM} paradigm [89]. Recirculating memory T-cells rely on a finely tuned equilibrium between quiescence and homeostatic proliferation, in several tissues wherein these cells can

temporarily stop. In contrast, T_{RM} cells live permanently as non-migratory cells within some sites, including BM, wherein they survive in a quiescent state.

It appears that recirculating memory T-cells and T_{RM} cells provide respectively systemic immunity and immediate protection against pathogens. The two types of memory T-cells act in concert for tissue protection, as recirculating memory T-cells are recruited with more efficient local effector response and boosting of systemic memory. Naturally, recirculating memory T-cells arrive with some delay in the tissue, while T_{RM} cells are already there for highly efficient immediate protection. As T_{RM} cells have been identified not only at the epithelial barriers of the body but also in lymphoid and/or internal organs, e.g., LN and brain, it is likely that each organ harbors some T_{RM} cells and that BM is not an exception to this rule. Despite their different migration pathways, positioning and role in immunity, T_{RM} cells participate in a local network of cellular and molecular interactions in the organ where they are located, influencing normal tissue homeostasis and organ function. In the case of BM T-cells, it has been shown that they normally regulate hematopoiesis, as well as bone metabolism. Instead, about T_{RM} cells located in barrier organs, it is conceivable that they protect host's health and sometimes contribute to disease in several manners, for example they might shape the gut microbiota composition, with a possible indirect impact on metabolic syndrome, obesity-related disorders, inflammatory bowel disease, and colorectal cancer [90].

2.6 Unsolved questions

Despite the current and specific knowledge regarding the characterization and role of memory T-cells, there are still unsolved questions. For example, what are the distinct signals regulating differentiation of either recirculating memory T-cells or T_{RM} cells? And where and when do they receive them?

Another question still open is that relating to intracellular networks orchestrating differentiation. We do not know yet the single molecules and/or molecular pathways involved in gene transcription, protein translation and metabolic state of T_{RM} and of recirculating memory T-cells.

Finally, are there changes occurring with aging in the number and activity between T_{RM} and recirculating memory T-cells?

Further investigation is needed in order to better understand the biology and specific functions of this memory subset of T-cells.

3. PHENOTYPICAL CHARACTERIZATION STUDY AND FUNCTIONAL ANALYSIS OF BONE MARROW TISSUE-RESIDENT MEMORY CD8 + T CELLS IN MULTIPLE MYELOMA PATIENTS

3.1 Background

One of the main features of the immune system is the "immunological memory", with the possibility, following exposure to a specific antigen, to enhance the future ability to respond to that same antigen with a more rapid secondary immune response and more intense. This immune memory is achieved because the memory cells, derived from both B and cellular T, are more efficient than virgin cells.

In the group of the T lymphocyte population, the naïve T-cells, once activated by an antigen, undergo an enormous clonal expansion and at the same time differentiate into a short-lived effector, which generally die at the end of the immune response, and in long-lived memory cells (long-lived memory T-cells), ready to provide protection in case of new contact with the same antigen. Within T-cell memory pool, we can distinguish two cellular subgroups, in relation to the phenotype and functional potential: T_{CM}, which express the chemokine receptor CCR7 at high intensity, and T_{EM}, which do not express the CCR7. Was also described a group of non-circulating resident T cells, T_{RM}, which live permanently in some peripheral tissues where they survive in a state of quiescence.

It appears that circulating memory T-cells are responsible for systemic immunity, while T_{RM} cells represent immediate protection in the main entry sites of the pathogen, mostly participating in local defence.

Recent studies have shown that also in the BM is possible to observe the presence of T_{RM} cells. Indeed, BM is able to provide survival signals by promoting the interaction among T_{RM} cells and stromal cells expressing homeostatic cytokines, such as IL-7 or IL-15.

In addition to their activity in local defence, has recently been highlighted the possibility that T_{RM} may contribute to the defence of the host in the neoplastic diseases, representing potentially reactive cells against some hematological tumors infiltrating the BM [91]. In relation to this last topic, we conducted a prospective population-based study aimed at evaluating the frequency, phenotype and functional analysis of T CD8 + memory cells residing in BM of subjects with a diagnosis of MM.

In functional analysis we evaluated the CD107a/b and NKG2D expression on the surface of BM CD8+ T_{RM}.

Cancer development is under surveillance by the immune system of the host and the tumor cells can be recognized and killed by the immune secretion of lytic granules from CD8+ T lymphocytes. This process involves the fusion of the granule membrane with the cytoplasmic membrane of the immune effector cell, resulting in surface exposure of lysosomal-associated proteins that are typically present on the lipid bilayer surrounding lytic granules, such as CD107a/b. Therefore, membrane expression of CD107a/b constitutes a marker of immune cell activation and cytotoxic degranulation [92]. For this reason, in order to evaluate the possible cytotoxic immune function of BM CD8+ T_{RM} against cancer cells we also evaluated the CD107a/b expression on their cell surface, after functional stimulation with activation molecules.

Finally, as several data in the literature highlight the presence of MICa and b ligands on myelomatous plasma cells [93], in order to evaluate one possible cytotoxicity pathways, we performed the cytometry evaluation of NKG2D on the surface of BM CD8+ T_{RM} isolated.

3.2 Materials and methods

3.2.1. Population

The study population was represented by 21 consecutive patients with new diagnosis of MM, with a diagnosis made between September 1, 2018 and August 31, 2019. All patients were followed in the Hematology Division of the AOUP "P. Giaccone" of Palermo". Patients with IgA, IgG and LCMM aged over 18 years were included. While, patients with liver cirrhosis, with another type of concomitant neoplasm or with another diagnosis of tumor disease made in the two years prior to hypothetical enrolment were excluded.

Clinical and laboratory data concerning the patient's characteristics and blood disease, also essential for assessing the patients eligibility, were collected through specific data bases and the consultation of medical records.

The biological study was carried out on bone marrow samples taken in quantities of 4 cc during the BM analysis phase, to which all patients were subjected for the suspected diagnosis.

Specific written informed consent was requested from the patients, in accordance with institutional and national requests, for the processing of personal data and above all for carrying out the planned biological investigations.

The laboratory part of the study, especially in relation to the flow cytometric analysis and the functional study was conducted at the "Central Laboratory for Advanced Diagnosis and Biomedical Research (CLADIBIOR) and Department Bi.N.D." of the AOUP "P. Giaccone" of Palermo.

3.2.2. Flow cytometry analysis

The evaluation *ex vivo* of CD8 T_{RM} frequency and phenotype in BM samples was performed using anti-human mAbs to CD3 (FITC, REA Clone 613 from Miltenyi Biotech), CD103 (PE, REA Clone 803 from Miltenyi Biotech), CD69 (PE-Vio615, REA Clone 824 from Miltenyi Biotech), CD45 (PerCP-Vio700, REA Clone 747 from Miltenyi Biotech), CD8 (PE-Vio770, REA Clone 734 from Miltenyi Biotech), CD45RA (Viogreen, REA Clone T6D11 from Miltenyi Biotech), CCR7 (CD197) (Vioblue, REA Clone 546 from Miltenyi Biotech), Ki67 (APC, REA Clone 183), Zombie NIR Fixable Violability kit Biolegend Cat.n. 423103. For the surface staining were used 2 ml of each monoclonal antibody. After 20 min. of incubation at RT the sample were washed and then were added 2 ml of 7AAD for the exclusion of death cells. After 5 min. the cells were re-suspended in PBS and acquired on FACSARIA I (BD Biosciences). The sequential gating strategy was: gate on lymphocytes population with CD45 vs SSC, 7AAD negative cells, exclusion of doublets with FSC-H vs FSC-A, $CD8^+CD3^+$ and evaluation of percentage of $CD103^+CD69^+$ cells. Was also established the subsets using CCR7 and CD45RA (Fig. 1).

3.2.3. In vitro assay

BM derived mononucleate cells from MM patients were then coltured in vitro in complete RPMI with 10% of human serum for 4 days with IL15 (25 ng/ml), IL7 (25 ng/ml) and TGFβ (2 ng/ml) in different combination and in RPMI alone. After colture we analysed the frequency of CD8 TRM and the proliferating fraction with intracellular staining with anti human Ki67 APC (from Miltenyi Biotech).

3.2.4. Functional analysis by cell stimulation

Fresh BM mononuclear cells (BM MNC) were isolated using Hypaque-Ficoll (Cederlane) density centrifugation. In some instances BM MNC were frozen (90% fetal calf serum (FCS)/10% DMSO) at −160 °C until use. 5x105 BM MNC were incubated with 750 ng/ml of ionomicin and 150 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich). FITC conjugated antibodies to the lysosome associated membrane proteins (LAMPs) CD107a (Anti-human CD107a -LAMP 1-, FITC, eBioH4A3 Clone, eBioscience) and CD107b (Anti-human CD107b -LAMP 2-, FITC, eBioH4B4 Clone, eBioscience) were added to the cells prior to stimulation according to the manufacturer instructions. A negative control was included to evaluate the spontaneous expression of CD107a/b. The cells were incubated for 1 h at 37 °C in a 5% CO2 incubator, followed by an additional 4h in the presence of the secretion inhibitor monensin (BD Pharmingen). After incubation samples were acquired on FACSAria flow cytometer (BD bioscence) and analyzed by Flowjo software.

Finally, in order to evaluate one possible cytotoxicity pathways, we studied the expression of NKG2D (CD314) (FITC, REA Clone 797 from Miltenyi Biotech) on BM CD8+ T_{RM} isolated from the samples under examination.

3.2.5. Statistical analysis

Non-parametric Mann-Whitney and Kruskall-wallis tests were performed to determine statistical differences in the distribution of the results using GraphPad Prism 7.00. Values of * $p<0.05$ were considered significant.

3.3 Results

In our study we collected data from 21 patients with new diagnosis of MM, followed in the Hematology Division of the AOUP "P. Giaccone" of Palermo. The study sample consisted of 11 males and 10 females, with a median age of 68 years (range 51 to 82). In the patients group, 7/21 had a multiple myeloma with isotype IgG k, 6/21 IgG λ, 2/21 IgA k, 2/21 IgA λ and 4/21 had LCMM λ.

In relation to the staging, 11/21 (52,4%) patients had ISS III, 5/21 (23,8%) had ISS II and 5/21 (23,8%) had ISS I.

The characteristics of patients are shown in table 1.

Cytofluorimetric analysis performed on bone marrow blood samples showed that the ex vivo average frequency of CD8+ T_{RM} was of 0.48% and the phenotype was represented mainly by T_{EM} (72,9%) followed by TEMRA (12.3%) and (7,6%) of naïve cells and (7,2%) of T_{CM} (Fig. 2). To evaluate factors capable of maintaining or to induce the expansion of these cells in vitro, we maintained BM-derived mononucleate cells from MM patients for 4 days in presence of homeostatic cytokines, IL-15, IL-7 plus IL-15 and IL-7 together with IL-15 and TGF-β. The result showed an increase of the percentage of CD8+ T_{RM} in all conditions tested, especially in presence of all cytokines (Fig. 3), with a percentage of CD8 T_{RM} of 2,74%. Regarding the phenotype distribution, we observed an expansion of CM compared to the other subsets (Fig. 4). We also analysed the percentage of CD8+ T_{RM} proliferating through the identification of Ki67 positive cells. Data highlight that IL-15 gives the strongest proliferative input, but also other cytokines contribute to the homeostatic maintenance of these cells (Fig. 5).

In order to evaluate the possible cytotoxic immune function of BM CD8+ T_{RM} against cancer cells we also evaluated the CD107a/b expression on their cell surface

after stimulation with activation molecules. This functional analysis highlights that BM CD8+ T_{RM}, after stimulation/activation, show degranulation, indirect signal of possible cytotoxic activity (Fig. 6).

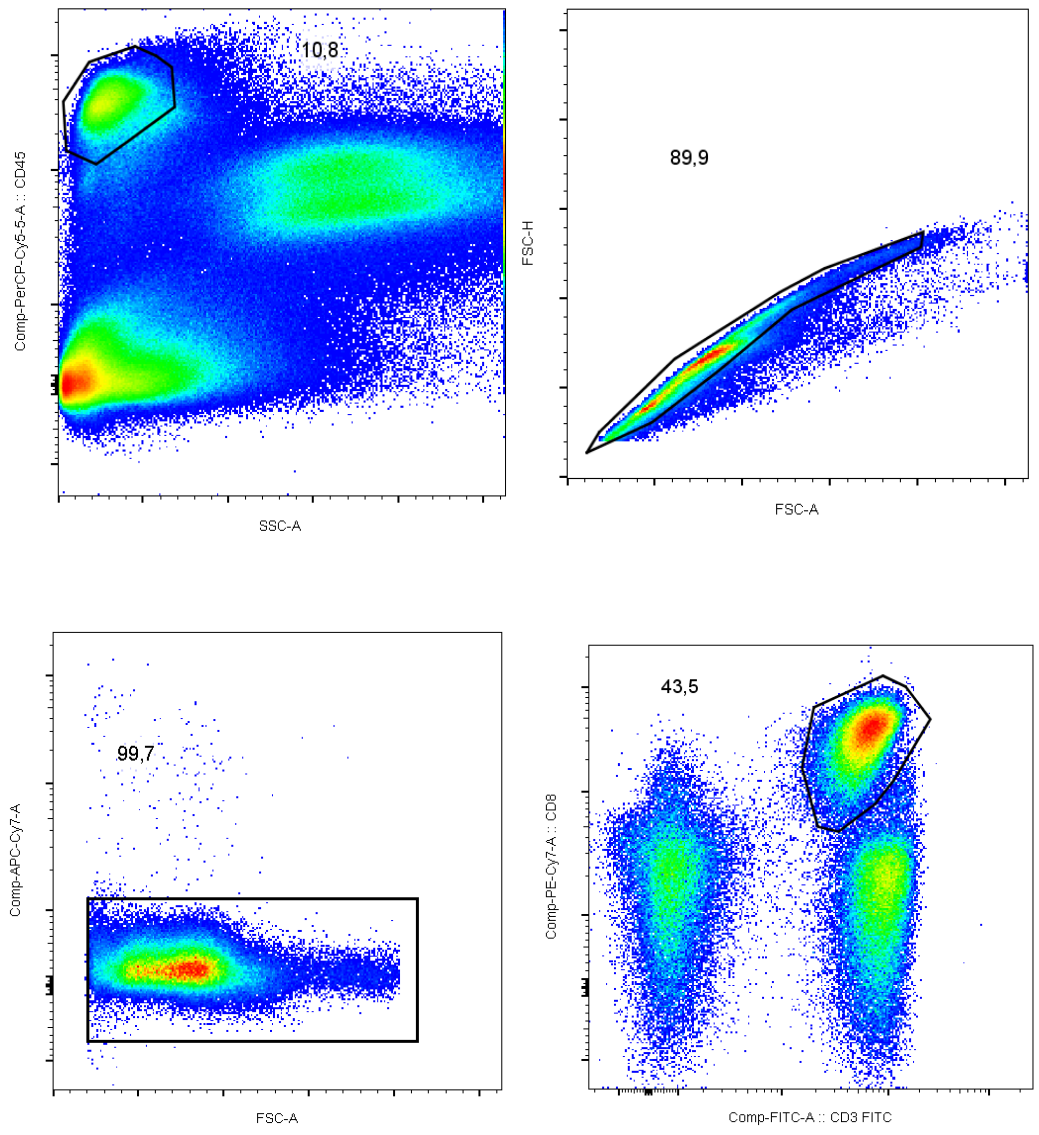
Finally, in order to explore one possible pathways of this cytotoxicity, we studied the cytofluorimetric expression of NKG2D on BM CD8+ T_{RM} with the evidence of expression of this marker on the surface of these cells (Fig. 7).

Table 1. Patients characteristics
(N= 21)

Characteristics	**N. patients**	**%**
Sex:		
M	11	52,4
F	10	47,6
Age:		
≤ 70 anni	12	57,2
> 70 anni	9	42,8
MM Isotype:		
IgG k	7	33,3
IgG λ	6	28,6
IgA k	2	9,5
IgA λ	2	9,5
Light chain k	0	0,0
Light chain λ	4	19,1
MDE (*Myeloma Defining Events*):		
HyperCalcemia	2	38,1
Renal Failure	4	19,0
Anemia	9	42,8
Bone Lesions	16	76,2
Bone Marrow Plasma cells ≥60%	11	52,4
FLC ratio ≥100	12	57,1

≥1 focal lesion in MRI (>5 mm)	8	38,1
ISS:		
I	5	23,8
II	5	23,8
III	11	52,4
ECOG:		
0-1	18	85,7
2-4	3	14,3

Fig 1: gating strategy



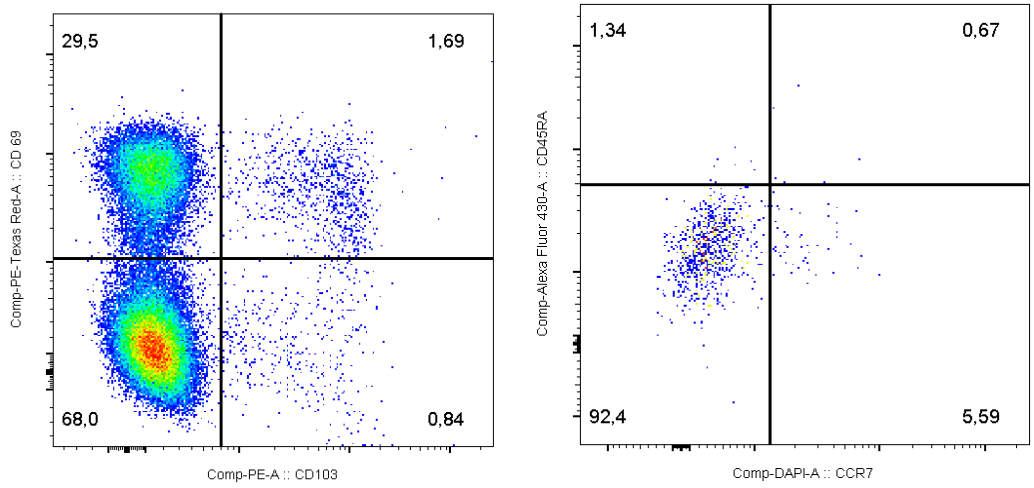


Fig. 2: Ex-vivo $CD8^+$ T_{RM} phenotype distribution

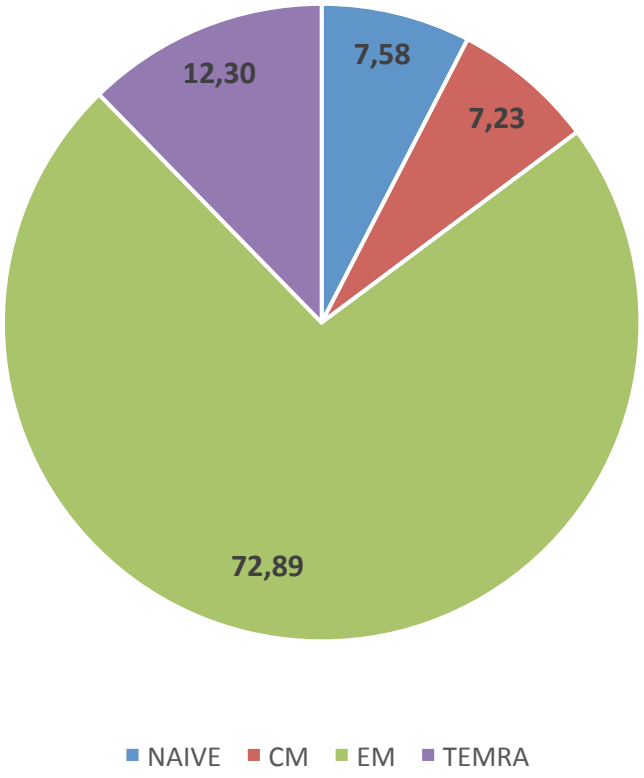


Fig. 3: Increase of $CD8^+$ T_{RM} after *in vitro* culture with IL7 + IL15 +TGF-β

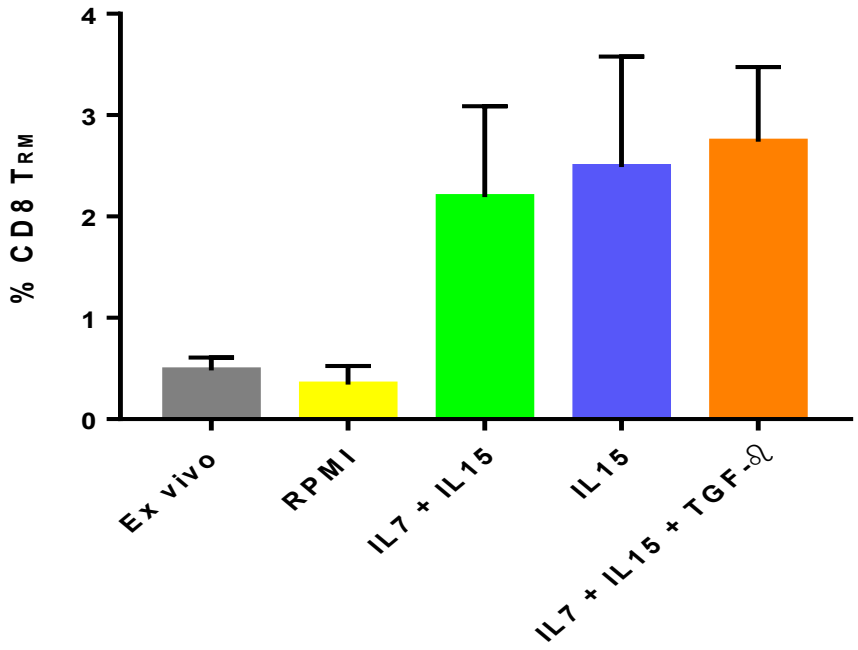


Fig. 4: $CD8^+T_{RM}$ phenotype distribution after 4 days culture with IL7 + IL15 +TGF-β

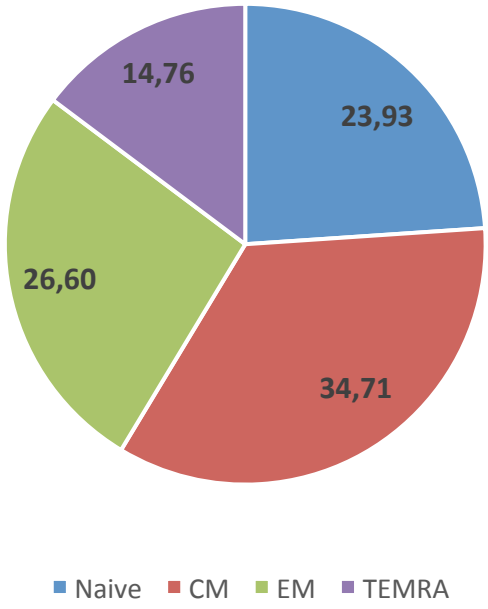


Fig. 5: proliferative input from homeostatic cytokines

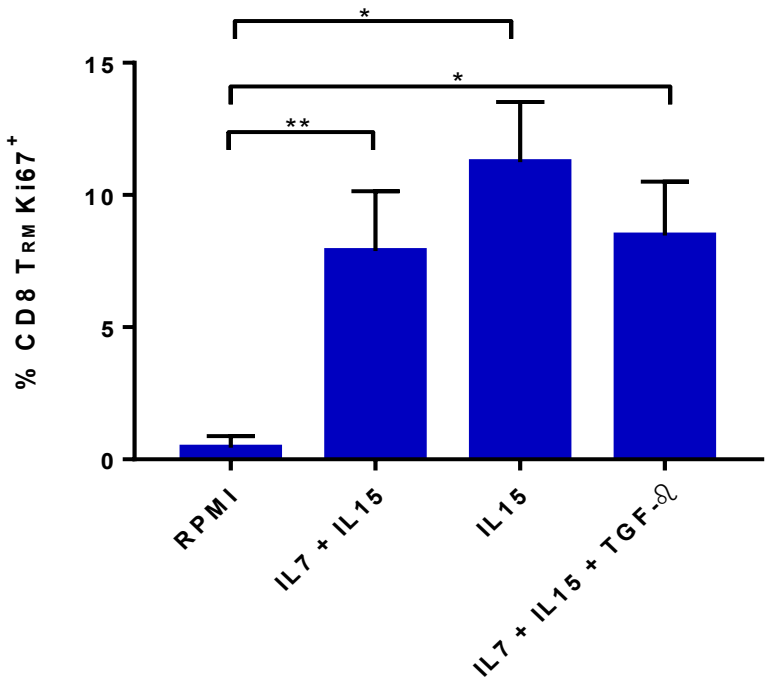
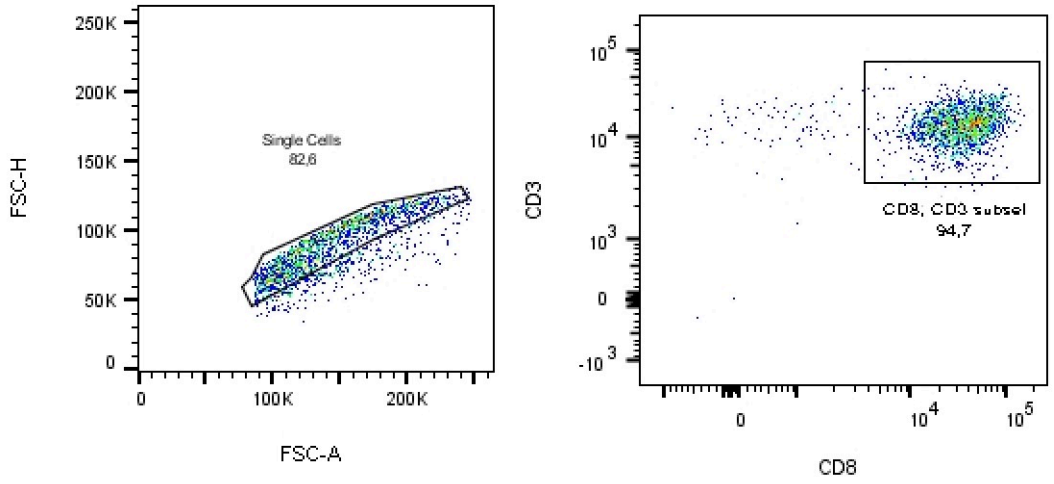
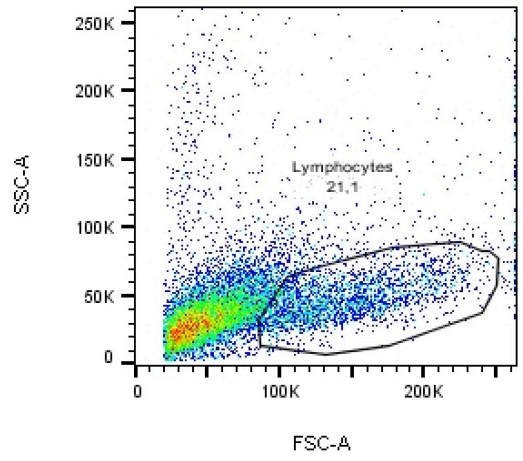
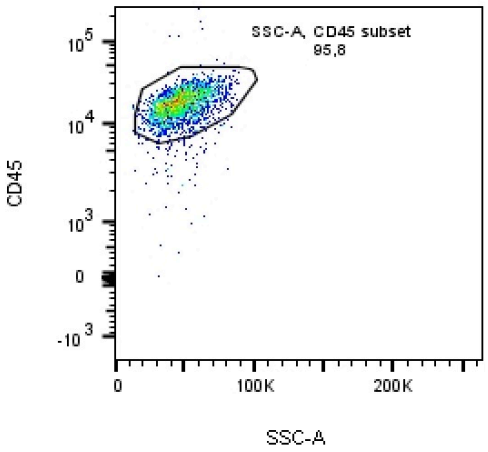
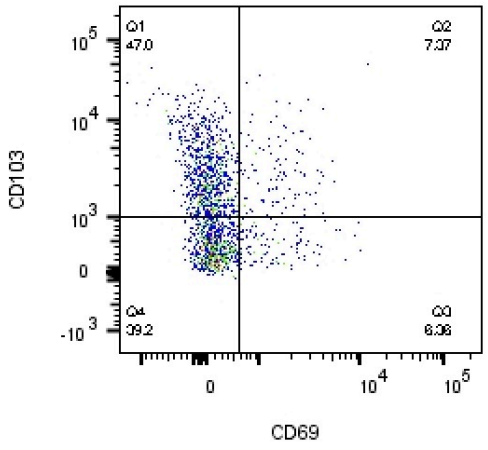


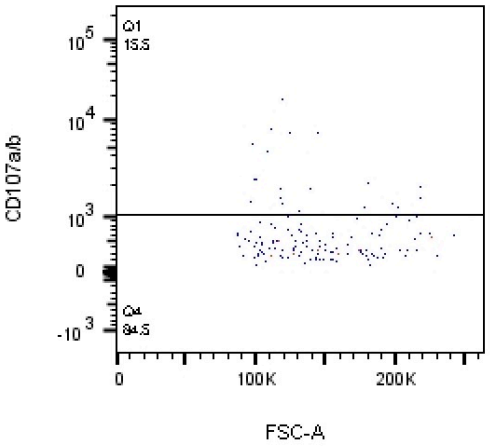
Fig. 6: increased expression of CD107 a/b on the surface of BM CD8+ TRM cells after incubation in the presence of the secretion inhibitor monensin

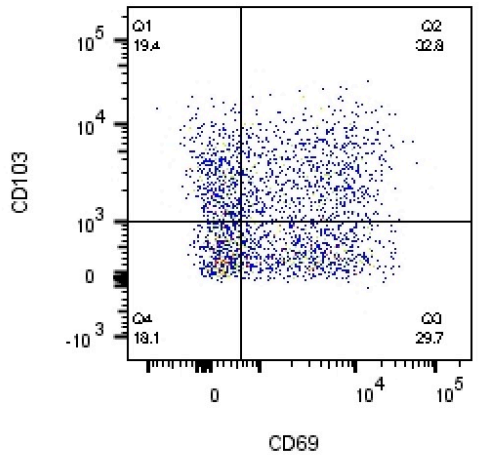



Lymphocytes
21,1
SSC-A
FSC-A


SSC-A, CD45 subset
95,8
CD45
SSC-A


Q1
47.0
Q2
7.07
Q4
39.2
Q3
6.68
CD103
CD69


Q1
15.5
Q4
84.5
CD107a/b
FSC-A


Q1
19.4
Q2
32.9
Q4
18.1
Q3
29.7
CD103
CD69

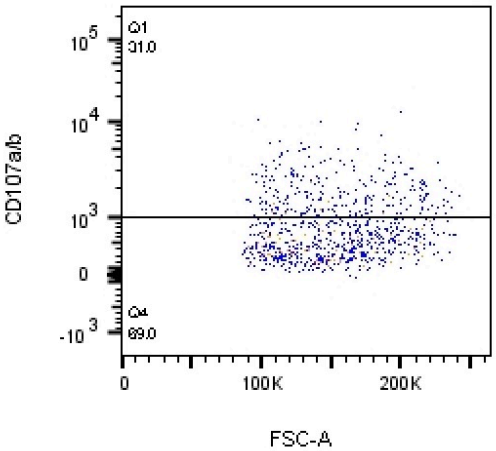
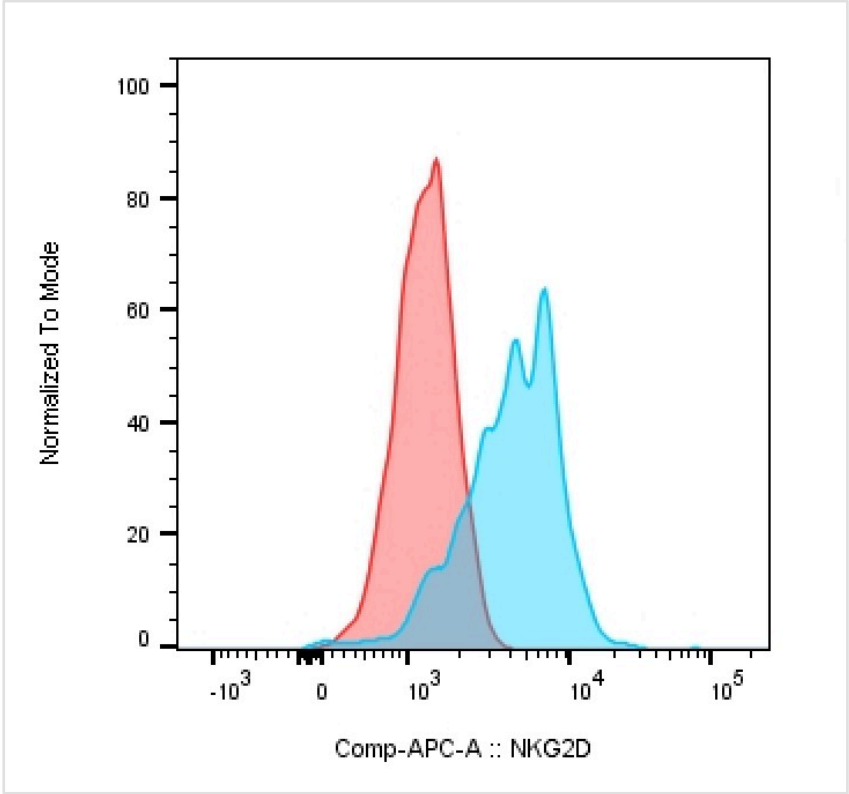

Q1
31.0
Q4
69.0
CD107a/b
FSC-A

Fig. 7: cytofluorimetric expression of NKG2D. *Red*: isotypic control; *Blue*: NKG2D expression on BM CD8+ TRM.



4. DISCUSSION

An important potential of our immune system is represented by "immunological memory", capable of inducing a faster and more intense secondary immune response towards an already known antigen. Both cell B and cell T cells mediate this immunological memory.

In the context of the lymphocyte T population, long-lived memory T-cells have been described and they provide protection in case of new contact with the same antigen. These memory T-cells can be localized in any part of the body and some of them, the T_{RM} cells, live permanently in some peripheral tissues, including the bone marrow.

In recent years, from some papers in the literature, data have emerged about the possible ability of these cells to contribute to the defence of the host in the neoplastic field, representing potentially reactive elements against hematological tumors infiltrating the marrow.

Therefore, in relation to this last topic, we conducted a prospective study in order to analyse the T_{RM} cells in the bone marrow of patients with a new diagnosis of multiple myeloma.

The main objective is to evaluate the frequency and phenotype of resident CD8 + memory T lymphocytes and also perform a functional study. In fact, CD8 + T_{RM} T lymphocytes may be able to control tumor growth and, therefore, they can represent a potential target in the context of the most modern immunotherapy.

At first, from the analysis we found that the ex vivo average frequency of CD8+ T_{RM} was of 0.48% and the phenotype was represented mainly by T_{EM} (72,9%). To evaluate factors capable of maintaining or to induce the expansion of these cells in vitro, we maintained BM-derived mononucleate cells in presence of homeostatic cytokines, IL-15, IL-7 plus IL-15 and IL-7 together with IL-15 and TGF-β, with an increase of the percentage of CD8+ T_{RM} in all conditions tested and in presence of all cytokines, especially under the proliferative input of IL-15.

In order to evaluate the possible cytotoxic immune function of BM CD8+ T_{RM} against cancer cells we also evaluated the CD107a/b expression on their cell surface after incubation with activation molecules. This functional analysis highlights that BM CD8+ T_{RM}, after stimulation/activation, show degranulation, indirect signal of possible cytotoxic activity.

Finally, in order to explore one possible pathways of this cytotoxicity, we studied the cytofluorimetric expression of NKG2D on BM CD8+ T_{RM} and the

cytofluorimetric analysis shows the expression of this marker on the surface of BM CD8+ T_{RM}.

5. CONCLUSIONS

It appears that circulating memory T-cells are responsible for systemic immunity, while T_{RM} cells represent immediate protection in the main entry sites of the pathogen, mostly participating in local defence.

Recent studies have shown that also in the BM is possible to observe the presence of T_{RM} cells, especially CD8+ T_{RM}, where, in addition to their activity in local defence, they may contribute to the defence of the host in the neoplastic diseases.

The latter possibility acquires considerable value in the therapeutic management of the patients with hematological neoplasms infiltrating the marrow, including MM. BM CD8+ T_{RM} cells could in fact represent an important therapeutic target, with the possibility of further improving the results of immunotherapy with the hope of increasing the response rate to the treatment of patients.

Therefore, we evaluated the frequency and the phenotype of CD8 T_{RM} in BM of MM patients and we performed a functional analysis of these cells. In MM patients, the increase of CD8+ T_{RM} cells with a CM phenotype after in vitro culture with the three cytokines could have an anti-tumor role in the control of MM. Furthermore, the expression of CD107 after functional stimulation and the NkG2D expression show the ability of CD8+ T_{RM} cells to perform cytotoxicity. Further studies are needed to investigate the cytotoxic capacity of these cells against myeloma cells, in order to study their functional role, also in the perspective of a possible use in future therapeutic programs.

References

1. Dispenzieri, A., et al., International Myeloma Working Group guidelines for serum-free light chain analysis in multiple myeloma and related disorders. Leukemia, 2009. 23(2): p. 215-24.

2. Waxman, A.J., et al., Racial disparities in incidence and outcome in multiple myeloma: a population-based study. Blood, 2010. 116(25): p. 5501-6.

3. Kumar, S.K., et al., Continued improvement in survival in multiple myeloma: changes in early mortality and outcomes in older patients. Leukemia, 2014. 28(5): p. 1122-8.

4. Vachon, C.M., et al., Increased risk of monoclonal gammopathy in first-degree relatives of patients with multiple myeloma or monoclonal gammopathy of undetermined significance. Blood, 2009. 114(4): p. 785-90.

5. Went, M., et al., Genetic correlation between multiple myeloma and chronic lymphocytic leukaemia provides evidence for shared aetiology. Blood Cancer J, 2018. 9(1): p. 1.

6. Carson, K.R., M.L. Bates, and M.H. Tomasson, The skinny on obesity and plasma cell myeloma: a review of the literature. Bone Marrow Transplant, 2014. 49(8): p. 1009-15.

7. Brown, L.M., et al., Risk of multiple myeloma and monoclonal gammopathy of undetermined significance among white and black male United States veterans with prior autoimmune, infectious, inflammatory, and allergic disorders. Blood, 2008. 111(7): p. 3388-94.

8. Kyrtsonis, M.C., et al., Genetic and molecular mechanisms in multiple myeloma: a route to better understand disease pathogenesis and heterogeneity. Appl Clin Genet, 2010. 3: p. 41-51.

9. Landgren, O., et al., Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. Blood, 2009. 113(22): p. 5412-7.

10. Hebraud, B., et al., Deletion of the 1p32 region is a major independent prognostic factor in young patients with myeloma: the IFM experience on 1195 patients. Leukemia, 2014. 28(3): p. 675-679.

11. Bezieau, S., et al., High incidence of N and K-Ras activating mutations in multiple myeloma and primary plasma cell leukemia at diagnosis. Hum Mutat, 2001. 18(3): p. 212-24.

12. Bartel, D.P., MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 2004. 116(2): p. 281-97.

13. Alsayed, Y., et al., Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. Blood, 2007. 109(7): p. 2708-17.

14. Gadó, K., et al., Role of interleukin-6 in the pathogenesis of multiple myeloma. Cell Biology International, 2000. 24(4): p. 195-209.

15. Roy, P., U.A. Sarkar, and S. Basak, The NF-kappaB Activating Pathways in Multiple Myeloma. Biomedicines, 2018. 6(2).

16. Kumar, S., et al., Prognostic value of bone marrow angiogenesis in patients with multiple myeloma undergoing high-dose therapy. Bone Marrow Transplant, 2004. 34(3): p. 235-9.

17. Rajkumar, S.V., et al., International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Lancet Oncol, 2014. 15(12): p. e538-48.

18. Al-Farsi, K., Multiple Myeloma: An Update. Oman Medical Journal, 2013. 28: p. No. 1:3-11.

19. Dimopoulos, M.A., et al., Oligosecretory and Non-Secretory Multiple Myeloma: Incidence, Clinical Characteristics and Outcomes. Clinical Lymphoma, Myeloma and Leukemia, 2017. 17(1): p. e115.

20. Heaney JLJ, Campbell JP, Iqbal G, Cairns D, Richter A, Child JA, Gregory W, Jackson G, Kaiser M, Owen R, Davies F, Morgan G, Dunn J, Drayson MT. Characterisation of immunoparesis in newly diagnosed myeloma and

its impact on progression-free and overall survival in both old and recent myeloma trials. Leukemia. 2018 Aug;32(8):1727-1738.

21. Bataille, R., J. Grenier, and J. Sany, Beta-2-microglobulin in myeloma: optimal use for staging, prognosis, and treatment--a prospective study of 160 patients. Blood, 1984. 63(2): p. 468-76.

22. Berggard, I. and A.G. Bearn, Isolation and properties of a low molecular weight beta-2-globulin occurring in human biological fluids. J Biol Chem, 1968. 243(15): p. 4095-103.

23. van Dongen, J.J.M., et al., EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia, 2012. 26(9): p. 1908-1975.

24. Caers, J., et al., European Myeloma Network recommendations on tools for the diagnosis and monitoring of multiple myeloma: what to use and when. Haematologica, 2018. 103(11): p. 1772-1784.

25. Durie, B.G. and S.E. Salmon, A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment, and survival. Cancer, 1975. 36(3): p. 842-54.

26. Greipp, P.R., et al., International staging system for multiple myeloma. J Clin Oncol, 2005. 23(15): p. 3412-20.

27. Palumbo, A., et al., Revised International Staging System for Multiple Myeloma: A Report From International Myeloma Working Group. J Clin Oncol, 2015. 33(26): p. 2863-9.

28. Engelhardt, M., et al., Geriatric assessment in multiple myeloma patients: validation of the International Myeloma Working Group (IMWG) score and comparison with other common comorbidity scores. Haematologica, 2016. 101(9): p. 1110-1119.

29. Schuster, S.R., et al., The clinical significance of cereblon expression in multiple myeloma. Leuk Res, 2014. 38(1): p. 23-8.

30. Mendoza, T.R., et al., Changes in pain and other symptoms in patients with painful multiple myeloma-related vertebral fracture treated with kyphoplasty or vertebroplasty. J Pain, 2012. 13(6): p. 564-70.

31. Mill, W.B. and R. Griffith, The role of radiation therapy in the management of plasma cell tumors. Cancer, 1980. 45(4): p. 647-52.

32. Maria Castella, Carlos Fernández de Larrea, and Beatriz Martín-Antonio. Immunotherapy: A Novel Era of Promising Treatments for Multiple Myeloma. Int J Mol Sci. 2018 Nov 15;19(11).

33. Seckinger A., Delgado J.A., Moser S., Moreno L., Neuber B., Grab A., Lipp S., Merino J., Prosper F., Emde M., et al. Target Expression, Generation, Preclinical Activity, and Pharmacokinetics of the BCMA-T Cell Bispecific Antibody EM801 for Multiple Myeloma Treatment. Cancer Cell. 2017;31:396–410.

34. Barrett D.M., Singh N., Porter D.L., Grupp S.A., June C.H. Chimeric antigen receptor therapy for cancer. Annu. Rev. Med. 2014;65:333–347.

35. Iijima N, Iwasaki A. Tissue instruction for migration and retention of TRM cells. Trends Immunol (2015) 36(9):55664.

36. Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. Nat Rev Immunol (2012) 12(11):749–61.

37. Iborra S, Martinez-Lopez M, Khouili SC, Enamorado M, Cueto FJ, Conde-Garrosa R, et al. Optimal generation of tissue-resident but not circulating memory T cells during viral infection requires crosspriming by DNGR-1+ dendritic cells. Immunity (2016) 45(4):847–60.

38. Takamura S. Persistence in temporary lung niches: a survival strategy of lung-resident memory CD8(+) T_cells. Viral Immunol (2017) 30(6):438–50.

39. Bergsbaken T, Bevan MJ, Fink PJ. Local inflammatory cues regulate differentiation and persistence of CD8(+) tissue-resident memory T cells. Cell Rep (2017) 19(1):114–24.

40. Iijima N, wasaki A. T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. Science (2014) 346(6205):93–8.

41. Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. Nat Rev Immunol (2016) 16(2):79–89.

42. Okhrimenko A, Grün JR, Westendorf K, et al. Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. Proc Natl Acad Sci U S A. 2014;111(25):9229–9234.

43. Reading JL, Gálvez-Cancino F, Swanton C, Lladser A, Peggs KS, Quezada SA. The function and dysfunction of memory CD8+ T cells in tumor immunity. Immunol Rev. 2018 May;283(1):194-212.

44. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon ML, et al. The developmental pathway for CD103 (+) CD8+ tissue-resident memory T cells of skin. Nat Immunol (2013) 14:1294–301.

45. David J. Topham and Emma C. Reilly. Tissue-Resident Memory CD8+ T Cells: From Phenotype to Function. Front Immunol. 2018 Mar 26;9:515.

46. Djenidi F, Adam J, Goubar A, Durgeau A, Meurice G, De Montpreville V, et al. CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. J Immunol (2015) 194:3475–86.

47. Franciszkiewicz K, Le Floc'h A, Boutet M, Vergnon I, Schmitt A, Mami-Chouaib F. CD103 or LFA-1 engagement at the immune synapse between cytotoxic T cells and tumor cells promotes maturation and regulates T-cell effector functions. Cancer Res (2013) 73:617–28.

48. Le Floc'h A, Jalil A, Vergnon I, Le Maux Chansac B, Lazar V, Bismuth G, et al. Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. J Exp Med (2007) 204:559–70.

49. Le Floc'h A, Jalil A, Franciszkiewicz K, Validire P, Vergnon I, Mami-Chouaib F. Minimal engagement of CD103 on cytotoxic T lymphocytes

with an E-cadherin-Fc molecule triggers lytic granule polarization via a phospholipase Cgamma-dependent pathway. Cancer Res (2011) 71:328–38.

50. Adachi T, Kobayashi T, Sugihara E, Yamada T, Ikuta K, Pittaluga S, et al. Hair follicle-derived IL-7 and IL-15 mediate skin-resident memory T cell homeostasis and lymphoma. Nat Med (2015) 21(11):1272–9.

51. Davies B, Prier JE, Jones CM, Gebhardt T, Carbone FR, Mackay LK. Cutting edge: tissue-resident memory T cells generated by multiple immunizations or localized deposition provide enhanced immunity. J Immunol (2017) 198(6):2233–7.

52. Casey KA, Fraser KA, Schenkel JM, Moran A, Abt MC, Beura LK, et al. Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. J Immunol (2012) 188(10):4866–75.

53. Shin H, Iwasaki A. Tissue-resident memory T cells. Immunol Rev (2013) 255(1):165–81.

54. Schenkel JM, Fraser KA, Casey KA, Beura LK, Pauken KE, Vezys V, et al. IL-15-independent maintenance of tissue-resident and boosted effector memory CD8 T cells. J Immunol (2016) 196(9):39206.

55. Pizzolla A, Nguyen THO, Smith JM, Brooks AG, Kedzieska K, Heath WR, et al. Resident memory CD8(+) T cells in the upper respiratory tract prevent pulmonary influenza virus infection. Sci Immunol (2017) 2(12):eaam6970.

56. Thom JT, Oxeius A. Tissue-resident memory T cells in cytomegalovirus infection. Curr Opin Virol (2016) 16:63–9.

57. Smith CJ, Caldeira-Dantas S, Turula H, Snyder CM. Murine CMV infection induces the continuous production of mucosal resident T cells. Cell Rep (2015) 13(6):113748.

58. Shiki Takamura. Niches for the Long-Term Maintenance of Tissue-Resident Memory T Cells. frontier in Immunology. May 2018.

59. Russo MV, McGavern DB. Immune surveillance of the CNS following infection and injury. Trends Immunol (2015) 36(10):63750.

60. Ellwardt E, Walsh JT, Kipnis J, Zipp F. Understanding the role of T cells in CNS homeostasis. Trends Immunol (2016) 37(2):154–5.

61. Steinbach K, Vincenti I, Kreutzfeldt M, Page N, Muschaweckh A, Wagner I, et al Brain-resident memory T cells represent an autonomous cytotoxic barrier to viral infection. J Exp Med (2016) 213(8):1571–87.

62. Fernandez-Ruiz D, Ng WY, Holz LE, Ma JZ, Zaid A, Wong YC, et al. Liver-resident memory CD8(+) T cells form a front-line defense against malaria liver-stage infection. Immunity (2016) 45(4):889–902.

63. Frost EL, Kersh AE, Evavold BD, Lukacher AE. Cutting edge: resident memory CD8 T cells express high-affinity TCRs. J Immunol (2015) 195(8):3520–4.

64. Nizard M, Roussel H, Diniz MO, Karaki S, Tran T, Voron T, et al. Induction of resident memory T cells enhances the efficacy of cancer vaccine. Nat Commun (2017) 8:15221.

65. Woon HG, Braun A, Li J, Smith C, Edwards J, Sierro F, et al. Compartmentalization of total and virus-specific tissue-resident memory CD8+ T cells in human lymphoid organs. PLoS Pathog (2016) 12(8):e1005799.

66. Beura LK, Mitchell JS, Thompson EA, Schenkel JM, Mohammed J, Wijeyesinghe S, et al. Intravital mucosal imaging of CD8(+) resident memory T cells shows tissue-autonomous recall responses that amplify secondary memory. Nat Immunol (2018) 19(2):173–82.

67. Jelley-Gibbs DM, Brown DM, Dibble JP, Haynes L, Eaton SM, Swain SL. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. J Exp Med (2005) 202(5):697–706.

68. Schenkel JM, Fraser KA, Maopust D. Cutting edge: resident memory CD8 T cells occupy frontline niches in secondary lymphoid organs. J Immunol (2014) 192(7):2961–4.

69. Hofmann M, Oschowitzer A, Kurzhals SR, Kruger CC, Pircher H Thymus-resident memory CD8+ T cells mediate local immunity. Eur J Immunol (2013) 43(9):2295–304.

70. Bonasio R, Scimone ML, Schaerli P, Grabie N, Lichtman AH, von Andrian UH. Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. Nat Immunol (2006) 7(10):1092–100.

71. Westermann J, Pabst R. Distribution of lymphocyte subsets and natural killer cells in the human body. Clin Investig (1992) 70:539–44.

72. Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. Trends Immunol (2005) 26:360–6.

73. Di Rosa F, Gebhardt T. Bone Marrow T Cells and the Integrated Functions of Recirculating and Tissue-Resident Memory T Cells. Front Immunol. 2016 Feb 16;7:51.

74. Di Rosa F. Two Niches in the Bone Marrow: A Hypothesis on Life-long T Cell Memory. Trends Immunol. 2016 Aug;37(8):503-512.

75. Mazo IB, Honczarenko M, Leung H, Cavanagh LL, Bonasio R, Weninger W, et al. Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. Immunity (2005) 22:259–70.

76. Zhang X, Dong H, Lin W, Voss S, Hinkley L, Westergren M, et al. Human bone marrow: a reservoir for “enhanced effector memory” CD8+ T cells with potent recall function. J Immunol (2006) 177:6730–7.

77. Schenkel JM, Fraser KA, Masopust D. Cutting edge: resident memory CD8 T cells occupy frontline niches in secondary lymphoid organs. J Immunol (2014) 192:2961–4.

78. Racanelli V, Frassanito MA, Leone P, Brunetti C, Ruggieri S, Dammacco F. Bone marrow of persistently hepatitis C virus-infected individuals accumulates memory CD8+ T cells specific for current and historical viral antigens: a study in patients with benign hematological disorders. J Immunol (2007) 179:5387–98.

79. Guerreiro M, Na IK, Letsch A, Haase D, Bauer S, Meisel C, et al. Human peripheral blood and bone marrow Epstein-Barr virus-specific T-cell repertoire in latent infection reveals distinct memory T-cell subsets. Eur J Immunol (2010) 40:1566–76.

80. Quinci AC, Vitale S, Parretta E, Soriani A, Iannitto ML, Cippitelli M, et al. IL-15 inhibits IL-7Ralpha expression by memory-phenotype CD8(+) T cells in the bone marrow. Eur J Immunol (2012) 42:1129–39.

81. Slifka MK, Whitmire JK, Ahmed R. Bone marrow contains virus-specific cytotoxic T lymphocytes. Blood (1997) 90:2103–8.

82. Di Rosa F, Santoni A. Bone marrow CD8 T cells are in a different activation state than those in lymphoid periphery. Eur J Immunol (2002) 32:1873–80.

83. Jahn B, Bergmann L, Weidmann E, Brieger J, Fenchel K, Schwulera U, et al. Bone marrow-derived T-cell clones obtained from untreated acute myelocytic leukemia exhibit blast directed autologous cytotoxicity. Leuk Res (1995) 19:73–82.

84. Noonan K, Matsui W, Serafini P, Carbley R, Tan G, Khalili J, et al. Activatedmarrow-infiltrating lymphocytes effectively target plasma cells and their clonogenic precursors. Cancer Res (2005) 65:2026–34.

85. Takizawa H, Boettcher S, Manz MG. Demand-adapted regulation of early hematopoiesis in infection and inflammation. Blood (2012) 119:2991–3002.

86. Li Y, Toraldo G, Li A, Yang X, Zhang H, Qian WP, et al. B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. Blood (2007) 109:3839–48.

87. Monteiro JP, Benjamin A, Costa ES, Barcinski MA, Bonomo A. Normal hematopoiesis is maintained by activated bone marrow CD4+ T cells. Blood (2005) 105:1484–91.

88. Kim S, Park K, Choi J, Jang E, Paik DJ, Seong RH, et al. Foxp3+ regulatory T cells ensure B lymphopoiesis by inhibiting the granulopoietic activity of effector T cells in mouse bone marrow. Eur J Immunol (2015) 45:167–79.

89. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature (1999) 401:708–12.

90. Hand TW, Dos Santos LM, Bouladoux N, Molloy MJ, Pagan AJ, Pepper M, et al. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. Science (2012) 337:1553–6.

91. Kimberly A. Noonan, Carol A. Huff, Janice Davis, M. Victor Lemas, Susan Fiorino, Jeffrey Bitzan, Anna Ferguson, Amy Emerling, Leo Luznik, William Matsui, Jonathan Powell, Ephraim Fuchs, Gary L. Rosner, Caroline Epstein, Lakshmi Rudraraju, Richard F. Ambinder, Richard J. Jones, Drew Pardoll, and Ivan Borrello. Adoptive transfer of activated marrow-infiltrating lymphocytes induces measurable antitumor immunity in the bone marrow in multiple myeloma. Sci Transl Med. 2015 May 20; 7(288): 288ra78.

92. Seila Lorenzo-Herrero, Christian Sordo-Bahamonde, Segundo Gonzalez, and Alejandro López-Soto. CD107a Degranulation Assay to Evaluate Immune Cell Antitumor Activity. Methods Mol Biol. 2019;1884:119-130.

93. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D, Groh V, Spies T, Pollio G, Cosman D, Catalano L, Tassone P, Rotoli B, Venuta S. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. Blood. 2005 Jan 1;105(1):251-8.