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"Unveiling the Relevance of Immune Dysregulation in Disease Pathogenesis: the $T_{R3\text{-}56}$ cell subtype as a Novel Immune-Regulatory Candidate"

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XXXVII CYCLE

Al mio tutor, Professore Giuseppe Terrazzano, ormai josè, senza il cui sostegno questo percorso non sarebbe certamente iniziato né mai giunto alla sua conclusione.

A lui, la mia sincera gratitudine per aver condiviso con me passione scientifica e vita accademica, per aver valorizzato in me potenzialità ed ambizioni, per aver dedicato a me dedizione di maestro, anche al di là dei confini di questo lavoro.

Mai potrò colmare il debito culturale e di riconoscenza maturato nei suoi riguardi.

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General Premises and Summary

The immune system protects the body from infections and maintains overall health. This protection involves the activation of a complex process by which the immune response is triggered in response to the presence of pathogens (such as viruses, bacteria, fungi, etc.), foreign substances, or abnormal cells (e.g., cancer). This process, known as "immune activation", involves several cells and immune molecules working in harmony to defend against the pathological threats.

Immune activation is expressed through the innate and adaptive immune responses, each with distinct roles and mechanisms for defending the body.

The innate immune response acts rapidly as a first line of defence, providing immediate but relatively non-specific protection. It does not differentiate among specific pathogens but recognizes common characteristics shared by many, such as certain molecules on their surfaces. Components of the innate immune system include physical barriers like the skin and mucous membranes, as well as cellular and biochemical elements such as phagocytes (white blood cells that engulf and digest pathogens) and natural killer (NK) cells, which target infected or abnormal cells. The innate response often promotes pro-inflammatory phases and is itself triggered by inflammation, aiding in the recruitment of immune cells to infection sites and enhancing overall defence.

The adaptive immune response develops more slowly but is highly specific, targeting particular pathogens and adapting to the microenvironmental conditions during the response. This response is characterized by "immunological memory," a fundamental feature of the adaptive immune system that offers a more effective response upon subsequent encounters with the same pathogen. Indeed, after encountering a specific pathogen, the adaptive immune system "remembers" it and responds more effectively to subsequent exposures. The adaptive response involves specialized white blood cells called B and T lymphocytes. B cells produce and secrete antibodies, which recognize and bind to specific antigens on pathogens, either directly neutralizing them or flagging them for elimination by other immune cells. T cells perform various functions, including assisting B cell functions (T helper, Th), directly killing infected cells (Cytotoxic T Lymphocytes, CTL), and

regulating the immune response. The versatility of T cells is crucial for coordinating immune responses, adapting to different challenges, and ensuring an effective but controlled defence against infections and other threats.

Therefore, immune activation is essential for defending the body against infections and other pathological threats. However, it is equally critical that this immune activation is followed by a "controlled shutdown" of the immune responses once the initial threat is neutralized. Without this regulation, prolonged immune activity can lead to unintended damage to the body's own cells, tissues, and organs, resulting in harmful autoimmune responses. This process is called "immune regulation" and plays a vital role in ensuring that the immune system recognizes and tolerates self-components, preventing auto-reactivity and the onset of autoimmune diseases. Therefore, a properly regulated immune system prevents both excessive immune reactions, which can lead to autoimmune diseases or insufficient responses, which can result in chronic infections or cancer. This regulatory process involves multiple mechanisms, including the suppression of overactive immune cells, the production of inhibitory cytokines, and the activation of regulatory cells, all of which are essential for maintaining immune homeostasis and protecting the body from self-inflicted damage.

The main regulatory mechanisms involve immune cells such as regulatory T cells (Tregs), cytokines, and immune checkpoints, which ensure the proper "homeostasis of immune responses". These elements act in synergy to prevent excessive or autoimmune reactions, ensuring a delicate balance between immune activation and immune tolerance. When these systems malfunction, it leads to dysregulation, contributing to various pathologies, including autoimmune disorders, chronic inflammation, and immune evasion by tumours. Understanding the factors that influence immunoregulation is essential for identifying therapeutic targets to treat immune-related diseases.

This thesis explores the role of the novel candidate for immune-regulation, the $T_{R3\text{-}56}$ cells, in four pathological models: the myelodysplastic syndromes (MDS), chronic lymphocytic leukaemia (CLL), COVID-19, and kidney transplantation, all of which involve balancing immune activation and regulation. The research during the PhD course has been highlighting $T_{R3\text{-}56}$ involvement in

immune regulation and disease determinism: i) these cells appear to contribute to immune dysregulation and disease progression by decreasing CTL activation and facilitating immune escape of myelodysplastic clones in MDS haematopoiesis; ii) Expansion of $T_{R3\text{-}56}$ cells is correlated to immune evasion and leukemic spread in CLL, marking them as potential targets for new therapies; iii) Elevated $T_{R3\text{-}56}$ levels in severe cases are associated with high CTL levels and inflammation in COVID-19, suggesting their role in modulating immune responses during infections; iv) Increased $T_{R3\text{-}56}$ levels correlate with unstable graft control in kidney transplantation, potentially serving as early indicators of immune-mediated graft issues.

Overall, $T_{R3\text{-}56}$ cells play a critical role in managing immune homeostasis and influencing disease outcomes, making them important for understanding and developing targeted therapies for these conditions.

Therefore, the $T_{R3\text{-}56}$ lymphocyte population emerges as a promising candidate for immune regulation. A deeper understanding of their role and functions may offer valuable insights into correcting immune imbalances, potentially making a significant impact in immunotherapy and disease management.

1. Introduction

1.1. The Immune System Profile and Role

The immune system is a complex network of cells, tissues, and organs that act together to defend the body against infections and maintain overall health [1-3]. This defence is achieved through a process called "immune activation", which is triggered in response to the presence of pathogens (such as viruses, bacteria, fungi, etc.), foreign substances, or abnormal cells (e.g., cancer) in the body [1-4]. Immune activation involves various cells and immune molecules working in harmony to create a defence against these threats [1-5].

The immune system activation processes are divided into two main responses (figure 1): *innate* and *adaptive* immune responses, each with distinct roles and mechanisms for defending the body [1-5].

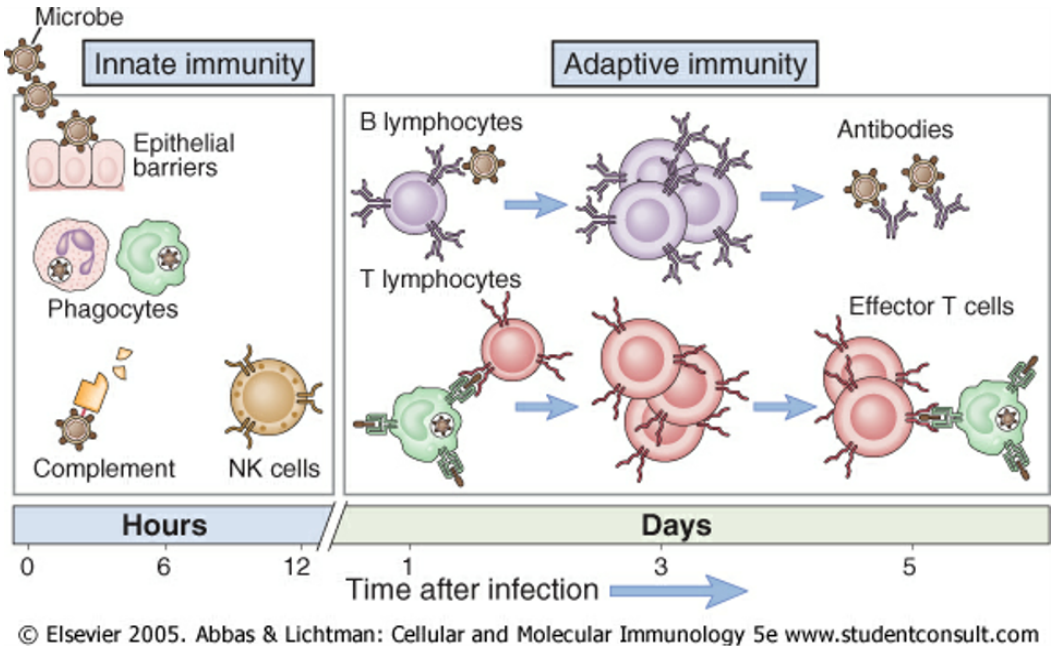

Figure 1. Simplified innate and adaptive immune responses

The innate immune response acts rapidly and is the body first line of defence, providing immediate but relatively non-specific protection [1-6]. It does not

discriminate among specific pathogens, but recognizes common characteristics shared by many pathogens, such as certain molecules on their surfaces [7-11].

Components of the innate immune system include:

- Physical Barriers: skin and mucous membranes that prevent pathogens from entering the body.
- Biochemical Elements: molecules that aid in pathogen destruction.
- Cellular Elements: phagocytes (white blood cells that engulf and digest pathogens), dendritic cells (DC) [7-11] and Natural Killer (NK) cells (which target infected or abnormal host cells) [12-14] (Figure 3).

The innate response often promotes pro-inflammatory phases and is triggered by inflammation, which helps recruit immune cells to the site of infection and enhances the body's overall defence [15-17].

The adaptive immune response develops more slowly but is highly specific, precisely targeting particular pathogens and adapting to the microenvironmental conditions during the immune response itself [1-3]. This response is characterized by "immunological memory," a key feature that provides an effector advantage upon subsequent encounters with the same pathogen [1-3,18,19]. After encountering a specific pathogen, the adaptive immune system "remembers" the previous encounter and responds more effectively upon subsequent exposures [1-3,18,19].

The adaptive response involves specialized white blood cells called B and T lymphocytes [1-3] (figure 2):

- B Cells: These immune cells produce and secrete antibodies, proteins capable of recognizing and binding to specific antigens expressed by pathogens such as bacteria or viruses [1-3,20,21]. This binding can directly neutralize pathogens or flag them for elimination by other immune cells, contributing to the fight against infections and maintaining immune balance in the body [1-3,20,21].
- T Cells: T cells perform various functions, including assisting B cell activity (T helper cells, Th) [1-3,22,23], directly killing infected cells (cytotoxic T lymphocytes, CTL) [1-3,24], and regulating the immune response [25]. T cells exhibit marked versatility (or *plasticity*), which is crucial for coordinating immune

responses, adapting to different challenges, and ensuring an effective but controlled defence against infections and other threats [1-3,22-25].

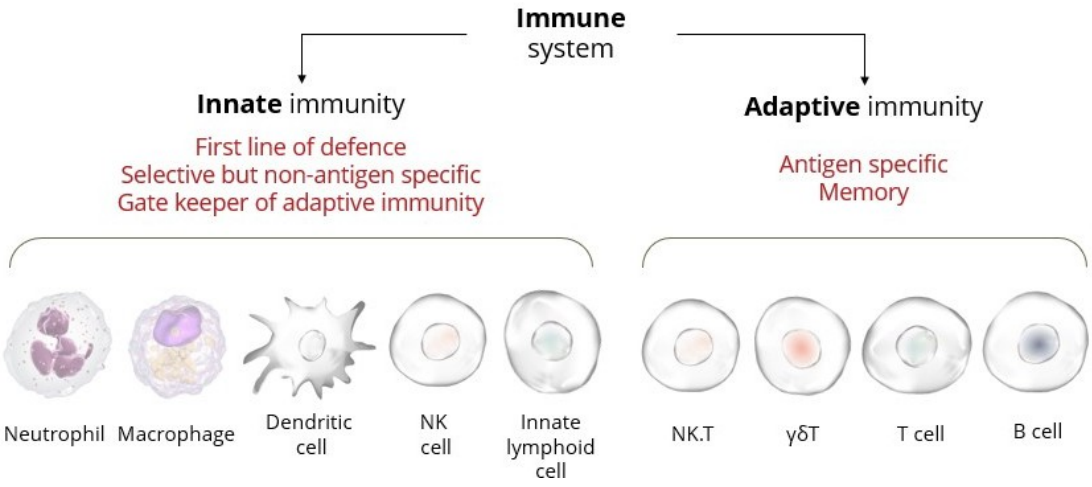

Figure 2. Innate and adaptive immune cells (https://www.innate-pharma.com/science/innate-immunity-nk-cells)

In summary, immune cells have several highly specialized roles in the body, including the identification and neutralization of threats (*the effector functions of immune activation*) and the ability to activate or inhibit the response itself (*the regulatory mechanisms*) [1-3,15-26]. This intricate balance and coordination (*homeostasis*) between the innate and adaptive immune responses is vital for maintaining overall health and effectively defending the body against a wide array of pathogens and diseases.

1.2. Immune Regulation

The management of the immune response is a complex and carefully coordinated process that ensures the immune system operates efficiently, without causing excessive or harmful reactions [25-30]. This regulation involves a vast network of immune cells, signalling molecules, and regulatory mechanisms working together to maintain balance and prevent immune-related diseases [25-30]. Inappropriate initiation or improper termination of the immune response can lead to serious health issues, such as chronic conditions, autoimmune diseases, and cancer [31-35].

According to the "danger model" proposed by P. Matzinger, initiating immune responses without an actual threat or harmful pathogens increases the risk of immune-mediated diseases [36-38]. Ideally, after eliminating a pathogen, the immune system should return to its baseline state without lingering effects that could harm the host's own tissues [1-9]. Prolonged immune activation in the absence of a threat can damage the molecular and cellular components of body tissues (self) [1-9]. Failures in regulatory mechanisms can lead to inappropriate initiation and non-termination of immune functions, causing immune cells to attack healthy tissues and generate damage [38-41].

1.2.1. The Interplay Between Immune Activation and Regulation

From a broader perspective, the prevailing hypothesis has been suggesting that immune responses are remarkably flexible and adaptable [42-44]. Individual immune cells can “set” their functional capabilities over time in response to specific microenvironmental demands, whether to initiate an active response (*the effector or activation phase*) [1-24] or maintain control through immune regulation [25-30].

This delicate balancing act is known as "immune plasticity" (figure 3) [45,46]. Immune plasticity refers to the immune system ability to adapt and respond flexibly to various stimuli, changing its behaviour based on the specific context of a challenge. This dynamic adaptability allows the immune system to switch between pro-inflammatory and anti-inflammatory states, *fine-tuning* its responses according to the nature of the threat. For example, immune cells such as T cells and

macrophages exhibit plasticity by adopting different functional profiles depending on signals they receive from their environment [45,46]. Such occurrence enables them to combat infections, promote tissue repair, or suppress inflammation when necessary. However, *disruptions in immune plasticity* can lead to pathological conditions, where the immune system either fails to mount a proper defence or becomes overactive, contributing to autoimmunity, chronic inflammation, or cancer progression [45,46].

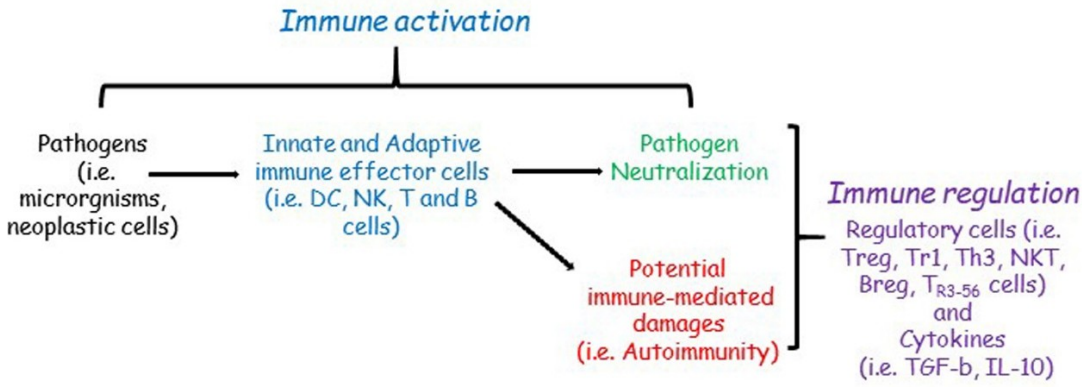

Figure 3. Simplified Immune plasticity network (Carriero F., Rubino V., Leone S. et al. Regulatory $T_{R3\text{-}56}$ Cells in the Complex Panorama of Immune Activation and Regulation. *Cells*. **2023**, *12(24)*, 2841.)

Understanding immune plasticity is critical for developing therapies that can modulate immune responses in a targeted and flexible manner, improving outcomes in diseases where immune dysregulation is a factor. Disruptions in immune plasticity can contribute to failures in both immune activation and regulation, leading to disorders such as immunodeficiencies and autoimmune diseases [38-41,47]. Current research focuses on understanding the mechanisms governing immune regulation and exploring new therapies for immune system-related conditions [48].

1.2.2. The Main Features of Immune Regulation

a) General aspects

Immune regulation is a complex and finely orchestrated process, encompassing a wide array of aspects and key mechanisms that are crucial for achieving a delicate balance between mounting an effective defence against pathogens and ensuring the restoration of health. This balance is essential to avoid excessive immune reactions that could cause harm to the host own tissues [25-48]. These mechanisms include the precise activation and suppression of various immune cells, the intricate signalling pathways that guide their actions, and the feedback loops that ensure the immune response is appropriately scaled and terminated once a threat has been neutralized. Furthermore, immune regulation faces the challenge of distinguishing between harmful invaders and the body own cells: the failure in this challenge can result in autoimmune diseases or chronic inflammation [25-48]. Therefore, the immune system ability to *finely tune* its responses is critical for maintaining overall health and preventing immune-related disorders.

A unique and critical feature of the immune system is its ability to distinguish between the body own cells and tissues (*self*) and foreign invaders (*non-self*), a process that is fundamental to its proper functioning [1-9]. This ability to discriminate is pivotal for preventing the immune system from erroneously attacking the self, an occurrence that can result in the development of autoimmune diseases, where the immune system mistakenly targets healthy tissues, causing inflammation and tissue damage [31-33].

The basis of this discrimination lies in the *immune tolerance* (or tolerance) mechanisms, which are essential for maintaining self-recognition and avoiding autoimmunity [42-44,49]. Tolerance is primarily achieved through two key processes: central and peripheral tolerance.

Central tolerance takes place during the early development of immune cells. In the thymus, T cells undergo rigorous selection processes to eliminate those that strongly react to self-antigens. Similarly, in the bone marrow, B cells that recognize self-antigens are either eliminated or undergo receptor editing to change their specificity [30,49-54]. This early screening is crucial for ensuring that self-reactive cells are either deleted or rendered non-functional before they can cause harm.

However, central tolerance alone is not sufficient to prevent autoimmunity, as some self-reactive cells inevitably escape from the primary lymphoid organs and migrate to peripheral tissues and organs. To address this, peripheral tolerance mechanisms are in place to regulate or suppress any self-reactive immune cells that circulate in the body after their development [55].

In this regard, several molecules and cells are involved in preserving and maintaining the peripheral tolerance mechanisms.

b) Molecules

Cytokines are signalling molecules, produced by immune cells, that regulate the immune response [56]. They can be pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, interferon-gamma (IFN-γ), and tumour necrosis factor-alpha (TNF-α), promote inflammation and immune activation [56]. Conversely, anti-inflammatory cytokines, such as IL-10 and transforming growth factor-beta (TGF-β), dampen immune responses and promote tolerance [57].

Checkpoint molecules, including programmed cell death protein 1 (PD-1) [58] and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [59], regulate immune responses and prevent excessive activation [60]. They act as "brakes" on immune cells, inhibiting their activation and effector functions [60]. Targeting these checkpoint molecules has been successful in immunotherapy, particularly for treating autoimmunity and cancer [60].

The immune system employs feedback mechanisms to regulate its activity. Various immune cells and molecules produce inhibitory or activating signals that modulate the response [23-30,56-60]. These feedback mechanisms help maintain balance and prevent excessive or prolonged activation [44-46]. The local tissue environment can significantly influence immune responses. Specific molecules or cells in tissues can enhance or suppress immune reactions [8-11,36-38,41-44]. Environmental factors play a significant role in determining immune plasticity and, consequently, influencing immune regulation positively or negatively [42-46].

c) Regulatory Cells

Numerous cells have regulatory roles in the immune response. *Regulatory T cells* (Tregs) are a specialized subset of $CD3^+$ $CD4^+$ T lymphocytes that play a crucial role in immune regulation and maintaining tolerance [26,29,30,55,61,62]. They are essential in preventing excessive responses and controlling diseases, including autoimmune disorders, allergies, and graft rejection [61-63]. Tregs cells are characterized by the expression of the transcription factor FoxP3, a master regulator of their development and function [62,63]. Mutations or deficiencies in FoxP3 lead to severe autoimmune diseases, highlighting the critical role of Tregs in homeostasis [61-63].

Two subtypes of Tregs have been described: natural constitutive (nTreg) [29,61-63] and inducible (iTreg) cells (figure 4) [61-64]. nTregs develop in the thymus and derive from progenitor T cells undergoing a selection process that confers regulatory properties [29,61-63]. They are characterized by specific surface markers, such as CD4 and CD25 (interleukin-2 receptor alpha chain) [29,61-63]. nTregs naturally suppress the activation and proliferation of other immune cells, helping maintain homeostasis and prevent autoimmune reactions [29, 61-63]. iTregs are generated in peripheral tissues in response to specific environmental cues [61-64]. They arise from the differentiation of conventional $CD4^+$ T cells (non-regulatory T cells) due to signals from the local tissue microenvironment and cytokines like TGF-β [61-64]. iTregs tailor their regulatory functions to specific tissues [61-64].

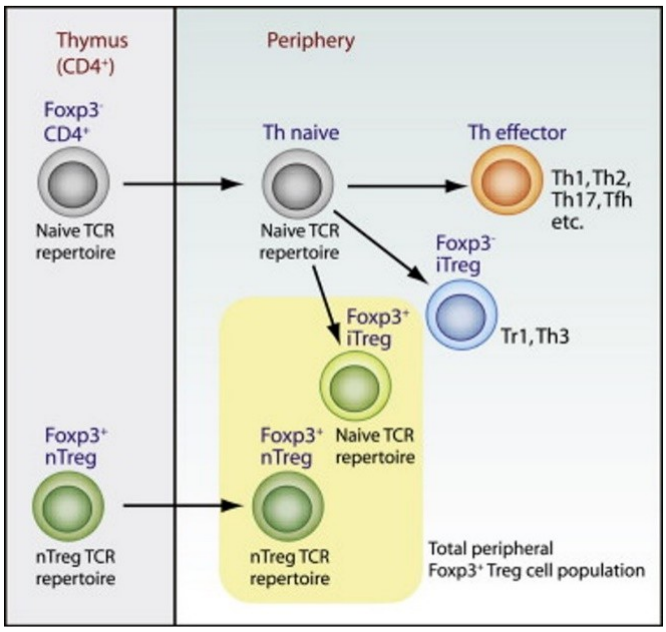

Figure 4. Immune regulatory T cell subtypes (https://www.semanticscholar.org)

Tregs suppress immune responses through various mechanisms: they secrete immunosuppressive cytokines like IL-10 and TGF-β, which suppress the activity and proliferation of other immune cells, such as T cells, B cells, and antigen-presenting cells [29,61-64]. Tregs also directly interact with and suppress the function of other immune cells through *cell-to-cell contact* [29,61-64], involving molecules such as CTLA-4 and LAG-3 on Treg surfaces, which interact with ligands on target cells, leading to inhibition of immune responses [63-65]. Finally, Tregs modulate the metabolic environment to suppress immune responses, using pathways like increased adenosine production or IL-2 consumption to create an immunosuppressive *milieu* [61].

Tregs are crucial for maintaining self-tolerance and preventing autoimmune diseases [29,61-65]. They recognize self-antigens and suppress the activation and function of autoreactive T cells that could harm the body's tissues [29,61-65]. However, the balance between Tregs and effector T cells can be disrupted in certain conditions, leading to *immune dysregulation* [29,41,42,44,55,61-65]. Treg deficiency and/or dysfunction can result in uncontrolled activation and the development of autoimmune diseases [29,41,42,44,55,61-65]. Conversely,

excessive or overactive Treg responses can contribute to immune suppression and hinder effective responses against infections or cancer [29,41,42,44,55,61-65].

Research on Tregs and their role in immune regulation is rapidly evolving. Approaches to harness the therapeutic potential of these cells in treating autoimmune diseases, allergies, transplant rejection, and other disorders are being evaluated [66,67]. Strategies include Treg-based cellular therapies and the modulation of Treg function and stability for therapeutic interventions [66,67].

$CD8^+$ suppressor T cells represent a subtype of Tregs with a unique ability to suppress responses, potentially useful in preventing autoimmune reactions [68-70]. $CD8^+$ Tregs are a specialized subset of cytotoxic T cells with unclear functions and mechanisms, but they play crucial roles in regulation [68-70].

Type 1 (*Tr1*) and Type 2 (*Tr2*) regulatory T cells are also noteworthy (figure 4). Tr1 cells produce anti-inflammatory cytokines IL-10 [71-73], which suppress the activity of other immune cells, including T cells and macrophages, thereby reducing inflammation [71-73]. Tr1 ability to regulate responses makes them interesting targets for potential therapeutic interventions in conditions involving immune dysregulation, such as autoimmune diseases and allergies [72,73,74]. Tr1 cells contribute to tumour immune evasion by suppressing the antitumor response [72,73]. Tr2 cells, also known as Th3 cells, produce TGF-β, which has anti-inflammatory properties and inhibits various immune cells [75-76]. Tr2 cells are involved in regulating responses in various contexts, including allergic reactions, autoimmune diseases, and tissue inflammation [75-76]. Their differentiation and development are influenced by the cytokine environment and interactions with other cells [77].

Natural killer T (NKT) cells characterized by the expression of both T cell receptors and natural killer cell markers, are pivotal in integrating the innate and adaptive immune systems [78-81]. These cells possess a unique ability to recognize lipid antigens presented by CD1d molecules, bridging the gap between the rapid, non-specific responses of innate immunity and the more specific, slower responses of adaptive immunity. NKT cells contribute to immune regulation and can modulate responses to infections, tumours, and autoimmune diseases through their diverse functional capabilities, including cytokine production and direct cytotoxic activity.

Their role in orchestrating both immediate and long-term immune responses underscore their importance in maintaining immune balance and health (figure 5).

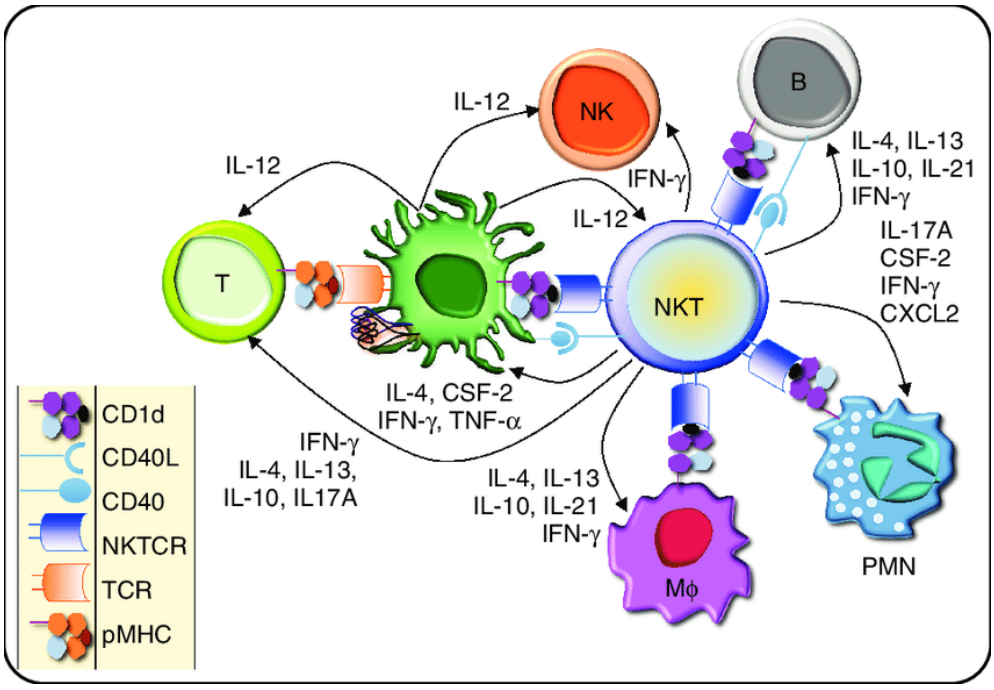

Figure 5. Natural Killer T (NKT) cells (https://www.researchgate.net)

1.3. New Cell Candidates for Immune Regulation

Emerging research is constantly describing novel cell types with potential roles in immune regulation, expanding our understanding of the intricate network that governs immune responses. These newly identified cell types, such as various subsets of regulatory T cells, innate lymphoid cells, and myeloid-derived suppressor cells, contribute to the complexity and depth of immune system modulation. They are involved in *fine-tuning* immune responses, preventing excessive inflammation, and maintaining tolerance to self-antigens.

The discovery of additional immune regulatory cell types not only deepen the complexity to our understanding of immune regulation, but also paves the way for novel therapeutic intervention. By targeting these cells or their signalling pathways, it may be possible to develop innovative treatments for a range of immune-related diseases. These innovative therapeutic approaches could potentially offer more

precise and effective ways to modulate the immune system, reducing the risk of side effects associated with broader immunosuppressive therapies and improving patient outcomes.

As research advances, the ongoing discovery and characterization of novel immune cell types will likely provide a more comprehensive and detailed understanding of immune regulation. This could ultimately transform the treatment of immune-related diseases, shifting the focus toward more personalized and targeted therapies that harness the unique functions of these newly discovered cells.

1.3.1. Mucosal-Associated Invariant T (MAIT) cells

MAIT cells are a unique subset of T cells that recognize metabolites of microbial vitamin B2 biosynthesis. They are predominantly found in mucosal tissues and are involved in both antimicrobial responses and immune regulation [82-83]. MAIT cells can produce a wide range of cytokines, including pro-inflammatory and regulatory cytokines, which positions them as potential modulators of both inflammation and tolerance [82-83]. Their role in maintaining mucosal immunity and modulating systemic inflammation makes them intriguing candidates for therapeutic targeting in autoimmune diseases and chronic inflammatory conditions [82-83].

1.3.2. Regulatory B Cells (Bregs)

Bregs represent a subset of B cells with immunosuppressive capabilities. They produce anti-inflammatory cytokines such as IL-10 and TGF-β, which contribute to the regulation of immune responses and maintenance of tolerance [84-86]. Bregs play a significant role in modulating autoimmune diseases, chronic infections, and transplantation tolerance [84-86]. Their ability to influence T cell responses and maintain immune homeostasis highlights their potential as therapeutic targets in various immune disorders [84-86].

1.3.3. T Helper 17 (Th17) Cells

Th17 cells are traditionally known for their role in promoting inflammation and autoimmunity through the secretion of IL-17. However, recent studies suggest that a subset of Th17 cells can acquire regulatory functions under certain conditions [87-88]. These regulatory Th17 cells (also known as Th17.1) can produce anti-inflammatory cytokines and contribute to tissue repair and tolerance, challenging the conventional view of Th17 cells solely as pro-inflammatory [87-88]. Understanding the conditions that lead to the generation of regulatory Th17 cells could provide new insights into managing autoimmune diseases and chronic inflammation [87-88].

1.3.4. Plasmacytoid Dendritic Cells (pDCs)

pDCs are known for their ability to produce large amounts of type I interferons in response to viral infections. Recent research has highlighted their role in immune regulation beyond their antiviral functions [89]. pDCs can influence T cell responses, contribute to immune tolerance, and modulate autoimmune diseases through their production of regulatory cytokines and interaction with other immune cells [89]. Their multifaceted role in both immunity and regulation makes them an area of active investigation for therapeutic modulation [89].

1.3.5. Exhausted T Cells

Exhausted T cells, typically characterized by high expression of inhibitory receptors like PD-1, are often found in chronic infections and cancer [90]. While traditionally viewed as dysfunctional, recent research suggests that exhausted T cells can have regulatory functions in certain contexts [90]. They may contribute to immune regulation by suppressing excessive inflammation and promoting tissue repair. Understanding their dual roles in exhaustion and regulation could lead to novel strategies for enhancing immune responses in cancer and chronic infections [90].

1.3.6. Regulatory Eosinophils

Eosinophils are traditionally associated with allergic reactions and parasitic infections. However, recent findings suggest that a subset of eosinophils exhibits regulatory properties [91]. These regulatory eosinophils produce anti-inflammatory cytokines and can modulate T cell responses, contributing to tissue homeostasis and immune regulation [91]. Their emerging role in regulating immune responses beyond traditional eosinophil functions presents new opportunities for targeting eosinophils in allergy and autoimmune disease therapy [91].

1.3.7. Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are a heterogeneous group of cells that can suppress T cell responses and promote immune tolerance [92-95]. They are known to accumulate in various pathological conditions, including cancer and chronic inflammation. MDSCs can influence immune responses through various mechanisms, such as the production of immunosuppressive cytokines and the modulation of metabolic pathways [92-95]. Their potential to modulate immune responses makes them a promising target for therapeutic intervention in cancer and autoimmune diseases [92-95].

These emerging cell types offer exciting new avenues for understanding and manipulating immune regulation. Their diverse roles in maintaining balance between immune activation and tolerance could lead to innovative approaches for treating immune-mediated diseases. Further research into these cells will help clarify their mechanisms of action and potential therapeutic applications.

2. In Search of New Regulatory Cell Subset: the aim of this thesis

Recent advances in immunology have allowed the identification and characterization of previously unrecognized cell subsets with regulatory functions [96]. These emerging regulatory cell subsets are crucial for maintaining immune balance, preventing autoimmune diseases, and modulating inflammatory responses.

The aim of this thesis is to identify and characterize the $T_{R3\text{-}56}$ cells, a new T lymphocyte subtype co-expressing CD3 and CD56 molecules, with regulatory functions within the immune system. This thesis aims to deepen our understanding of immune regulation through the exploration of $T_{R3\text{-}56}$ cells, potentially identifying new therapeutic targets for the treatment of autoimmune disorders, transplantation conditions, infections and haematological disorders.

Key objectives include:

- Investigating and characterizing the potential involvement of $T_{R3\text{-}56}$ cells in immune-mediated conditions;

- Assessing the potential significance of $T_{R3\text{-}56}$ cells in maintaining immune balance and preventing pathological immune responses;

- Contributing to the broader understanding of immune regulation by integrating findings into the existing framework of immune cell functions and interactions.

As perspectives and in future studies:

- Understanding the mechanisms of action of this newly identified cell subset

- Evaluation of targeting this regulatory cell subset for therapeutic purposes in various immune-related diseases.

In summary, this doctoral research aimed to contribute to the field of immunology by advancing our understanding of immune regulation through the exploration of key mechanisms governing immune balance, while also identifying potential targets for the development of innovative therapeutic strategies.

2.1. $CD3^+CD56^+$ co-expressing T cell populations

Several distinct subpopulations of T lymphocytes co-expressing CD3 and CD56 molecules have been identified, highlighting the diversity within the immune system [78-82,97-112]. These cells exhibit both T cell characteristics (marked by the presence of CD3) and NK cell features (marked by CD56 expression) [78-82,97-112]. This dual expression suggests a multifaceted role for these cells in immune responses, blending aspects of both adaptive and innate immunity.

Within the $CD3^+$ $CD56^+$ T cell population, NKT cells stand out due to their well-characterized function of bridging innate and adaptive immunity (figure 6).

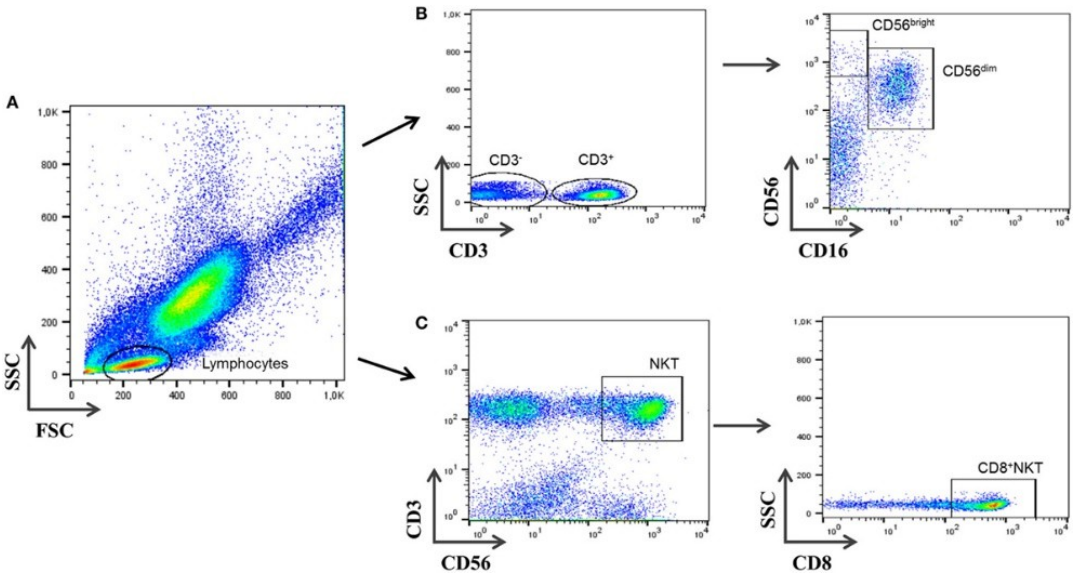

Figure 6. Classical *gating strategy* of NK and NKT cells by Flow Cytometry approach (de Andrés C., Fernández-Paredes L., Tejera-Alhambra M. et al. Activation of Blood $CD3^+CD56^+CD8^+$ T Cells during Pregnancy and Multiple Sclerosis. Front Immunol. **2017**, *8*. 196.)

As previously reported in this thesis, NKT cells are capable of recognizing lipid antigens presented by CD1d molecules, a process that is distinct from the peptide recognition by conventional T cells. Upon activation, NKT cells rapidly produce a broad range of cytokines, including IFN-γ and IL-4, which play crucial

roles in modulating immune responses and influencing the activity of other immune cells [78-82, 97-100]. Through these actions, NKT cells contribute to the regulation of immune responses, inflammation, and the defence against certain infections and tumours (see figure 5).

While NKT cells have been extensively studied, the functions and significance of other $CD3^+$ $CD56^+$ T cell subtypes remain less understood. These cells are thought to be involved in a variety of immune activities, including cytotoxicity, cytokine production, and potentially in the broader regulation of immune responses and disease mechanisms [100-108]. Their ability to engage in cytotoxic activity suggests they might play a direct role in the elimination of infected or malignant cells. However, the exact mechanisms through which these cells contribute to immune regulation and the development or progression of diseases are still being investigated.

Elevated levels of $CD3^+$ $CD56^+$ T cells have been observed in various pathological conditions, including solid tumours, non-alcoholic fatty liver disease (NAFLD), autoimmune disorders, and haematological malignancies [100-108]. In these contexts, they may contribute to disease pathology, either through their cytotoxic effects or by modulating the local immune environment. Their presence in such conditions raises questions about whether they play a protective role by targeting diseased cells, or if they contribute to tissue damage and disease progression through dysregulated immune responses [100-108]. Moreover, these cells are often recognized as potent cytotoxic effectors, capable of inducing cell death in target cells, which further underscores their potential impact in both protective and pathological immune processes [109-111].

The investigation into the roles of $CD3^+$ $CD56^+$ T cell subtypes is crucial for understanding their contributions to immunity and disease. As research advances, these cells may emerge as significant targets for therapeutic interventions aimed at modulating immune responses in a variety of diseases, potentially offering new avenues for treatment strategies.

In the context of haematological malignancies, $CD3^+CD56^+$ T cells have been shown to play varied roles. Their dysfunction has been suggested to contribute to the impaired immune response against leukemic blasts in patients with acute

myeloid and acute lymphocytic leukaemia [105]. Additionally, $CD3^+CD56^+$ T cells are expanded in the bone marrow of patients with Chronic Myeloid Leukaemia (CML) [106], and their numbers decrease in CML patients treated with tyrosine kinase inhibitors [107]. An increased proportion of $CD3^+CD56^+$ lymphocytes has also been observed in lymph nodes affected by Large B Cell Lymphoma [108].

Overall, these findings indicate a general increase in $CD3^+CD56^+$ T lymphocytes in cancer patients, but they do not provide a clear explanation for this phenomenon. Thus, the current understanding of these cells remains incomplete. A more thorough phenotypic and functional characterization of $CD3^+CD56^+$ lymphocyte subtypes is essential to elucidate their role and potential involvement in effector functions and immune regulation mechanisms.

Nevertheless, it remains possible that $CD3^+CD56^+$ cell phenotypes are more diverse than currently recognized or that their functions may be influenced by plasticity. This highlights our curiosity to further investigate these cells, as a more comprehensive understanding of their diverse roles and functional adaptations could provide deeper insights into their potential in cancer immunotherapy and immune regulation.

Therefore, this thesis summarizes the results of the research conducted during the PhD training to understand the potential role and involvement of $CD3^+CD56^+$ cells in human disease models.

The pathological models addressed include Myelodysplastic Syndromes, Chronic Lymphocytic Leukaemia, COVID-19, and kidney transplants.

2.2. The original description on $T_{R3\text{-}56}$ as a unique regulatory cell population

In 2020, the research group of Prof. Terrazzano (which includes the author of this doctoral thesis) originally studied the regulatory role of $CD3^+$ $CD56^+$ T cells in the progression of type 1 diabetes (T1D) [112].

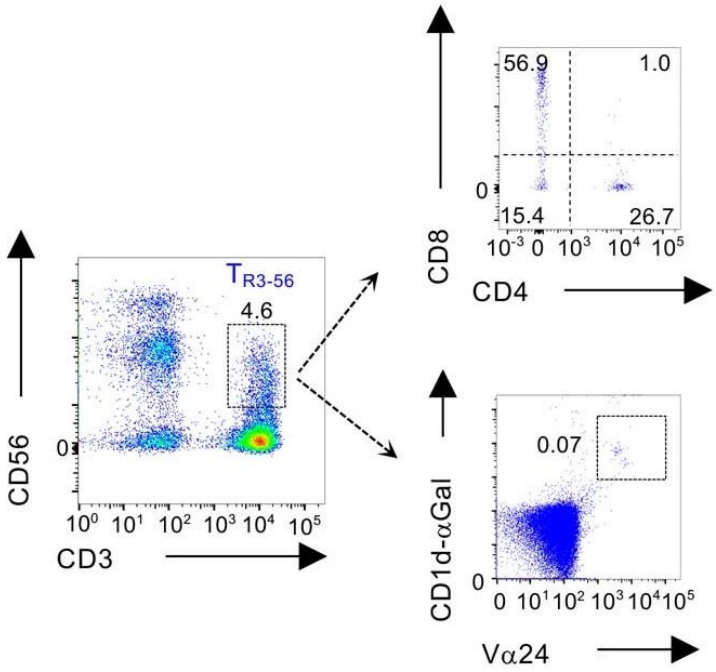

Figure 7. Gating strategy of T_{R3-56} cells by Flow Cytometry approach (Terrazzano, G.; Bruzzaniti, S.; Rubino, V. et al. T1D progression is associated with loss of CD3+CD56+ regulatory T cells that control CD8+ T cell effector functions. *Nat. Metab.* **2020**, *2*(2), 142-152.)

The research demonstrated that individuals with T1D had a significant reduction in the number of $CD3^+CD56^+$ regulatory T cells compared to healthy individuals [112]. This reduction was associated with an increase in the activation and effector functions of $CD8^+$ T cells, which contribute to the destruction of insulin-producing beta cells in the pancreas [112]. Additionally, the study showed that the reduced number of $CD3^+CD56^+$ regulatory T cells was correlated with disease progression in T1D patients. The decline in these regulatory T cells was linked to increased insulin requirements, indicating a worsening of the disease [112]. Overall, the study suggested that the loss of $CD3^+CD56^+$ regulatory T cells contributes to the progression of T1D by allowing for the activation and effector functions of $CD8^+$ T cells. These findings highlight the importance of these regulatory T cells in maintaining immune tolerance and controlling autoimmune responses in type 1 diabetes.

Moreover, such research demonstrated that the $CD3^+CD56^+$ T regulatory subset differs from NKT cells [112]. Specifically, $CD3^+CD56^+$ regulatory cells (i) are not CD1d-restricted, (ii) do not express Valpha24/Vbeta11 chains (figure 6) but display a heterogeneous V-beta repertoire, (iii) are unable to kill K562 cells in vitro, and (iv) only 1-5% of CD1d-restricted T cells are positive for the CD56 molecule. Furthermore, this $CD3^+CD56^+$ regulatory subset is genetically, metabolically, and functionally distinct from NKT cells and other cells co-expressing CD3 and CD56 [112]. Prof. Terrazzano originally named this subset as $T_{R3\text{-}56}$ cells [112].

This PhD thesis focuses on expanding the research into the involvement and role of $T_{R3\text{-}56}$ cells in various pathophysiological models. Specifically, it aims to investigate the relationship between $T_{R3\text{-}56}$ cells and other immune system cells in the context of disease determinism within inflammatory and immune-mediated processes. The broader objective is to deepen the understanding of the regulatory functions of this lymphocyte subset and to explore potential use of $T_{R3\text{-}56}$ cells as disease biomarkers, as well as their use in therapeutic strategies for modulating these cells in human diseases.

3. Study Models: Diseases and Pathophysiological Contexts

3.1. Myelodysplastic Syndromes

Myelodysplastic syndromes (MDS) represent an ample and heterogeneous group of acquired hematopoietic clonal disorders, characterized by ineffective haematopoiesis, leading to peripheral cytopenia, and an elevated risk of progression to acute myeloid leukaemia (AML). These disorders are marked by a disruption in the normal production of blood cells, resulting in a range of symptoms related to anaemia, infection due to neutropenia, and bleeding caused by thrombocytopenia [113].

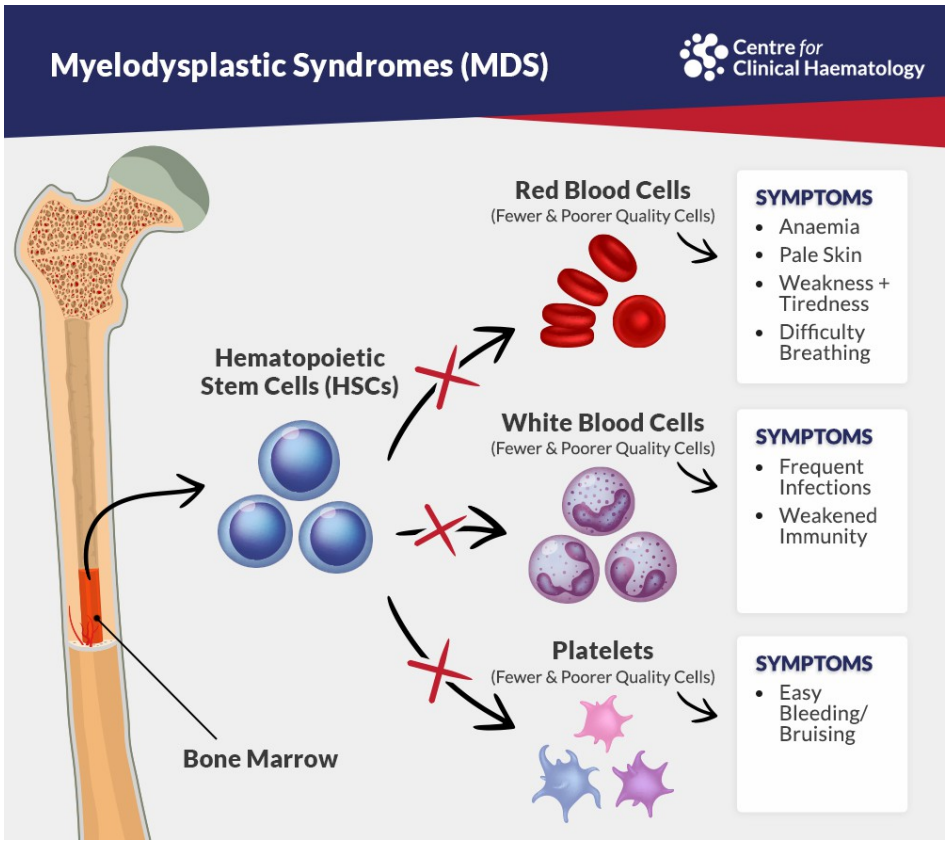

Figure 8. Myelodysplastic Syndromes (MDS) (https://cfch.com.sg/myelodysplastic-syndromes-mds)

Recent research has increasingly focused on the complex pathogenesis of MDS, which appears to involve a multifaceted interplay between several key factors. Genetic alterations in hematopoietic stem cells (HSCs) are at the core of the disease, with mutations in genes related to DNA methylation, histone modification, splicing factors, and signal transduction being frequently identified in MDS patients. These genetic changes lead to clonal expansion and the dominance of abnormal HSCs, disrupting normal blood cell formation [113].

However, the pathogenesis of MDS extends beyond these genetic factors. In this regard, an abnormal pro-inflammatory microenvironment within the bone marrow has been identified as a significant contributor to disease progression [113, 114]. This microenvironment, often characterized by increased levels of inflammatory cytokines and altered interactions between hematopoietic cells and the bone marrow stroma, creates conditions that exacerbate ineffective haematopoiesis and support the survival and expansion of malignant clones (figure 9).

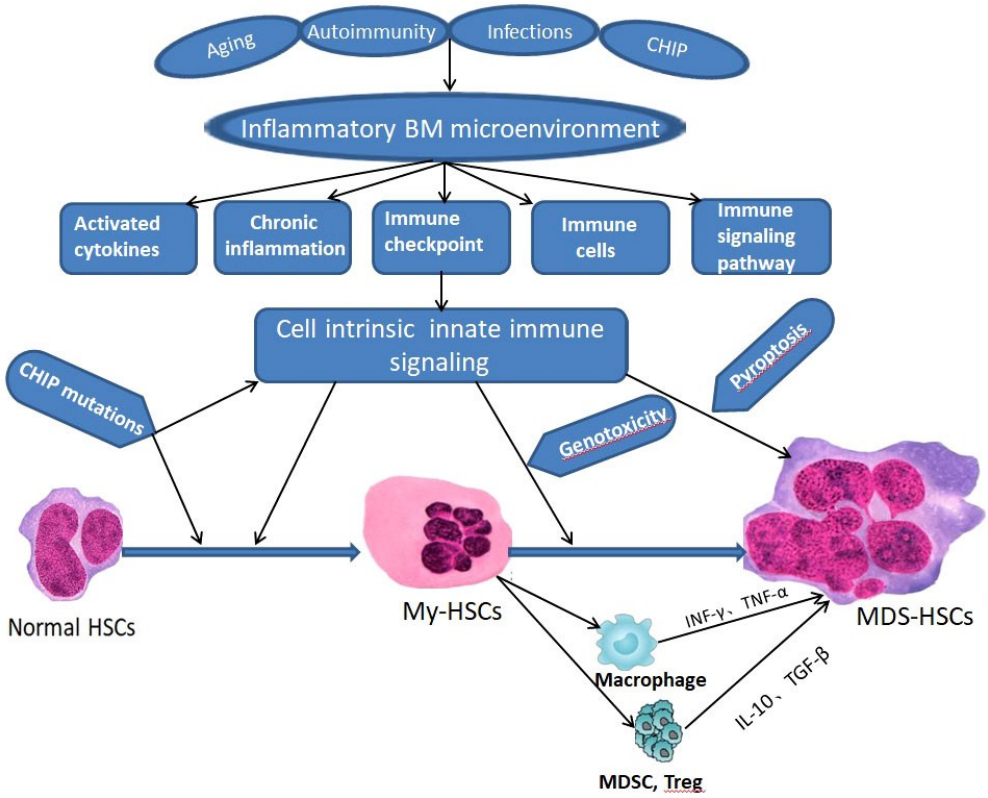

Figure 9. MDS and immune mediated mechanism (Peng X., Zhu X., Di T. et al. The yin-yang of immunity: Immune dysregulation in myelodysplastic syndrome with different risk stratification. *Front Immunol*. **2022**, *13*, 994053.)

In addition, immune dysregulation plays a crucial role in MDS [114] The immune system, which typically functions to eliminate abnormal cells, may become dysregulated in MDS, leading to either an inadequate response that allows malignant cells to persist or an overactive response that further damages normal hematopoietic cells. T-cell mediated autoimmunity, altered expression of immune checkpoint molecules, and dysfunctional macrophages are some of the immune-related abnormalities observed in MDS patients.

Together, these factors - genetic alterations in HSCs, an abnormal pro-inflammatory microenvironment, and immune dysregulation - interact in a complex manner to drive the development and progression of MDS [114]. Understanding these interactions not only provides insights into the underlying mechanisms of the disease but also opens up potential avenues for novel therapeutic strategies aimed at targeting these specific pathogenic processes.

Notably, a subset of patients exhibits alterations in immune regulation

mechanisms.

In this regard, the immune-mediated mechanisms and the role of bystander T cells, which may be recruited by dysplastic antigens and related to immunogenic acquired somatic mutations, are thought to contribute to the selection and progression of dysplastic clones that evade immune-mediated damage [115,116].

A key feature in MDS pathogenesis is the abnormal activation and clonal expansion of CTL in the bone marrow. In early stages of MDS (very low and low risk), activated CTLs and a pro-inflammatory environment can damage polyclonal haematopoiesis, promoting the selection of dysplastic clones that escape immune attack. Conversely, in late MDS stages, an immune-suppressive environment may impair CTL function and support the expansion and progression of dysplastic clones [117].

Additional factors such as hematopoietic stem cell pyroptosis [118], a disturbed inflammatory cytokine profile [119], an oligoclonal CD8+ and CD4+ T

cell repertoire [120], ineffective tolerance control [117,121], and associations with autoimmune disorders [122,123] have been frequently observed in MDS patients.

In response to these findings, various immune-modulating strategies have been proposed for MDS management. Immunosuppressive therapies (IST) have been tested in multiple clinical trials, showing a broad range of responses (0%–66%) [124-127]. The limited efficacy in some patients highlights the need for more effective selection criteria, which are currently lacking.

IST approaches targeting molecules involved in immune-mediated pathways are under investigation in several clinical trials [128].

A deeper understanding of immune-mediated determinism in MDS could enhance both our comprehension of MDS pathogenesis and patient management. In this context, the potential involvement of $T_{R3\text{-}56}$ cells may help clarify the broader landscape of dysregulation in MDS.

3.2. Chronic Lymphocytic Leukaemia (CLL)

Chronic Lymphocytic Leukaemia (CLL) is the most prevalent leukaemia in adults, characterized by the proliferation of mature B cells expressing CD5, CD19, and CD23 [129-131]. The clinical progression of CLL varies widely among patients. Asymptomatic individuals in the early stages may derive minimal benefit from early drug interventions, whereas those with advanced disease typically require immediate first-line therapy to manage disease progression [129-131].

Several factors, including p53 mutations, immunoglobulin heavy-chain variable gene (IGHV) mutational status, and various cytogenetic and epigenetic changes [132-134], as well as micro-environmental stimuli [135], have been proposed as predictive and diagnostic biomarkers for CLL. Despite these advancements, there is a recognized need for additional markers to enhance diagnostic and prognostic accuracy for the disease. Recent successes with immunotherapy in refractory or relapsed CLL [136] underscore the potential for harnessing immune effectors to target leukaemia cells effectively.

Some immunological changes, such as the expansion of T and NK cells and a reduction in circulating normal B cells, are associated with monoclonal B-cell lymphocytosis (MBL), a premalignant condition that precedes CLL. However, most immune surveillance dysfunctions develop as the disease progresses, likely contributing to the transition from MBL to CLL [137]. Clinically, these immunologic dysregulations lead to increased susceptibility to infections and secondary malignancies, the occurrence of autoimmune phenomena, and a failure to control disease progression [137] (figure 10).

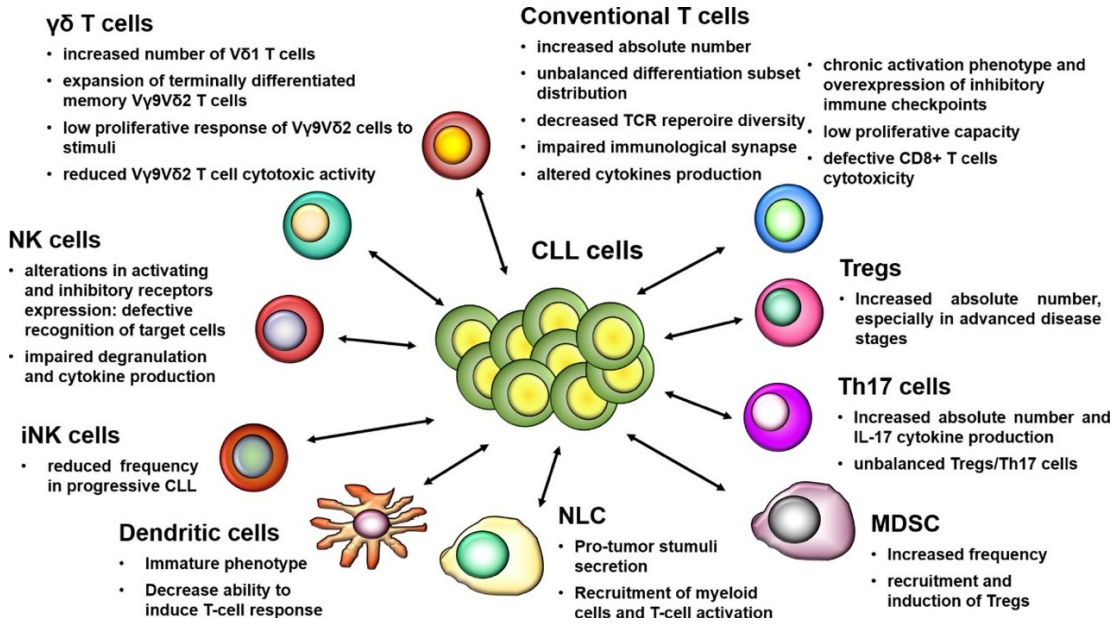

Figure 10. Chronic lymphocytic leukaemia (CLL) and immune cells (Griggio V., Perutelli F., Salvetti C. et al. Immune Dysfunctions and Immune-Based Therapeutic Interventions in Chronic Lymphocytic Leukemia. *Front Immunol*. **2020**, *11*, 594556.)

Investigating the immune profiles of patients with clinically stable CLL could yield valuable insights into the interplay between immune response, immune-regulation and disease progression. While the mechanisms by which the immune system controls tumour cells remain largely undefined, the roles of NK cells, CTLs, and IFN-γ dependent pathways in tumour control have been well-documented [138-147].

Patients with CLL often exhibit a functional T-cell compartment with notable defects, including impaired proliferation, reduced cytotoxicity, and compromised formation of effective immune synapses [148-151]. Similarly, NK cell activity analysis has revealed defects in lysis and antibody-dependent cellular cytotoxicity (ADCC), although some NK functions may remain unaffected [152-156].

The functional alterations observed in CTL and NK cells may be correlated to the patterns of immune response dysregulation, particularly affecting the cells responsible for maintaining immune system balance. This disruption could

influence not only the activity of these cytotoxic cells but also the broader regulatory network of immune interactions.

In this context, it is reasonable to hypothesize that $T_{R3\text{-}56}$ cells might also play an altered or dysregulated role, contributing to the overall immune imbalance. Investigating their involvement could provide valuable insights into the mechanisms underlying immune dysfunction and offer potential avenues for therapeutic intervention in CLL.

3.3. COVID-19

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has posed an unprecedented global health challenge since its initial identification in December 2019 [157-158]. With a spectrum of clinical manifestations ranging from mild respiratory symptoms to severe pneumonia and multiorgan dysfunction, *COronaVIrus Disease 19* (COVID-19) rapidly spread across all continents, causing significant morbidity and mortality worldwide [159-161].

As scientific and medical communities continue to grapple with the complexities of this pandemic, understanding the intricate interaction between new SARS-CoV-2 variants and the human immune system remains crucial for developing effective diagnostic biomarkers, therapeutic interventions, vaccines, and preventive strategies [161-165].

Following the virus entry [157-161,166], several immunological events occur that activate innate and adaptive immune responses aimed at controlling viral replication and eliminating infected cells [167-171]. As of mid-2024, COVID-19 persists as a global health challenge, and advancements in vaccines [172] and ongoing public health efforts are essential to manage its impact in 2024.

In this context, scientific literature has highlighted various immune responses during SARS-CoV-2 infection and described extensive involvement of the immune system in countering this infection, offering new insights for a comprehensive understanding of the processes and mechanisms of susceptibility and resistance to infection (Figure 11) [167-171,173]. Of particular relevance is the phenomenon known as the "cytokine storm", characterized by an excessive release of pro-inflammatory cytokines, which can lead to endothelial and tissue damage, exacerbating symptoms to the point of acute respiratory distress syndrome (ARDS) and multiorgan failure in COVID-19 [167-171,173].

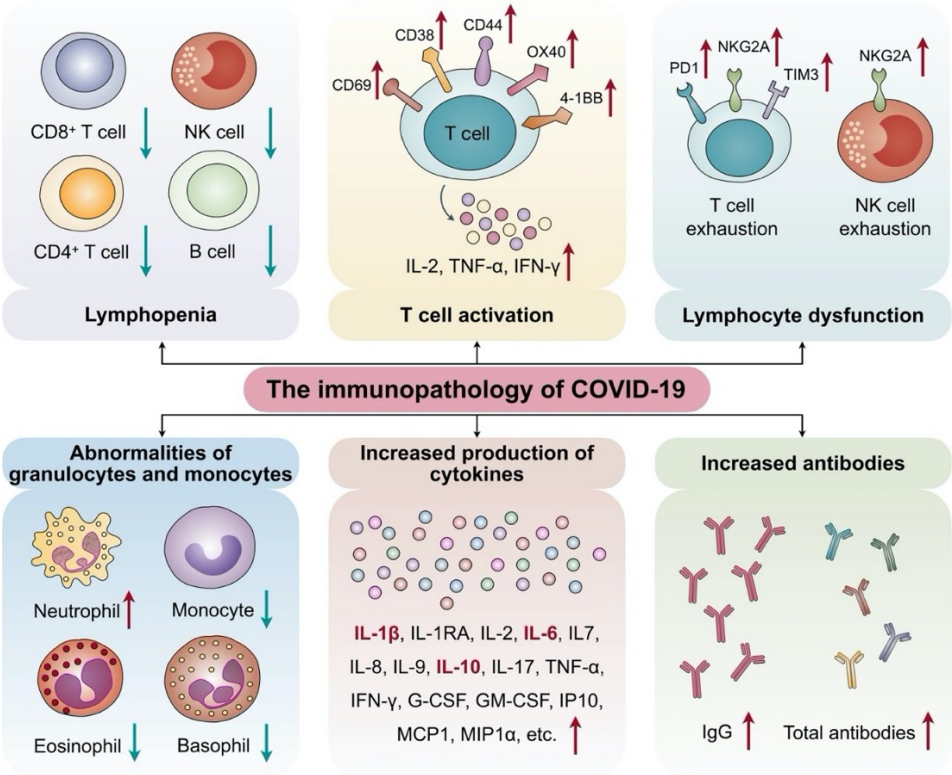

Figure 11. Simplified sketch over the immunopathology of COVID-19 (Yang, L., Liu, S., Liu, J. et al. COVID-19: immunopathogenesis and Immunotherapeutics. *Sig Transduct Target Ther*. **2020**, *5*, 128.)

NK cells play a crucial role in the innate immune response against SARS-CoV-2 [167-171,173]. NK cells are essential components of the immune response against SARS-CoV-2, contributing to early antiviral defence, cytokine production, and modulation of the immune response. CTLs directly eliminate virus-infected cells. Th cells play a critical role in coordinating immune responses [23,167-171]. Th1 cells are fundamental in the adaptive immune response against SARS-CoV-2, as they activate the immune response by producing cytokines such as IFN-γ, which enhances macrophage phagocytic activity and antigen presentation and supports the differentiation of $CD8^+$ cytotoxic T cells into CTLs [23,167-171,174].

Several cytokines are described as primarily involved in COVID-19 [167-171]. In this regard, TNF-α contributes to the cytokine storm [167-171,173,175]. IL-6 drives the inflammatory response, with elevated levels linked to severe COVID-19 and complications such as ARDS [167-171,176,177]. While IL-10

helps attenuate excessive inflammation [178], its role in the immune response to COVID-19 remains controversial [167-171]. Th17 cells may contribute to the immune response against SARS-CoV-2 by producing IL-17 and other cytokines that recruit neutrophils and help combat the viral infection to preserve mucosal integrity [88,167-171,179]. IL-17 can also amplify inflammatory responses by inducing the production of other pro-inflammatory cytokines (e.g., IL-6, TNF-α) [88,179].

B lymphocytes and antibodies are essential components of the immune response against COVID-19, playing a critical role in defence against SARS-CoV-2 and potential immune system-related issues [167-171]. Rapid production of IgM and IgG antibodies occurs at the onset of infection [180,181], although cellular immunity plays a significant role in response to vaccines [182]. Dysregulated immune responses, characterized by hyperinflammation and cytokine storms, have been implicated in the pathogenesis of severe COVID-19, leading to tissue damage, vascular dysfunction, and multiorgan failure [157-163,183,184]. Conversely, an effective and coordinated immune response involving both innate and adaptive immune mechanisms is crucial for viral clearance and infection resolution.

As stated in this PhD thesis, the immune system comprises a complex network of cells and molecules that protect the host from pathogens, including viruses. Within this intricate system, various subsets of immune cells play a fundamental role in orchestrating immune responses [28,30]. Among lymphocytes, Tregs have attracted significant attention for their ability to modulate immune function and maintain tolerance to autoantigens while preventing excessive immune responses to foreign invaders [28,30].

In the complex framework of COVID-19 pathophysiology, characterizing the dysregulation of the immune response appears to be of potential interest. In this regard, the study on the potential $T_{R3\text{-}56}$ cell involvement within the context of this viral infection could be of particularly relevance.

3.4. Kidney Transplantation

Immune-mediated processes are widely observed to be fundamental in maintaining renal homeostasis, and they also play a key role in the progression of chronic kidney disorders. Indeed, the imbalance of the complex equilibrium involving subsets of regulatory cells and the activity of adaptive immune effectors has been considered critical for the pathogenesis of renal failure or rejection upon the transplantation [185].

Kidney transplantation represents the primary therapeutic option for controlling end-stage renal disease [186]. In this context, the recognition of allo-specificities by the recipient immune effectors, despite advances in immunomodulatory approaches, remains one of the main causes of damage and loss of the allograft [187].

While antibody-mediated (humoral) rejection is considered the primary cause of dysfunction and progressive graft loss [188,189], the involvement of T cell-mediated immunity in early and late events contributing to graft rejection remains a critical issue [188]. Indeed, the key role of helper T cells in activating an effective humoral response has been well established [190,191]. In this context, the availability of valuable criteria to promptly identify early immune-mediated lesions in kidney transplant recipients represents an unmet goal.

As stated in this PhD thesis, the *fine-tuning* of the immune response is usually achieved through multiple regulatory processes, all belonging to the network of immune tolerance [192-194], whose disruption is associated with immune-mediated tissue lesions. In this context, the key role of regulatory populations in preventing harmful immune-dependent events has been widely demonstrated, as for the role of Tregs [195].

CTLs play a significant role in mediating transplant damage [196-198]. In this context, the expression of activation molecules such as CD25, CD69, CD154, and CD95 on the surface of circulating CTLs in kidney recipients, the increased frequency of terminally differentiated memory CD8 T cells [197], and an altered Treg/CTL ratio in transplanted tissue [199,200] have already been proposed as relevant markers for identifying early immune-mediated lesions in kidney transplant recipients.

This PhD thesis investigated the potential involvement of $T_{R3\text{-}56}$ cells in regulating immune-mediated damage in kidney transplants. Specifically, it explored the hypothesis that TR3-56 cells might play a crucial role in modulating the immune response to prevent or mitigate transplant-related injury. In perspective, this research could shed light on novel regulatory mechanisms that could inform therapeutic strategies to enhance graft survival and minimize immune-mediated complications in kidney transplant recipients.

4. Materials and Methods

4.1. Myelodysplastic Syndromes (MDS)

4.1.1. Individuals and Controls

Fifty-eight consecutive patients with a newly diagnosis of MDS were enrolled in the study by the clinicians of Division of Haematology, Department of Clinical Medicine and Surgery, University of Naples "Federico II", Naples, Italy (Dr. G. Cerciello, Prof. F. Pane). Bone marrow and peripheral blood sample collection, haematological investigation, and cytogenetic characterization were performed according to the recommendations of the World Health Organization (WHO). Patients were categorized according to WHO 2016 and Revised-IPSS (IPSS-R) score [201]. 14 were classified as very-low risk, 32 as low risk, 5 as intermediate risk, 3 as high risk and 4 as very high risk. A detailed description of the clinical characteristics of our MDS cohort is provided in Table 1.

Table 1. Patient clinical characteristics

Total number of MDS patients	58
Age	
Years, median (range)	77 (40–89)
Sex, *n* (%)	
Male	36 (62)
Female	22 (38)
Blood routine, median (range; interquartile range)	
Haemoglobin, g/dl	10.25 (7.8–13.2; 9.1–12.25)
Platelet count, $\times 10^9$/L	140 (30–383; 104–197)
Neutrophil count, $\times 10^6$/L	2061 (320–7588; 1008–3190)
Bone marrow blasts, median (range; interquartile range)	
Bone marrow blasts, %	0.4 (0–18; 0–2)
Cytogenetic by IPSS-R criteria, *n* (%)	
Very good	0 (0)
Good	55 (95)
Intermediate	3 (5)
Poor	0 (0)
Very poor	0 (0)
WHO 2016 classification, *n* (%)	
MDS-SLD	26 (45)
MDS-MLD	14 (24)
MDS-RS-SLD	3 (5)
MDS-RS-MLD	6 (10)
MDS-del(5q)	3 (5)
MDS-EB1	2 (3)
MDS-EB2	4 (7)
IPSS-R classification, *n* (%)	
Very-low risk	14 (24)
Low risk	32 (55)
Intermediate risk	5 (9)
High	3 (5)
Very-high risk	4 (7)

Bone marrow (BM) and peripheral blood (PB) samples were obtained during routine diagnostic procedures. For T cell receptor (TCR) repertoire analysis 10 healthy donors, matched for sex/age with MDS subjects, were enrolled in the study [202]. Additionally, to identify the occurrence of BM preferential T cells expansions, BM and PB Vβ TCR repertoire analysis was performed in 5 very low and 16 low-risk MDS patients, as previously described [202].

Informed consent was obtained from each individual before peripheral blood and bone marrow sample collection. Study was approved by the local Ethical Committee. None of the recruited patients was receiving medical treatments that could have an impact on their immune condition. Enrolled patients were not affected by immune-mediated diseases and acute or chronic viral infections.

4.1.2. Monoclonal Antibodies, Immunofluorescence and Flow Cytometry

FITC, PE, PEcy5, PEcy7 and APC labelled mAbs against CD3, CD4, CD8, CD56, CD25, CD45, CD54, PE anti-γδ TCR and isotype-matched controls were purchased from BD PharMingen. Anti-Vβ14, anti-Vβ12, anti-Vβ7.2, anti-Vβ20, anti-Vβ18, anti-Vβ7.1, anti-Vβ22, anti-Vβ13.2, anti-Vβ1, anti-Vβ17, anti-Vβ5.3, anti-Vβ5.1, anti-Vβ23, anti-Vβ4, anti-Vβ2, anti-Vβ13.1, anti-Vβ5.2, anti-Vβ8, anti-Vβ9, anti-Vβ11, anti-Vβ3, anti-Vβ13.6, anti-Vβ21.3F, anti-Vβ16 and anti-TCR monoclonal antibodies were from Beckman-Coulter. PE-labelled CD1d tetramer loaded with alpha-galactosyl-ceramide and PE-labelled CD1d negative control tetramer were from ProImmune. $T_{R3\text{-}56}$ lymphocytes were identified by co-staining with anti-human CD3 and anti-human CD56 monoclonal antibodies as described [112]. The phenotypes are referred to flow cytometric analysis of the lymphocyte population gated by using FSC and SSC parameters, as well as CD45 labelling. Flow cytometry and data analysis were performed by a FACScalibur apparatus equipped with two lasers and CellQuest analysis software (Becton Dickinson). For the comparative analysis of CD54 expression on BM CTL, immunofluorescence data were expressed as ratio between the mean intensity fluorescence (MIF) value for the CD8 population and the control MIF value obtained after staining the same cell population with the isotype control mAb, as described [202]. To define CD4 and CD8 TCR skewing, we considered the

occurrence of a percentage of expression higher than three standard deviations observed, for each Vβ family analysed, in 10 healthy controls sex/age-matched with the MSD cohort, as described [202,203]. The occurrence of a skewed BM CD4 and CD8 repertoire with an expression frequency higher than 20% compared to PB was considered as BM preferential skewing, as described [202]. This approach could be useful to identify T cell clone expansions potentially associated with the recognition of BM antigens likely relevant for MDS pathogenesis/progression.

4.1.3. Statistical analysis

Statistical evaluation of data, using InStat 3.0 software (*GraphPad Software Inc*.), was performed by *Mann-Whitney* or *Spearman's correlation test*. Two-sided p-values less than 0.05 were considered significant.

4.2. Chronic Lymphocytic Leukaemia (CLL)

4.2.1. Individuals and Controls

Twenty-six patients, diagnosed as stage 0-1 CLL, according to the *Rai* system, and as stage A, according to the *Binet* system [204], all belonging to the Low Risk category, according to the CLL-IPI score [205], were enrolled in the study. A detailed description of the clinical characteristics of our cohort is reported in Table 2.

Table 2. Patient clinical and haematological characteristics

Parameters	Mean ± SD	Range
Male/Female ratio 15:11		
Age (years)	68 ± 7.23	45 - 74
Duration of illness (years)	8.91± 4.46	3 - 18
Haemoglobin (g/dL)	13.79 ± 1.26	11.9 - 16.5
Platelet Count (X 10^9/L)	188 ± 65.85	107 - 384
White blood cell count (X 10^9/L)	24.82 ± 22.92	5.86 - 109.2
Neutrophil count (X 10^9/L)	9.97 ± 8.59	0.61 - 34.94
Lymphocyte count (X 10^9/L)	15.97 ± 15.59	3.96 - 74.26

Patients did not show lymphadenopathy greater than 2 cm in all the superficial lymph-node stations; the splenic dimensions are stable over time, with a splenic volume ranging between 250 and 400 mL.

Twenty healthy donors, sex/age matched with CLL subjects, were also enrolled in the study.

The clinical management of patients and healthy subjects was performed by the clinicians of the Division of Haematology, Department of Clinical Medicine and Surgery, University of Naples "Federico II", Naples Italy (Dr. F. Chiurazzi, Prof. F. Pane).

Informed consent was obtained from each individual before each sample collection. Study was approved by the local Ethical Committee. None of the patients recruited was receiving immune-modifying medical treatments. Enrolled patients were not affected by immune-mediated diseases and acute or chronic viral infections.

4.2.2. Monoclonal Antibodies, Immunofluorescence and Flow Cytometry

FiTC, PE, PEcy5, PEcy7, and APC labelled anti-CD3, -CD4, -CD8, -CD56, -CD25, -CD45 and control isotype-matched monoclonal antibodies (mAb) were purchased from BD PharMingen (San Jose, CA). To analyse Foxp3 expression, intracellular staining was performed using the anti-human Foxp3 kit (eBioscience, San Diego, CA, USA), following the manufacturer's instructions.

$T_{R3\text{-}56}$ lymphocytes were identified by co-staining with anti-human CD3 and anti-human CD56 mAbs, as described [112]. All phenotypes referred to flow cytometric analysis of the lymphocyte population gated by using *Forward Scatter* (FSC) and *Side Scatter* (SSC) parameters, as well as CD45 labelling.

Flow cytometry and data analysis were performed by a two-laser equipped FACScal- ibur apparatus and the CellQuest analysis software (Becton Dickinson).

4.2.3. Statistical Analysis

Statistical evaluation of data, using InStat 3.0 software (GraphPad Software Inc., San Diego, CA, USA) was performed by *Mann-Whitney*, *Wilcoxon matched-pairs signed-rank test or Spearman's correlation test*, as indicated. Two-sided p-values less than 0.05 were considered significant.

4.3. COVID-19

4.3.1. Individuals and Controls

All patients were clinically classified upon hospitalization according to the WHO classification [206]. Briefly, COVID infection was diagnosed using molecular analysis (RT-PCR) on nasopharyngeal swabs. Patients were classified according to the WHO ordinal scale, which categorizes their condition into Groups 1 to 7: 1) not hospitalized with normal activities; 2) not hospitalized but unable to resume normal activities; 3) hospitalized without supplemental oxygen; 4) hospitalized with supplemental oxygen; 5) hospitalized with high-flow oxygen, non-invasive ventilation, or both; 6) hospitalized with ECMO, invasive ventilation, or both; and 7) death [206]. Recruited patients belonged to the COVID groups requiring hospitalization, thus falling into symptomatic groups ranging from 3 to 7.

The study was a retrospective analysis of a cohort of hospitalized COVID-19 patients with varying degrees of clinical severity. Patients were admitted to the Infectious Diseases Section of Federico II University, Naples, Italy (clinicians: Prof. I. Gentile and Prof. B. Pinchera) during the first and second waves of the pandemic (2020-2021). Whole blood samples were collected at admission of hospitalization in EDTA-containing tubes and then immediately analysed by flow cytometry. Serum samples were separated from blood cells after collection.

Ethical approval for the study was obtained from the Ethics Committee of the Federico II University of Naples. The study was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

To ensure that the patient population was not influenced by treatments, in the current retrospective evaluation we selected only the analysis of data from those hospitalized patients who had not previously undergone anti-inflammatory steroid therapy or azithromycin (n = 106) [207].

Furthermore, none of the patients had received the anti-SARS-CoV-2 vaccine at the time of enrolment.

4.3.2. Monoclonal Antibodies, Immunofluorescence and Flow Cytometry

Immunophenotyping analysis was performed by multicolour flow cytometry, as described [208]. Briefly, CD45 was used to gate viable lymphocyte cells. From this gate, $CD3^+$ $CD4^+$ cells were identified as Th, while $CD3^+$ $CD8^+$ cells were identified as CTL. Between Th, Th1 and Th17 were distinguished by specific surface markers, namely CXCR3 and CCR6, respectively. Furthermore, the human leukocyte antigen DR (HLA-DR) molecule was used as activation markers expressed on activated T lymphocytes. CD3 and CD45, CD56 and CD19 were used to distribute T ($CD3^+$ $CD45^+$), NK ($CD45^+$ $CD3^-$ $CD56^+$) and B ($CD45^+$ $CD19^+$) cells for each patient. Treg cells were identified as $CD3^+$ $CD4^+$ $CD25^{High}$ $CD127^{low}$ and $T_{R3\text{-}56}$ as $CD3^+$ $CD56^+$.

4.3.3. Serum cytokine analysis

Serum IL-17A, IL-6, IL-10 and TNF-α levels were analysed using human-specific enzyme-linked immunosorbent assay (ELISA) Max™ Set Deluxe kits (BioLegend, Inc., San Diego, USA). The concentration values (pg/mL) of each cytokine were obtained by interpolating the absorbance values on the respective calibration curve.

4.3.4. Statistical Analysis

Statistical differences between three groups were assessed by *Mann-Whitney test* to compare the differences between Group 1 and Group 2, Group 1 and Group 3, and Group 2 and Group 3. The correlations between variables were evaluated by *Spearman's rank-order* correlation and *Spearman's rank correlation coefficient* (rs) was calculated. Statistical analysis and Graphics have been performed by Prism 9, GraphPad Inc (San Diego, CA USA). P values <0.05 were considered as significant.

4.4. Kidney transplants

4.4.1. Individuals and Controls

The study was conducted on 53 renal transplant recipients, all first-time cadaver donor transplant recipients, under regular follow-up at the Clinical Care Pathway in Nephrology and Renal Transplantation, University Hospital "Federico II", c/o the Department of Public Health of the Federico II University, Naples, Italy (clinicians: Prof. M. Sabbatini, Dr. R. Carrano). Inclusion criteria were: age 18-65; transplant year: >1 year, with a bimonthly clinical and laboratory check-up in the last 6 months; plasma creatinine <3 mg/dl; haemoglobin value >11 g/dl; white blood cell count >4000/μL (neutrophils >2000/μL); platelet count >75,000/μL; absence of clinical signs of transplant rejection, of infectious episodes and no change in immunosuppressive regimen in the last 6 months. Exclusion criteria were: previous or combined transplant; Panel Reactive Antibodies (PRA) >25% and/or presence of Donor Specific Antibodies (DSA) at transplantation; presence of proteinuria greater than 300 mg/day on 24-hour samples; presence of hyperlipidaemia (baseline cholesterol and/or triglycerides greater than 220 and 200 mg/dl, respectively); evidence of autoimmune diseases or viral infections. Based on these criteria, 53 consecutive patients, in regular follow-up, were included in the study. These subjects were subsequently divided into two groups based on their laboratory data: patients with stable renal function and urinary parameters (stable group) and patients showing changes ≥0.2 mg/dl in serum creatinine level and/or >100 mg/day in proteinuria on 24-hour urine samples in two consecutive evaluations, despite no clinical predisposing condition (worsening hypertension, recurrence of underlying renal disease, cardiovascular disease). These patients represented the unstable group. Plasma creatinine concentration was assessed by an autoanalyzer with a modified Jaffè method, urinary protein excretion by PCR ([urinary protein/urinary creatinine] × 1000, mg/mM) method; glomerular filtration rate was estimated by the EPI-CKD formula. Immunomodulatory treatments included corticosteroids and calcineurin inhibitors. Demographic and laboratory data of the enrolled subjects are reported in Table 3.

Table 3. Demographic and laboratory data of the patients enrolled in the study

Enrolled individuals (N=53)	
SEX (M/F)	30/23
AGE (Mean±SD)	51.83±14.04
TRASPLANT VINTAGE (years)	5.56±4.2
White Blood Cell count ($x10^9$/L) Mean±SD	8.278±2.57
Neutrophil count ($x10^9$/L) Mean±SD	5.308±2.15
Lymphocyte count ($x10^9$/L) Mean±SD	2.029±0.83

The study, conducted in accordance with the Good Clinical Practice guidelines, was approved by the Ethics Committee of the Federico II University of Naples (Protocol number: 66/11). All procedures were in accordance with the *Declaration of Helsinki*, as revised in 2008. Twenty blood donors, age and sex matched to the patients, were enrolled in the study as healthy controls. All patients and controls signed informed consent for the study.

4.4.2. Monoclonal Antibodies, Immunofluorescence and Flow Cytometry

Blood samples were analysed by immunofluorescence using FITC or Pe-Cy5 anti-human CD3 (BD Pharmingen, clone UCHT1), FITC anti-human CD4 (BD Pharmingen, clone RPA-T4), Pe-Cy7 anti-human CD8 (BD Pharmingen, clone RPA-T8), Pe-Cy5 or Pe-Cy7 anti-human CD56 (BD Biosciences, clone NCAM16.2), PE anti-human Vα24 (Beckman Coulter, clone C15), FITC anti-

human CD19 (eBioscience, clone HIB19), PE anti-human CD25 (BD, clone M-A251), PE anti-human CD54 (BD, clone HA58), FoxP3-all (eBioscience, clone PCH101), FITC anti-human Ki-67 (BD, clone B56). For intracellular detection of FoxP3-all and Ki-67 the BD fixation and permeabilization FoxP3 buffer kit (eBioscence), was employed according to the manufacturer's instructions. For analysis of CD54 expression level in T lymphocytes, fluorescence data were expressed as the ratio of mean intensity fluorescence (MIF) value for the $CD4^+$ or $CD8^+$ T cell population and the control MIF value obtained after staining of the same cell subset with the control isotype mAb, as described [209].

Treg cells were identified as $CD3^+$ $CD4^+$ $CD25^{High}$ $FoxP3^+$ and $T_{R3\text{-}56}$ as $CD3^+$ $CD56^+$ [112].

All phenotypes referred to flow cytometric analysis of the lymphocyte population gated using forward- (FSC) and side-scatter (SSC) parameters. Flow cytometric evaluation was performed using the *ATTUNE NxT* acoustic focusing cytometer (Life Technologies). Data analysis was performed using FlowJo software (FlowJo, LLC). PBMCs for the detection of FOXp3 and ki67 were isolated by centrifugation of peripheral blood on a Ficoll-Paque cushion gradient (GE Healthcare, Uppsala, Sweden). To assess possible variations in results, two independent samples obtained from each subject were analysed at 1-week intervals and yielded substantially comparable results (not shown).

4.4.3. Statistical Analysis

Statistical evaluation of data, by using GraphPad Prism 6.0 software (GraphPad Software, Inc, La Jolla, CA, USA) has been performed by *Mann-Whitney* test and *Fisher's two-tailed* exact test, as indicated. Two-sided p values of less than 0.05 were considered to indicate statistical significance.

5. Results and discussion

5.1. Myelodysplastic Syndromes (MDS)

5.1.1. $T_{R3\text{-}56}$ Lymphocytes, Activated CTLs, and Blasts in the Bone Marrow of MDS Subjects

To address the role of the $T_{R3\text{-}56}$ subset in the pathogenesis/progression of MDS, we first analysed their level in the bone marrow. Additionally, we assessed the activation of bone marrow (BM) CTLs by their expression of CD54 [209]; the correlation of bone marrow $T_{R3\text{-}56}$ lymphocytes with the number of bone marrow blasts was also analysed.

As shown in figure 12 panel A, an increasing trend in the percentage of BM $T_{R3\text{-}56}$ was observed from the very low/low-risk group to the high/very high-risk group (5.1 ± 0.39; median 4.65; interquartile range [IQR] 3.43-6.69 in the very low/low-risk group; 5.44 ± 1.4; median 3.67-9.46 in the intermediate-risk group; 8.22 ± 1.8; median 4.58; IQR 3.69-14.58 in the high/very high-risk group).

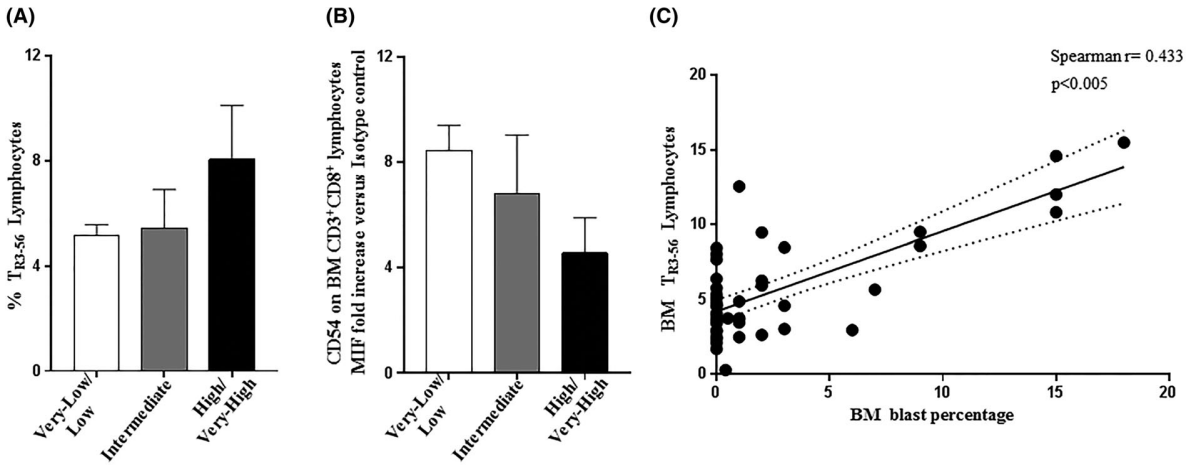

Figure 12. BM $T_{R3\text{-}56}$ lymphocyte percentage positively correlates with BM blast number and seems to be inversely associated with BM CD54 expression on BM CTL in MDS subjects. (**A**) White, grey and black columns indicate BM percentage of T_{R3-56} cell subset in very-low/low risk (N 46), intermediate risk (N 5) and high/very-high risk (N 7) MDS patients, respectively. (**B**) White, grey and black columns indicate CD54 expression on

MDS patients, respectively. As shown, an increasing trend of BM T_{R3-56} percentage accompanied by a decreasing trend for CD54 expression on CTL is observed from very-low/low risk to high/very-high risk group. (**C**) Spearman's evaluation of correlation between BM T_{R3-56} cell subset percentage and BM blast in MDS subjects. As shown, a significant ($p < 0.005$) positive correlation ($r = 0.433$) has been revealed.

Additionally, a decreasing trend for CD54 expression from the very low/low-risk group to the high/very high-risk group was observed (8.45 ± 0.9 in the very low/low-risk group; 6.81 ± 2.2 in the intermediate-risk group; 4.65 ± 1.3 in the high/very high-risk group) (figure 12 panel B).

The expression of CD54 on CTLs has been widely considered to be directly involved in antigen-dependent CTL activation processes [210,211]. Therefore, it could serve as a valuable marker for antigen-dependent CTL activation in the bone marrow of MDS patients.

Advanced stages of MDS have been associated with the occurrence of immunosuppressive mechanisms. To analyse the potential role of T_{R3-56} in mediating immunomodulation in the bone marrow microenvironment, we assessed, using the Spearman test, the relationship of T_{R3-56} with the number of bone marrow blasts in our cohort. As shown in figure 12 panel C, a significant positive correlation was revealed ($r = 0.433$; $p < 0.005$).

Since immuno-mediated mechanisms are relevant to the pathogenesis of MDS in the early stages of the disease [115,117,119,120], we focused on very low/low-risk MDS patients. We previously described that the level of CD54 expression on BM CTLs may identify two subgroups of MDS patients showing high (≥10 fold increase MIF compared to isotype control) or low (<10 fold increase MIF compared to isotype control) CD54 expression on BM CTLs [209]. Therefore, we analysed the BM immunological profile in very low/low-risk MDS patients categorized based on CD54 expression on BM CTLs. As shown in figure 13 panel C and D, lower CD54 expression in bone marrow CTLs is associated with an increase in bone marrow CTLs (33.32 ± 2.03% in the bone marrow vs. 25.38 ± 1.88% in the bone marrow; $p < 0.0001$) and with higher T_{R3-56} in the bone

marrow (5.07 ± 0.54% in the bone marrow vs. 4.05 ± 0.46% in the bone marrow; $p < 0.05$). No significant differences were observed in bone marrow CTLs and T_{R3-56}, nor in peripheral blood distribution in subjects showing higher CD54 expression on bone marrow CTLs (figure 13 panel A and B). These data add T_{R3-56} subset to the immune-regulatory network potentially involved in MDS pathogenesis/progression mechanisms. Indeed, a T_{R3-56}-dependent control of CTL activation in BM of MDS patients might be hypothesised.

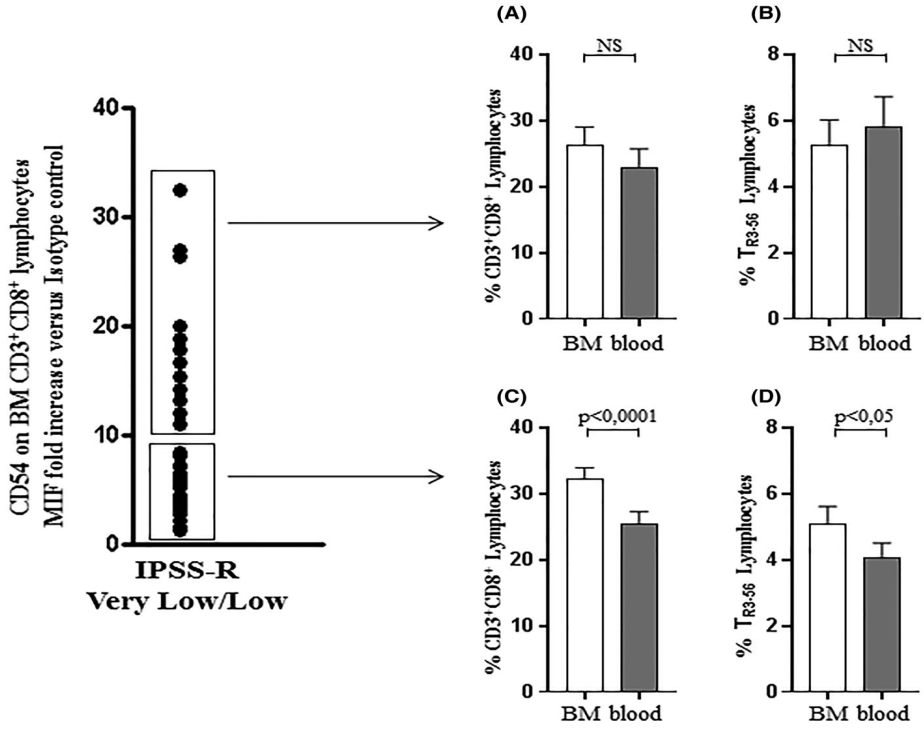

Figure 13. TR3-56 lymphocyte percentage in BM of very-low/low risk. MDS patients inversely correlates with CD54 expression on BM CTL. Left part of the figure shows very-low/low risk MDS patients grouping according to CD54 expression on BM CTL, as previously described. (**A–D**) Analysis of BM versus blood $CD3^+CD8^+$ and T_{R3-56} in the very-low/low risk MDS patients, grouped according to their CD54 expression on BM CTL. White and grey columns indicate BM and PB percentage, respectively. (**A and C**) Analysis of BM versus blood CTL distribution in the subjects categorised according their CD54 expression on BM CTL. (**B and D**) Analysis of BM versus blood T_{R3-56} distribution in the subjects categorised according their CD54 expression on BM CTL. As shown, significant BM recruitment of CTL ($p < 0.0001$) and T_{R3-56} ($p < 0.05$) lymphocytes has been observed only in the subjects with lower CD54 expression on BM CTL.

5.1.2. $T_{R3\text{-}56}$ Lymphocytes and CTL-Skewed T Cell Repertoire in Bone Marrow of Subjects with Very Low/Low-Risk MDS

The involvement of the Treg subset in controlling T cell expansion in the bone marrow of subjects with low-risk MDS has been described [202]. To assess whether $T_{R3\text{-}56}$ subset could also participate in controlling clonal expansion of bone marrow T cells, we analysed peripheral blood and bone marrow TCR repertoire in very low/low-risk MDS patients. This approach allowed the comparative assessment of TCR repertoire in bone marrow microsite versus peripheral blood and the identification of clonal expansions of T cells, potentially related to the recognition of bone marrow antigens.

Individuals with very low/low risk MDS were divided into two groups: those with <2 TCR preferential BM Vβ skewing versus those showing ≥ 2 TCR preferential BM Vβ skewing in the CTL repertoire, as previously described [202]. As shown in figure 14 panel A, a significant inverse correlation (Spearman $r = -0.723$; $p < 0.05$) was observed between BM CD54 CTL expression and the percentage of BM T_{R3-56}.

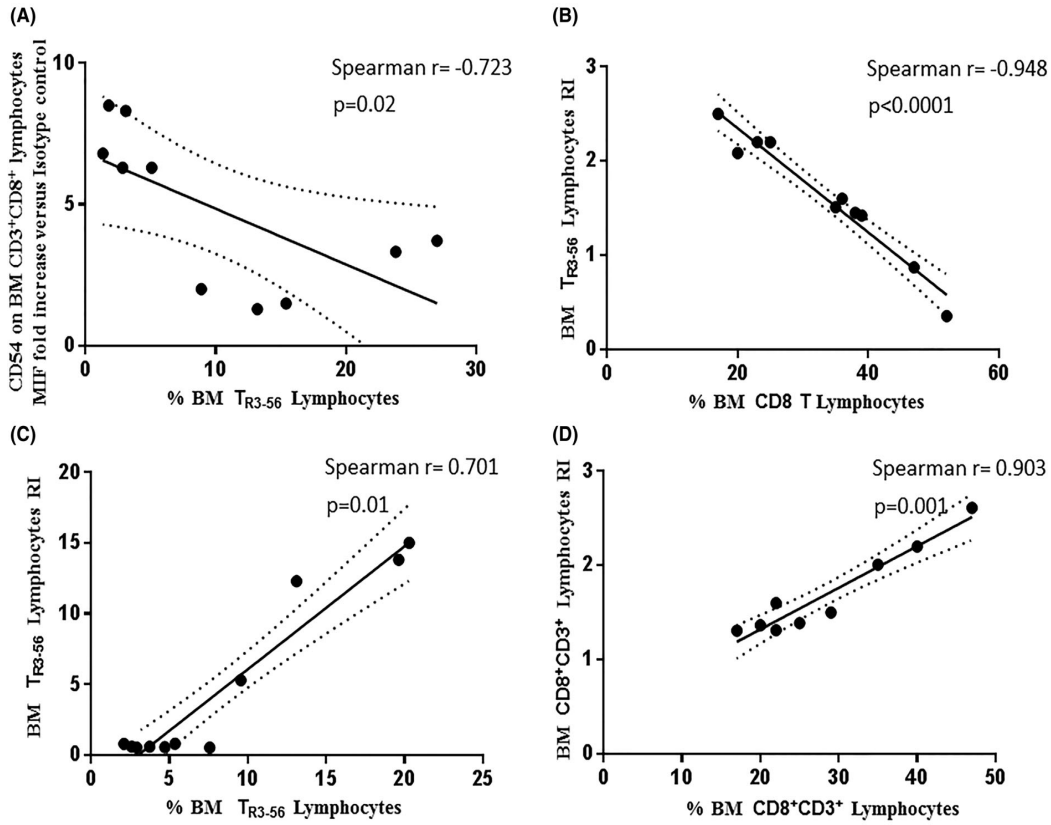

Figure 14. Spearman's correlation analysis of BM T_{R3-56} lymphocytes, BM CTL percentage and activation in very-low/low risk MDS patients categorised according to the presence of a preferential BM T cell skewed Vβ repertoire. (**A**) Spearman correlation analysis of CD54 expression on BM CTL versus BM T_{R3-56} percentage in MDS subjects with ≥2 CTL Vβ skewed clones in BM; significant negative correlation (Spearman $r = -0.723$; $p < 0.05$) is shown. (**B**) Spearman correlation analysis of BM T_{R3-56} RI (ratio between the percentage of T_{R3-56} lymphocytes in BM vs. their percentage in PB) versus BM CTL percentage in MDS subjects with ≥2 CTL Vβ skewed clones in BM; significant negative correlation (Spearman $r = -0.948$; $p < 0.0001$) is revealed. (**C**) Spearman correlation analysis of BM T_{R3-56} vs BM T_{R3-56} percentage in MDS subjects with <2 CTL Vβ skewed clones in BM; significant positive correlation (Spearman $r = 0.701$; $p < 0.05$) is observed. (**D**) Spearman correlation analysis of BM CTL RI versus BM CTL percentage in MDS subjects with ≥2 $CD4^+$ Vβ skewed clones in BM; significant positive correlation (Spearman $r = 0.903$; $p < 0.005$) is shown.

Immune response is a microsite process involving the recognition of specific antigens by resident and/or microsite-recruited immune effectors. Therefore, the

frequency of immune cells, specifically localized in the BM compared to the PB, could represent a valuable tool to analyse the mechanisms underlying the immune activation events occurring in the BM. With this aim, we analysed the ratio between the percentage of lymphocytes, belonging to the CTL and/or $T_{R3\text{-}56}$ subset, and the percentage of the same cell population in the PB (recruitment index [RI]). In this context, a ratio >1 indicates the preferential BM recruitment BM from PB of each immune subset.

We show (figure 14 panel B) a strong negative correlation between BM $T_{R3\text{-}56}$ RI and BM CTL percentage (Spearman $r = -0.948$; $p < 0.0001$) in very low/low risk MDS subjects showing ≥2 preferential TCR Vβ expansions in BM $CD8^+$ T cells. In contrast (figure 14 panel C), a significant correlation (Spearman $r = 0.701$; $p < 0.05$) was observed between BM $T_{R3\text{-}56}$ RI and their BM percentage in very low/low risk individuals with <2 preferential BM TCR Vβ skewing in $CD8^+$ T cells. Subjects with ≥2 preferential BM TCR Vβ expansions in $CD4^+$ T cells (figure 14 panel D) showed a positive correlation between BM CTL RI and their BM percentage (Spearman $r = 0.903$; $p < 0.005$). No significant correlations were observed in very low/low risk subjects characterized by the presence of <2 skewed TCR Vβ families in BM $CD4^+$ T cells (not shown).

These data inversely associate BM $T_{R3\text{-}56}$ level with BM CTL expansion and activation in our cohort. Furthermore, a correlation between $CD4^+$ T cell skewing and BM $CD8^+$ percentage was also revealed in very low/low risk MDS subjects with ≥2 TCR Vβ expansions in BM $CD4^+$ T cells.

5.2. Chronic Lymphocytic Leukaemia (CLL)

5.2.1. Circulating T, NK, Treg and T_{R3-56} cells in CLL patients

We analysed the number and percentage of circulating T and NK cells in CLL subjects showing stable disease, compared to the group of healthy subjects matched for sex and age.

According to the literature [148-156], we observed that the reduction in the percentages of T lymphocytes (18.54 ± 2.45 vs. 74.64 ± 0.67 in controls; $p < 0.0001$) and NK cells (3.90 ± 0.58 vs. 9.48 ± 0.45 in controls; $p < 0.0001$) (figure 15 panel A and C respectively) is accompanied by an increase in the absolute number of T cells (2268 ± 225.4 vs. 1456 ± 38.58 in controls; $p < 0.0001$) and NK cells (379.7 ± 44.94 vs. 197.5 ± 5.23 in controls; $p < 0.0001$) in CLL subjects (figure 15 panel B and D, respectively). Furthermore, we observed a significant decrease in the CD4/CD8 T cell ratio in CLL patients compared to healthy subjects (2.21 ± 0.42 vs. 2.53 ± 0.09 in controls; $p < 0.001$) (figure 15 panel E).

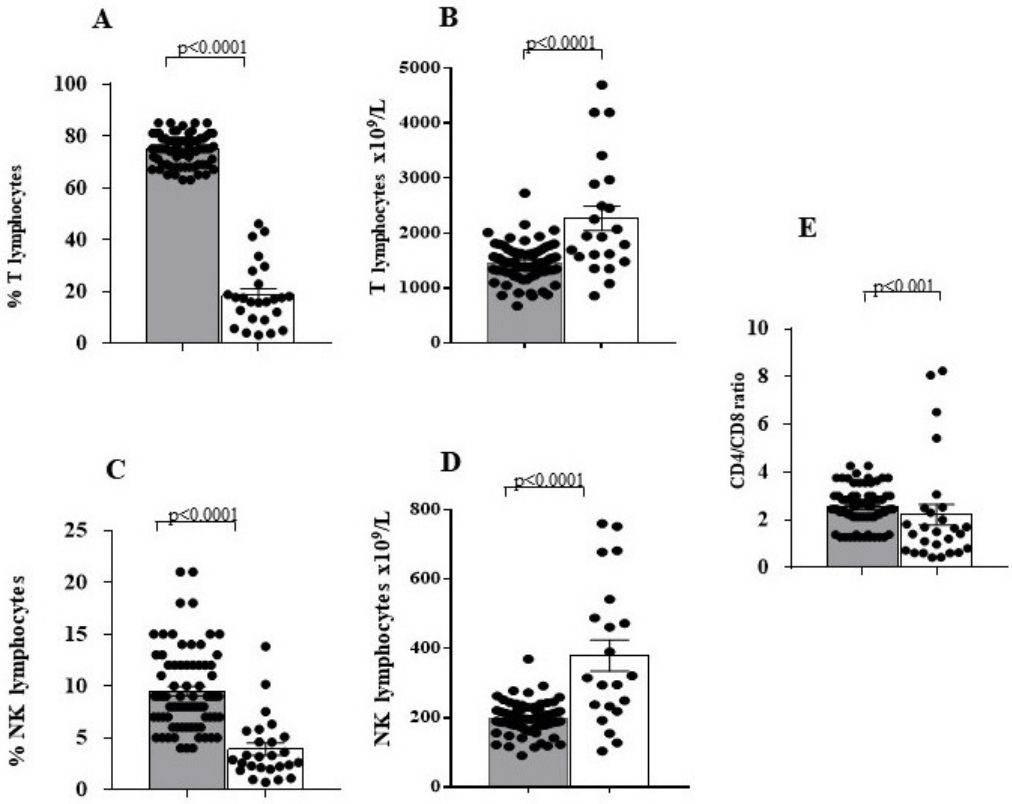

Figure 15. Significant decrease in the percentage, associated with increased number characterises circulating T and natural killer (NK) cells of chronic lymphocytic leukaemia (CLL) subjects with stable disease. Panels **A** and **C** indicate the percentage of circulating T and NK lymphocytes. Panels **B** and **D** indicate the number of T and NK cells in peripheral blood. Panel **E** shows the CD4/CD8 T-cell ratio of circulating lymphocytes. Comparative analysis of LLC and healthy controls shows decreased percentage of circulating T and NK effectors associated with a significant increase in their number. Grey and white columns indicate data obtained in healthy controls and CLL individuals, respective. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Statistical significance values are indicated.

Furthermore, we assessed the presence of both Tregs [212-214] and $T_{R3\text{-}56}$ [112] in the CCL subject cohort. As shown, the percentage of Tregs is reduced in

CLL subjects compared to healthy controls (1.31 ± 0.17 versus 2.43 ± 0.18 in healthy controls; $p < 0.0001$) (figure 16 panel A, B), while the absolute number of Tregs (figure 16 panel B) is increased (177.2 ± 25.8 versus 21.17 ± 1.01 in healthy controls; $p < 0.0001$). In contrast, the percentage of the $T_{R3\text{-}56}$ population shows no significant difference in the two groups (3.11 ± 0.47 versus 3.91 ± 0.39 in healthy controls) (figure 16 panel C). Furthermore, a higher number of circulating $T_{R3\text{-}56}$ cells was observed in CLL subjects (341.5 ± 61.99 vs. 91.31 ± 11.58 in healthy controls; $p < 0.001$) (figure 16 panel D). Furthermore, the level of circulating Treg and $T_{R3\text{-}56}$ cells directly correlated with the CLL cohort (Spearman r = 0.625; $p < 0.005$) (figure 16 panel E).

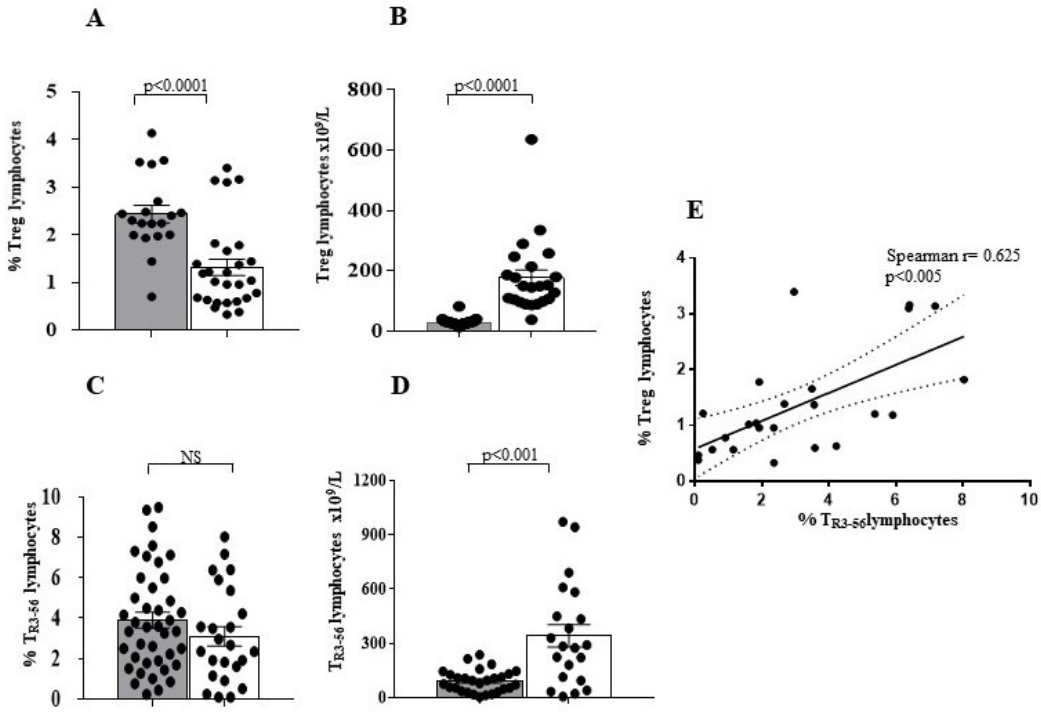

Figure 16. Analysis of circulating $CD4^+CD25^+$ (Treg) and $CD3^+CD56^+$ ($T_{R3\text{-}56}$) regulatory T-cell subsets in CLL subjects with stable disease. Panel A to D show comparative analysis of the percentage and the number of circulating Treg and $T_{R3\text{-}56}$, in

CLL subjects and healthy controls. Decreased percentage (**A**) and increased number (**B**) of the Treg cells has been shown to be associated with not significant difference in percentage of circulating $T_{R3\text{-}56}$ lymphocytes (**C**) and increased number of this T-cell subset (**D**) in the CLL cohort. Grey and white columns indicate data obtained in healthy controls and CLL individuals, respectively. Statistical evaluation of data has been performed by means of the *Mann Whitney* test. Panel (**E**) shows the significant correlation, as evaluated by *Spearman's* test, between percentage of circulating Treg and $T_{R3\text{-}56}$ lymphocytes in CLL subjects. Statistical significance values are indicated. NS indicates the not statistically significant value.

To describe the T cell profile of our CLL cohort, we analysed the percentage of Treg and $T_{R3\text{-}56}$ subset in the $CD4^+$ T cell population. In this regard, an increased percentage of Treg (13.81 ± 1.07 versus 7.68 ± 0.53 in healthy controls; $p < 0.0001$) and $T_{R3\text{-}56}$ (15.48 ± 2.34 versus 6.01 ± 0.63 in healthy controls; $p < 0.0001$) was observed in the $CD4^+$ T cell subset of CLL subjects (figure 17 panel A and B respectively).

Therefore, an increase in regulatory T-cell subsets appears to characterize the T-cell compartment in CLL patients, as already highlighted [215,216]. This evidence suggests that an increased rate of Treg and $T_{R3\text{-}56}$ differentiation within the T-cell compartment characterizes the CLL individuals with stable disease.

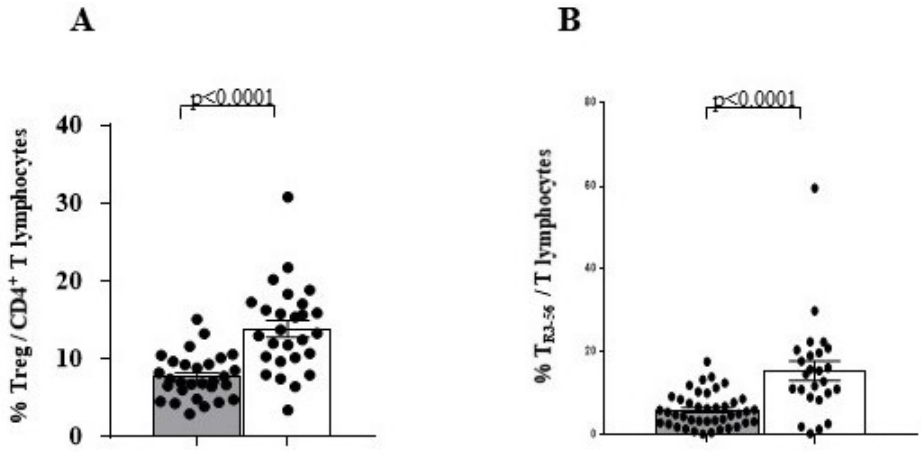

Figure 17. Percentage of Treg and TR3-56 lymphocytes are significantly increased in the T-cell compartment of CLL subjects. Panel **A** shows comparative analysis of the percentage of the Treg subset in the $CD4^+$ T cell population in the CLL subjects, as compared with controls. As shown, significant increase in Treg cells has been revealed in the CLL cohort. Panel **B** shows percentage of TR3-56 lymphocytes in the $CD4^+$ T cells of CLL subjects, as compared with healthy individuals. As shown, significant increase in T_{R3-56} cells has been revealed in the CLL cohort. Grey and white columns indicate data obtained in healthy controls and CLL individuals, respectively. Statistical evaluation of data has been performed by means of the Mann–Whitney test. Statistical significance values are reported.

5.3. COVID-19

5.3.1. Immune status in patients stratified by severity of COVID disease

We categorized COVID-19 patients into three groups based on severity: Group 1 (WHO 3), Group 2 (WHO 4), and Group 3 (WHO 5, 6, and 7), following the previously described stratification [207,208]. In the current study, we analysed data from hospitalized patients who had not received anti-SARS-CoV-2 therapy or vaccination prior to admission (see Materials and methods section).

Based on the assessment of the percentage of whole white blood cells (WBCs), lymphocytes are significantly reduced in Groups 2 and 3 with more severe clinical conditions (figure 18 panel A). No statistically significant differences are observed in the monocyte population (figure 18 panel B). Finally, neutrophils appear to show a slight increase in Group 2 compared to Group 1 (figure 18 panel C).

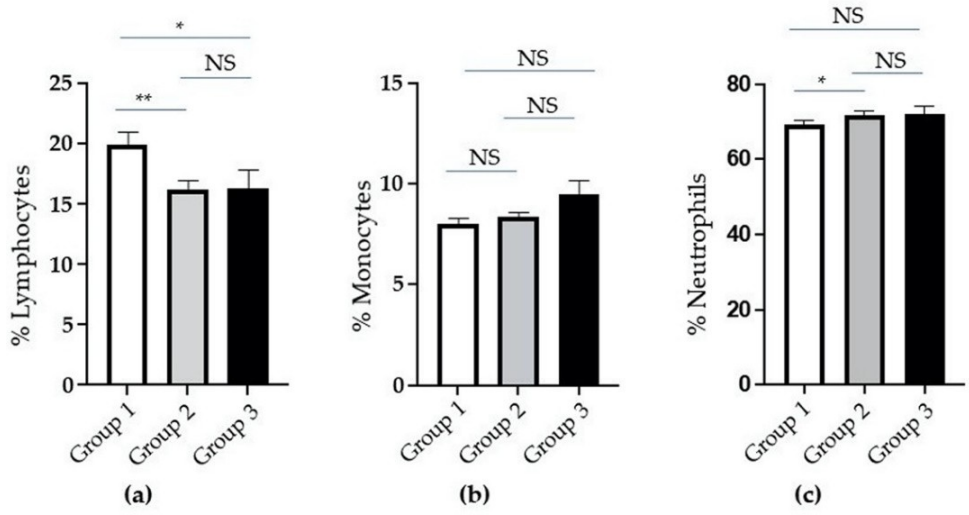

Figure 18. Analysis of WBCs in Groups of COVID-19 patients based on increasing severity. (**A**) The percentage of whole Lymphocytes, (**B**) Monocytes and (**C**) Neutrophils in Groups 1 (white columns), 2 (grey columns) and 3 (black columns) of patients. Statistical analysis (*Mann-Whitney test*) is reported: $p \leq 0.05$ (*); $p \leq 0.005$ (**); not significant (NS).

Focusing on the lymphocyte population among white blood cells, we observed a reduction in the percentage of T cells in groups 2 and 3 compared to group 1 (figure 19 panel A). It is noteworthy that although some fluctuations are observed, the percentage of CTLs remains substantially stable across all Groups (figure 19 panel B). Specifically, even though Group 2 shows a decrease compared to Group 1, and Group 3 shows an increase compared to Group 2, the percentage of CTLs in all groups stays constantly about 22%. Th cells decreased following the severity of COVID disease (figure 19 panel C). B cells progressively increased from group 1 to group 3 (figure 19 panel D). A significant increase is exhibited by NK cells (figure 19 panel E).

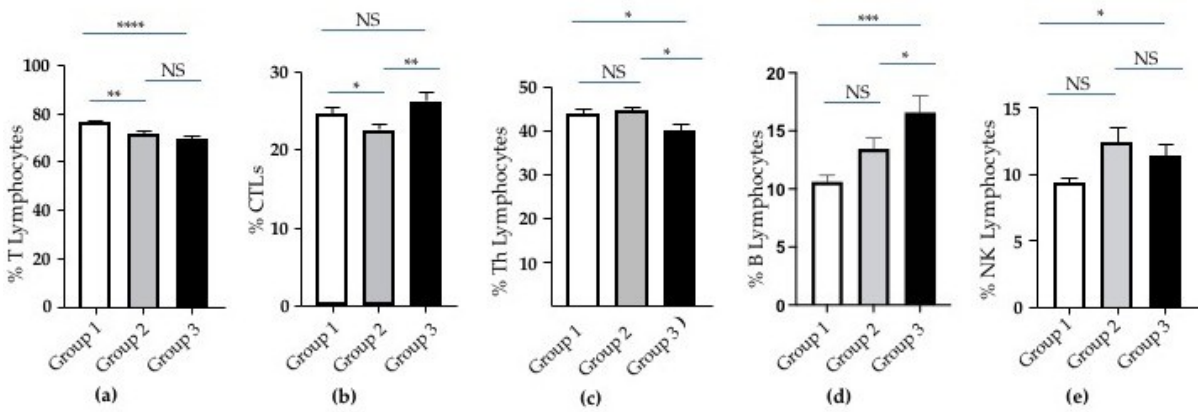

Figure 19. Analysis of lymphocyte subtypes in Groups of COVID-19 patients based on increasing severity. (**A**) The percentage of T, (**B**) CTLs, (**C**) Th, (**D**) B and (**E**) NK Lymphocytes in Groups 1 (white columns), 2 (grey columns) and 3 (black columns) of patients. Statistical analysis (*Mann-Whitney test*) is reported: $p \leq 0.05$ (*); $p \leq 0.005$ (**); $p \leq 0.0005$ (***); $p < 0.0001$ (****); not significant (NS).

5.3.2. Activated T cells, Treg and $T_{R3\text{-}56}$ cells in patients stratified by severity of COVID disease

Since T cells appeared to decrease with the severity of clinical conditions (from Group 1 to Group 3), with an increase in CTLs in Group 3, we assessed the activation status of T cells by evaluating HLA-DR expression on their cell surface [208], in the three groups. Interestingly, the percentage of activated T cells increased significantly in Group 2, compared to Group 1 (figure 20 panel A). In contrast, the percentage of the same cells in Group 3 was lower than in Group 2 and appeared similar to that of Group 1 (figure 20 panel A).

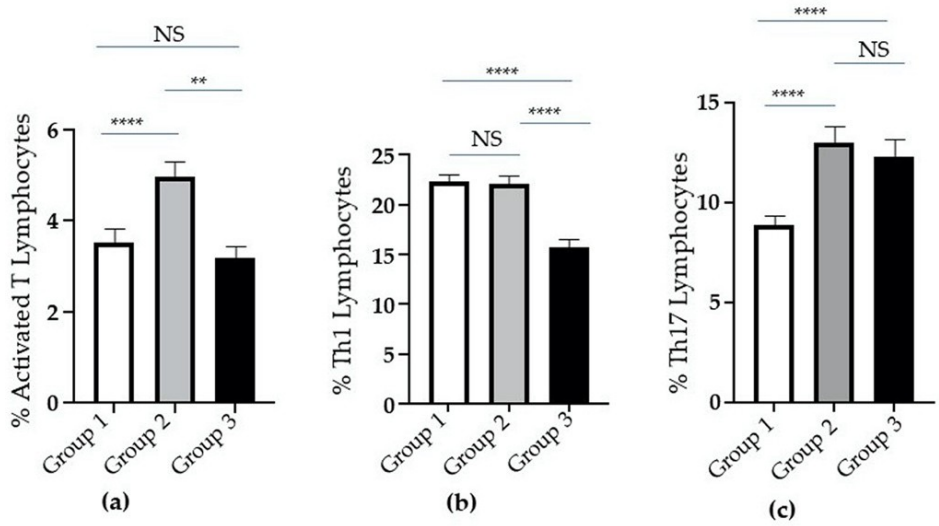

Figure 20. Analysis of activated T, Th1 and Th17 cells in Groups of COVID-19 patients based on increasing severity. (**A**) The percentage of activated T, (**B**) Th1 and (**C**) Th17 Lymphocytes in Groups 1 (white columns), 2 (grey columns) and 3 (black columns) of patients. Statistical analysis (*Mann-Whitney test*) is reported: $p \geq 0.01$ (*); $p \leq 0.005$ (**); $p \leq 0.0005$ (***); $p < 0.0001$ (****); not significant (NS).

Furthermore, the percentage of Th1 cells is reduced in Group 3, compared to Groups 1 and 2 (figure 20 panel B). Interestingly, the decrease in Th1 cells in Group 3 is accompanied by an increase in B cells in the same group (see figure 19 panel D), potentially indicating an immune shift toward humoral response. In contrast,

the percentage of Th17 cells increased significantly in Groups 2 and 3, compared to Group 1 (figure 20 panel C).

A significant reduction in the percentage of Treg cells is evident in Group 3 (figure 21 panel A).

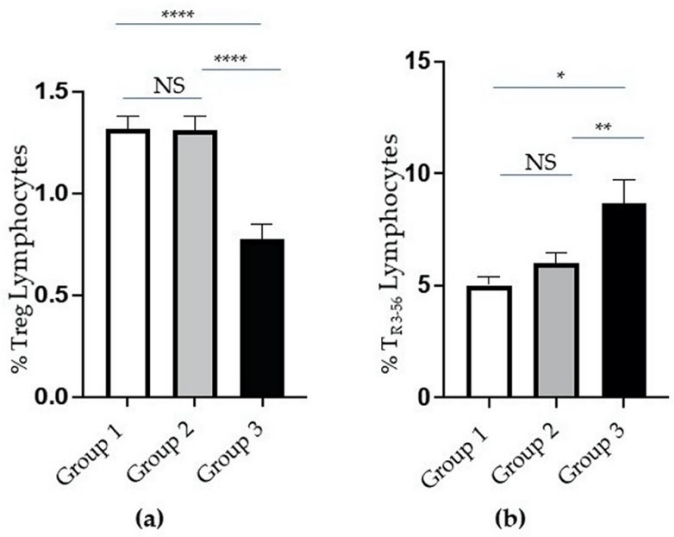

Figure 21. Analysis of Treg and T_{R3-56} cells in Groups of COVID-19 patients based on increasing severity. (**A**) The percentage of Treg and (**B**) T_{R3-56} Lymphocytes in Groups 1 (white columns), 2 (grey columns) and 3 (black columns) of patients. Statistical analysis (*Mann-Whitney test*) is reported: $p \leq 0.05$ (*); $p \leq 0.005$ (**); $p \leq 0.0005$ (***); not significant (NS).

Therefore, we focused our attention on the T_{R3-56} population. In this regard, it is interesting to note that the percentage of T_{R3-56} cells is significantly increased in Group 3, which presents more severe clinical conditions (figure 21 panel B).

5.3.3. Cytokines in patients stratified by severity of COVID disease

We analysed the serum concentration of cytokines in the three patient groups (Table 4). Interestingly, TNF-α was significantly different between Group 1 and Group 2 (p=0.0011) and between Group 1 and Group 3 (p=0.0075). No statistical

difference was observed between Group 2 and Group 3 (Table 4). Furthermore, no statistical differences were observed in the concentrations of IL-6, IL-17 and IL-10 between the patient groups (Table 4).

Table 4. Serum cytokine concentrations in Groups of COVID-19 patients based on increasing severity *

	Group 1	Group 2	Group 3	
	Mean ± SE	Mean ± SE	Mean ± SE	Mann-Whitney (P value)
TNF-α (pg/ml)	3,07 ± 0,04	9,45 ± 2,37	4,30 ± 0,61	**0,0011** group 1 *vs* group 2 **0,0075** group 1 *vs* group 3 NS group 2 *vs* group 3
IL-6 (pg/ml)	49,61 ± 6,24	170 ± 50,71	52,77 ± 13,74	NS group 1 *vs* group 2 NS group 1 *vs* group 3 NS group 2 *vs* group 3
IL-17 (pg/ml)	3,34 ± 0,27	4,86 ± 0,91	3,44 ± 0,61	NS group 1 *vs* group 2 NS group 1 *vs* group 3 NS group 2 *vs* group 3
IL-10 (pg/ml)	7,31 ± 0,45	10,71 ± 1,93	11,65 ± 3,70	NS group 1 *vs* group 2 NS group 1 *vs* group 3 NS group 2 *vs* group 3

* ELISA serum concentrations are reported. Significative values are reported in bold. Mean ± Standard Error (SE) and *p value* are reported.

5.3.4. Patients stratified based on a cut-off calculated on the distribution of $T_{R3\text{-}56}$ cells

Since the percentage of $T_{R3\text{-}56}$ cells is increased in patients with more severe clinical conditions, while the percentage of Treg cells appears reduced, we focused our attention on the $T_{R3\text{-}56}$ regulatory level in our patient cohort.

In this regard, the percentage of $T_{R3\text{-}56}$ cells in the enrolled patients varies widely from 0.3 to 20.5. The mean percentage value is 6.3, with a standard deviation (SD) of 4.5 and a standard error (SE) of 0.7. Therefore, it is a distribution with percentage values spread over a very wide range. To identify the most relevant existing correlations between $T_{R3\text{-}56}$ lymphocytes and cells and molecules involved in the antiviral response, we arbitrarily focused on those patients whose percentage value was above the 75th percentile (8.2%) and above the mean + 3 x SE (8.4%).

Adopting this criterion, we applied a cut-off of 8.4% to stratify patients, obtaining a small group of patients (n = 24), with very high levels of $T_{R3\text{-}56}$ cells ($T_{R3\text{-}56}$ High group).

Interestingly, this group is predominantly composed of individuals from group 2 (n = 8) and group 3 (n = 14), supporting the hypothesis that $T_{R3\text{-}56}$ cells are positively correlated with the severity of the clinical condition of COVID-19.

In patients belonging to the $T_{R3\text{-}56}$High group, we observed that higher percentages of $T_{R3\text{-}56}$ cells correspond to high levels of CTL (data not shown). This finding supports the increase in CTL observed in patients with more severe clinical conditions (figure 19 panel B) and the corresponding increase in $T_{R3\text{-}56}$ cells in group 3 (figure 21 panel B). Furthermore, we observed a positive correlation between the percentage of $T_{R3\text{-}56}$ cells and immune effector cells: CTL and NK cells (Table 5). Furthermore, the percentage of $T_{R3\text{-}56}$ is negatively correlated with monocytes in the $T_{R3\text{-}56}$High group (Table 5).

In particular, the percentage of $T_{R3\text{-}56}$ is positively correlated with IL-17A production (Table 5). The increase in IL-17A may indicate the establishment of an advanced inflammatory state in subjects of our cohorts with more severe COVID.

Table 5. T_{R3-56} cells positively correlate with IL-17, NK and CTLs in TR3-56^{High} Group of patients *.

	% TR3-56 lymphocytes	
	Slope	P value
TNF-α (pg/ml)	0,1336	0,6730
IL-17 (pg/ml)	0,6786	**0,0106**
% Lymphocytes	-0,3719	0,0735
% Monocytes	-0,6431	**0,0007**
% Neutrophils	0,2351	0,2688
% T lymphocytes	-0,1228	0,5675
% B lymphocytes	-0,3902	0,0594
% NK lymphocytes	0,4456	**0,0291**
% CTLs	0,4507	**0,0271**
% Th lymphocytes	-0,3846	0,0635
% Th1 lymphocytes	0,2662	0,2086
% Th17 lymphocytes	-0,1446	0,5001
% Treg lymphocytes	-0,2540	0,2311

* *Spearman correlation* is reported: *r = slope; p= p value.* The Spearman correlation coefficients range from -1 to +1. The sign of the coefficient (*r)* indicates whether it is a positive or negative monotonic relationship. A positive correlation means that as one variable increases, the other variable tends to increase as well. A negative correlation means that as one variable increases, the other tends to decrease. Values closer to -1 or +1 represent stronger relationships compared to values closer to zero. Significative values (*p*) are reported in bold.

No correlations were observed between T_{R3-56} and other cells (lymphocytes, neutrophils, T cells, Th, Th1, Th17, Treg and B cells) and TNF-α and.

Finally, no correlations were observed in the group of patients with percentages of T_{R3-56} below the cut-off (data not shown).

5.4. Kidney transplants

5.4.1. Increased circulating $T_{R3\text{-}56}$ regulatory T cells, associated with decreased Treg lymphocytes, characterizes a cohort of kidney transplant recipients

Figures 22 and 23 illustrates the complete immune profile analysis performed in the cohort of 53 kidney transplant recipients with no evidence of graft rejection, no infectious episodes, and no changes in immunosuppressive regimen in the last 6 months, compared to 20 age/sex-matched healthy controls.

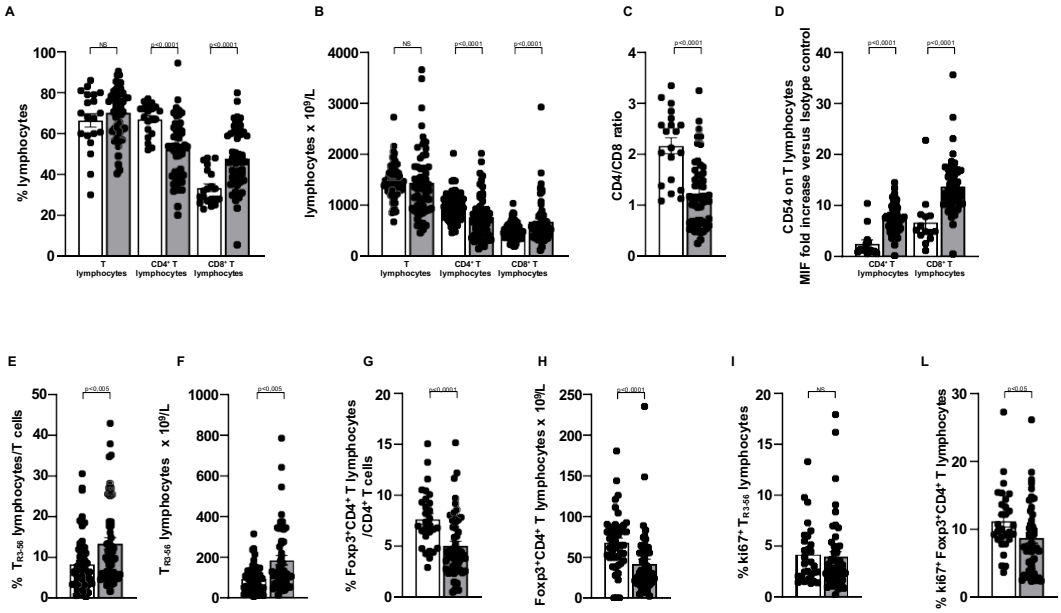

Figure 22. Increased amount of the circulating CTL and the $T_{R3\text{-}56}$ regulatory T cells, higher expression of the CD54 activation molecule on T cell effectors and decreased amount and growth ability of the Treg subset characterise a cohort of allograft kidney recipients showing no rejection episodes, no infections and no changes in immuno-suppression therapy in the previous six months. White and grey columns indicate data obtained in healthy controls and kidney transplanted subjects, respectively. (**A,B**) Indicate the percentage and number of circulating T, $CD4^+$ and $CD8^+$ T lymphocytes, as indicated; (**C**) Indicates CD4/CD8 ratio; (**D**) Refers CD54 expression level in $CD4^+$ and $CD8^+$ T lymphocytes, as indicated; as detailed in the Material and Method Section, CD54

expression on the T cell effectors has been expressed as ratio of the mean intensity fluorescence (MIF) value for $CD4^+$ and $CD8^+$ T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb. (**E,F**) Indicate percentage and number of the circulating $T_{R3\text{-}56}$ regulatory T cells, respectively; (**G,H**) Refer percentage and number of the circulating Treg population; (**I,L**) Indicate the growth ability of the circulating $T_{R3\text{-}56}$ and Treg populations, as represented by their intracellular expression of the ki67 molecule; Statistical evaluation of data has been performed by means of the Mann-Whitney test. Statistical significance values are indicated.

As shown in figure 22 panels A-C, comparative analysis with controls showed that $CD4^+$ T cells were significantly decreased in transplant recipients both in percentage (67.04±1.74 in controls versus 52.17±2 in kidney recipients; $p<0.0001$) and in absolute number (977±30.52 109/L in controls versus 765±59.11 109/L in kidney recipients; $p<0.0001$). At the same time, there was an increase in the percentage of circulating CTL (33.47±0.88 versus 47.83±2 in kidney recipients; $p<0.0001$) and number (486.5±18.19 109/L versus 678.3±60.96 109/L; $p<0.0001$) and a decrease in the CD4/CD8 ratio (2.16±0.15 in controls versus 1.23±0.09 in kidney recipients; $p<0.0001$). Analysis of the surface expression of the CD54 molecule (figure 22 panel D), widely associated with antigen-dependent T cell activation [217,218], revealed a significant activation of both $CD4^+$ (2.51±0.75 in controls versus 7.44±0.37 in kidney recipients; $p<0.0001$) and CTL (6.72±1.39 in controls versus 13.77±0.76 in kidney recipients; $p<0.0001$). Therefore, we focused on the regulatory subsets, represented by Tregs and the $T_{R3\text{-}56}$ T cell population. Compared with healthy subjects (figure 22 panels E and F), the $T_{R3\text{-}56}$ regulatory T cell subset was significantly increased in transplant recipients, both as percentage (8.32±0.89 in controls versus 13.40±1.43 in kidney recipients; $p<0.005$) and as number (94.05±10.61 109/L in controls versus 185.6±23.34 109/L in kidney recipients; $p<0.005$), while Treg lymphocytes (figure 22 panels G and H) were reduced in transplant recipients both as percentage (7.66±0.53 in controls versus 5.05±0.42 in kidney recipients; $p<0.0001$) and as number (68.37±5.24 109/L in controls versus 42.13±5.54 109/L in kidney recipients; $p<0.0001$).

A key feature of the Treg subset is its high growth capacity [219,220]. Therefore, we assessed this parameter in both subsets of circulating regulatory T cells (Treg and $T_{R3\text{-}56}$), detecting their intracellular expression of ki67. As shown, we observed (figure 22 panel L) a significant reduction in intracellular ki67 expression in circulating Treg lymphocytes from transplant recipients (11.23±0.89 in controls versus 8.73±0.77 in kidney recipients; $p<0.05$). However, (figure 22 panel I) no significant difference (4.17±0.54 in controls versus 3.93±0.51 in kidney recipients) was detected in the growth capacity of the $T_{R3\text{-}56}$ subset compared to healthy controls. As shown, significantly reduced levels of B lymphocytes (figure 23 panels A and B), a lower percentage of circulating iNKT cells (figure 23 panel C), no difference in the number of iNKT (figure 23 panel D), as well as in the percentage and amount of circulating NK effectors (figure 23 panels E and F) were observed in transplant recipients compared to controls. Furthermore, comparative analysis of the growth capacity of innate and adaptive lymphocytes between kidney recipients and controls, revealed no significant difference in intracellular ki67 expression by $CD4^+$ T cells and CTL (figure 23 panels G and H), nor in B lymphocytes (figure 23 panel I) or iNKT cells (figure 23 panel J).

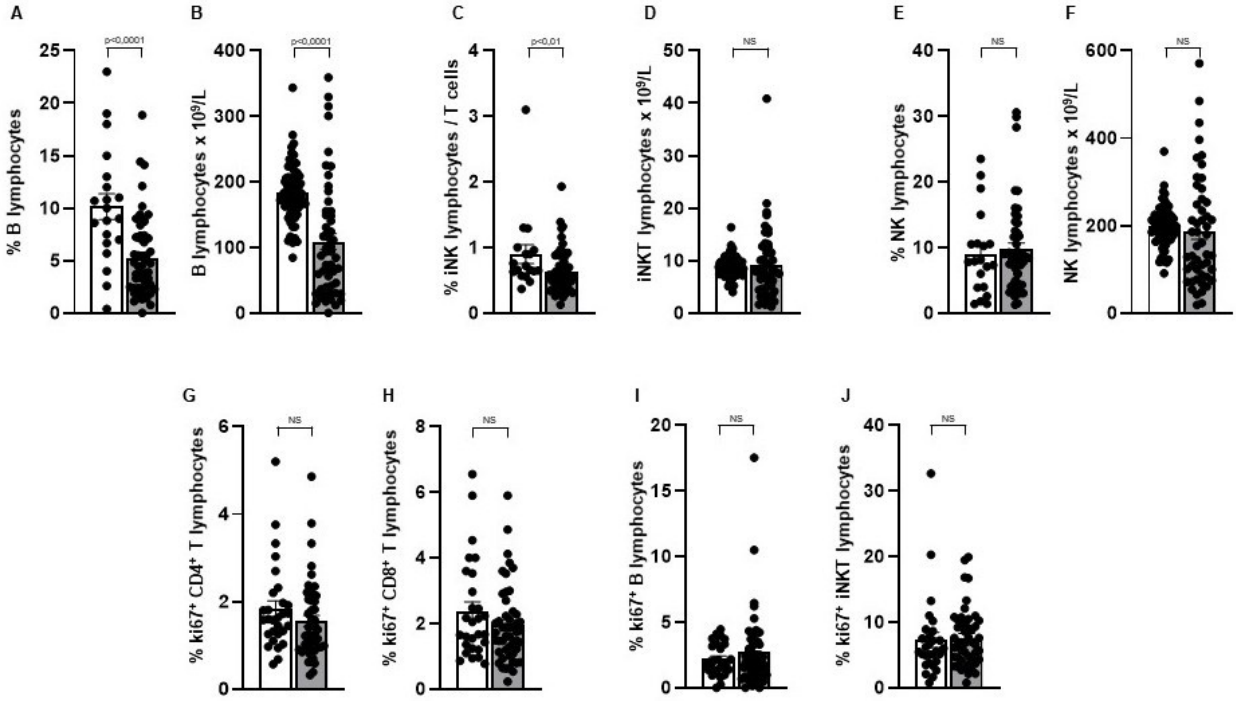

Figure 23. Reduced amount of the circulating iNKT and B lymphocytes characterise a cohort of allograft kidney recipients showing no rejection episodes, no infections and no changes in immuno-suppression therapy in the previous six months. White and grey columns indicate data obtained in healthy controls and kidney transplanted subjects, respectively. (**A,B**) indicate percentage and number of circulating B cells, as indicated; (**C,D**) indicate percentage and number of the circulating iNKT subset; (**E,F**) indicate percentage and number of the circulating NK cells, respectively; (**G,H,I** and **J**) refer growth ability, as represented by intracellular expression of the ki67 molecule, of the circulating CD4+, CD8+, iNKT T cells and of B lymphocytes, respectively; Statistical evaluation of data has been performed by means of the Mann-Whitney test. Statistical significance values are indicated.

Therefore, compared to healthy controls, the immune profile of the kidney transplant recipient cohort revealed the presence of activated adaptive effectors, of increased number of $T_{R3\text{-}56}$ lymphocytes, and reduced level and growth capacity of the Treg subpopulation. In addition, an increase in CTLs, a reduction in the levels of $CD4^+$ T cells, B cells and iNKT lymphocytes was observed.

5.4.2. Reduced Treg subset growth capacity characterizes the subgroup of transplant recipients with a higher amount of circulating $T_{R3\text{-}56}$ regulatory T cells

The cohort of kidney transplant recipients was characterized by increased CTL activation, higher levels of circulating $T_{R3\text{-}56}$ regulatory T cells, and reduced amount and growth capacity of the Treg population. Therefore, we focused our investigation on the immune profile associated, in our cohort, with highest levels of circulating $T_{R3\text{-}56}$ lymphocytes. In this context, kidney transplant recipients were categorized based on their level of circulating $T_{R3\text{-}56}$ in two subgroups characterized by a $T_{R3\text{-}56}$/T cell ratio higher or similar to controls. The cut-off value (9.16% of T cells) was arbitrarily established, as detailed in the Patients and Methods section, by increasing of three SEM the median value observed in healthy subjects enrolled in the study. Light and dark grey columns in figure 24 show the results obtained in the group of transplant recipients characterized by TR3-56 lymphocytes levels similar (≤9.16% of T cells) or higher (>9.16% of T cells) than controls, respectively.

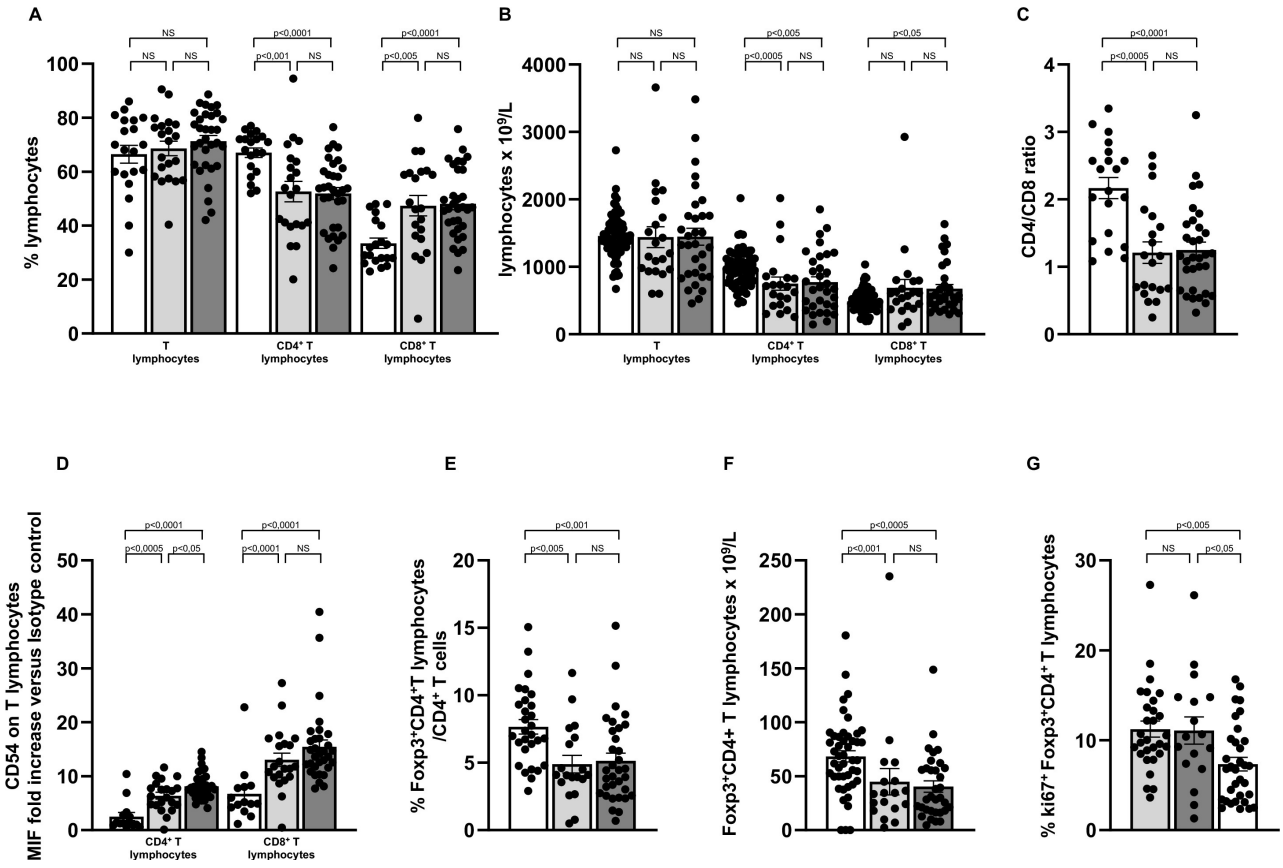

Figure 24. Allograft kidney recipients show association of highest level of circulating T_{R3-56} regulatory T cells with significant decrease of the Treg growth ability and increasing CD54 expression by the $CD4^+$ T cell population. White columns indicate healthy controls; light and dark grey columns indicate transplanted subjects showing circulating T_{R3-56} levels <9.16% or ≥ 9.16% of the T cell population, respectively; the 9.16 cut-off value has been obtained by increasing of three standard errors the median value observed in healthy controls (See patient and method section for details). (**A,B**) Indicate percentage and number of circulating T, $CD4^+$ and $CD8^+$ T lymphocytes; (**C**) Shows CD4/CD8 ratio; (**D**) Refers CD54 expression level in $CD4^+$ and $CD8^+$ T lymphocytes; as detailed in the Material and Method Section, CD54 expression on the T cell effectors has been expressed as ratio of the mean intensity fluorescence (MIF) value for $CD4^+$ and $CD8^+$ T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb. (**E,F**) Indicate percentage and number of the circulating Treg in the different cohorts; (**G**) Indicate the growth ability of the circulating Treg population, as represented by their intracellular expression of the ki67 molecule; Statistical evaluation of

data has been performed by means of the Mann-Whitney test. Statistical significance values are indicated.

Figure 24 panels A and B show that kidney transplant recipients, regardless of the amount of circulating $T_{R3\text{-}56}$ regulatory T cells, showed a reduced amount of $CD4^+$ T cells, increasing levels of CTL, reduced CD4/CD8 ratio (figure 24 panel C), and lower percentage and number of Treg population (figure 24 panels E and F). Furthermore, an increasing activation of T cell effectors, assessed by their CD54 expression, was found in patients compared to controls (figure 24 panel D).

However, a more significant increase in CD54 expression was observed in $CD4^+$ T cells from transplant recipients with a higher $T_{R3\text{-}56}$ level (6.39±0.61 in kidney recipients with $T_{R3\text{-}56}$ level ≤9.16% of T cells versus 8.13±0.43 in kidney recipients with $T_{R3\text{-}56}$ >9.16% of T cells; $p<0.05$). As shown (figure 25 panels A and B), the significant decrease in circulating B cells found in kidney transplant recipients compared with controls was most consistently found in the subgroup of transplant recipients characterized by higher circulating $T_{R3\text{-}56}$ regulatory T cells (6.70±0.95 in kidney recipients with $T_{R3\text{-}56}$ level ≤9.16% of T cells vs. 4.30±0.56 in kidney recipients with higher $T_{R3\text{-}56}$ level; $p<0.05$). In contrast, no difference was found in the level of iNKT (figure 25 panels C and D) and NK cells (figure 25 panels E and F), in patients compared with controls, regardless their level of circulating $T_{R3\text{-}56}$ cells. Similarly (figure 25 panels G-J), no difference in the growth capacity of $CD4^+$, CTL, iNKT and B lymphocytes was detected between controls and patients, regardless of the level of circulating $T_{R3\text{-}56}$ lymphocytes.

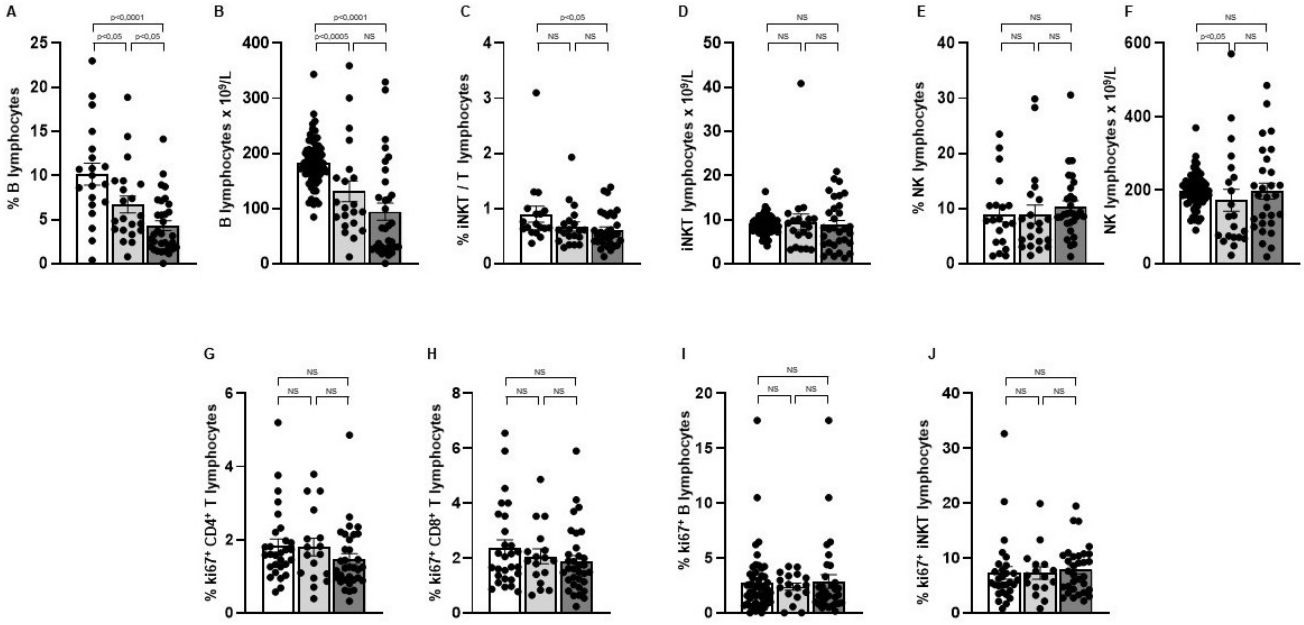

Figure 25. Allograft kidney recipients with highest level of circulating $T_{R3\text{-}56}$ regulatory T cells show significant decreased percentage of circulating B lymphocytes when compared with the counterpart. White columns indicate healthy controls; light and dark grey columns indicate transplanted subjects showing circulating $T_{R3\text{-}56}$ levels <9.16% or ≥ 9.16% of the T cell population, respectively; the 9.16 cut-off value has been obtained by increasing of three standard errors the median value observed in healthy controls (See patient and method section for details). (**A-F**) indicate percentage and number of the circulating B, iNKT and NK lymphocytes, as indicated; (**G-J**) show comparative analysis of growth ability of $CD4^+$, $CD8^+$, iNKT and B lymphocytes, between healthy controls and kidney transplant recipients showing $T_{R3\text{-}56}$ levels <9.16% or ≥ 9.16% of the T cell population, respectively; Statistical evaluation of data has been performed by means of the Mann-Whitney test. Statistical significance values are indicated.

Notably (see figure 24 panel G), only renal transplant recipients showing a higher $T_{R3\text{-}56}$ level (>9.16% of T cells) showed a significant decrease in the growth capacity of the Treg subset (11.08±1.50 in renal recipients with a $T_{R3\text{-}56}$ level ≤9.16% of T cells versus 7.34±0.75 in renal recipients with the highest $T_{R3\text{-}56}$ level; $p<0.05$).

Thus, in renal transplant recipients, higher $T_{R3\text{-}56}$ levels are preferentially associated with increased activation of the $CD4^+$ T and decreased growth ability of the Treg subset.

5.4.3. Higher levels of $T_{R3\text{-}56}$ are associated with early signs of unstable graft tolerance

Our immune profiling analysis showed that, in our cohort of kidney transplant recipients, increased circulating $T_{R3\text{-}56}$ regulatory T cells preferentially associated with reduced numbers and growth capacity of Tregs, as well as with increased activation of $CD4^+$ T cells. Therefore, we investigated whether circulating $T_{R3\text{-}56}$ could be a valuable criterion for identifying kidney transplant recipients with early signs of unstable graft control. For this purpose, kidney recipients were classified, based on their laboratory and clinical data, into the Stable and Unstable group, as detailed in the Patient and Method section. Briefly, the stable group was represented by subjects with stable renal function and urinary parameters, while the unstable group included patients who showed changes in serum creatinine level ≥0.2 mg/dl and/or proteinuria >100 mg/day in 24-hour urine samples in two consecutive evaluations, despite the absence of predisposing clinical conditions. As shown in Figure 26, the comparative analysis of the immune profile of kidney recipients, grouped according to their stable versus unstable clinical conditions, revealed significant difference between the two patient subgroups in the number of CTLs (546.89±38.84 109/L in kidney recipients with stable disease versus 713.8±80.43 109/L in kidney recipients with unstable disease; $p<0.05$), which was higher in individuals belonging to the unstable disease group, also showing (figure 26 panels B and C) a lower CD4/CD8 ratio (1.45±0.14 in kidney recipients with stable disease versus 0.99±0.09 in the counterpart with unstable disease; $p<0.05$). As shown (figure 26 panels E and F), significant increase of circulating $T_{R3\text{-}56}$ cells as percentage (9.75±1.31 in kidney recipients with stable disease versus 17.04±2.36 in the counterpart with unstable disease; $p<0.05$) as well as number (120.6±19.27 109/L in kidney recipients with stable disease versus 254.8±40.32 109/L in the subgroup with unstable disease; $p<0.01$), associated with (figure 26 panel L), significant reduced Treg growth capacity (10.77±1.14 in kidney recipients with

stable disease versus 6.29±0.78 in kidney recipients with unstable disease; p<0.005), characterize the subgroup of patients with unstable disease.

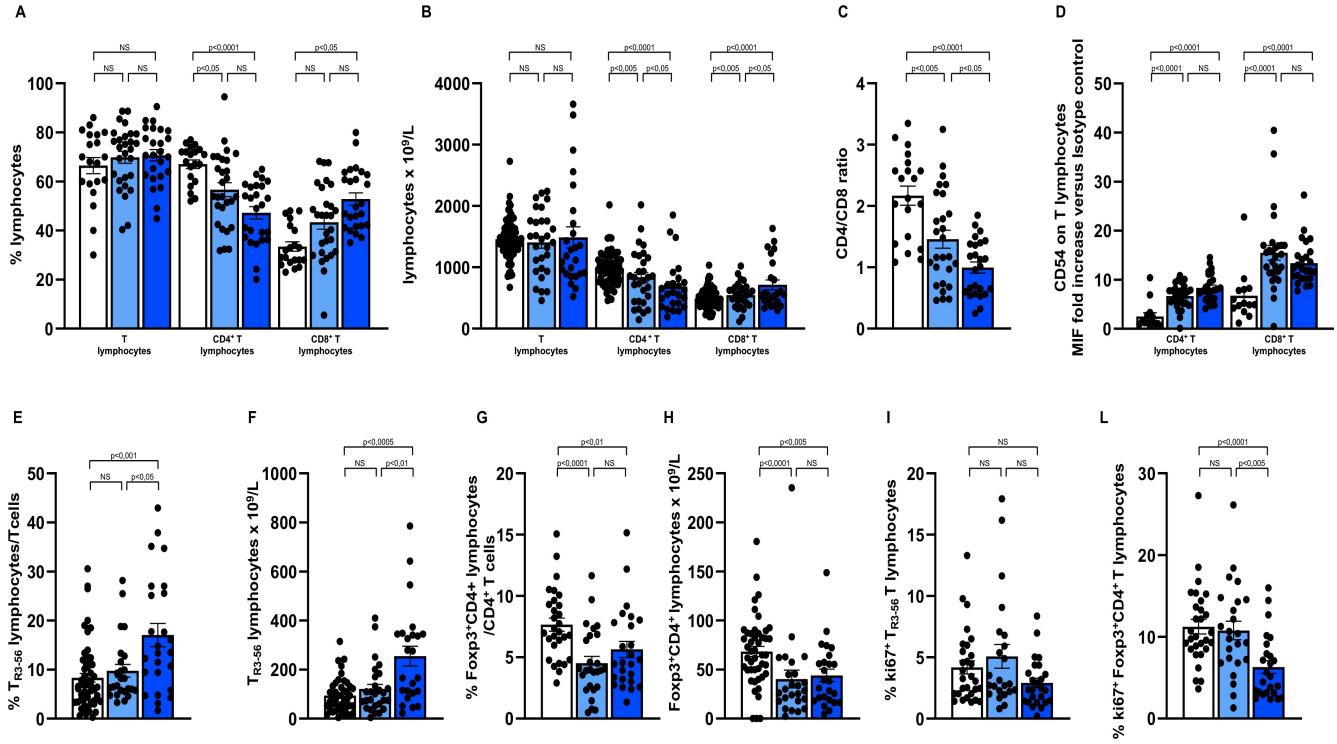

Figure 26. Increasing amount of circulating T_{R3-56} lymphocytes and reduced growth ability of the Treg population characterise kidney transplant recipients showing unstable control of the graft. White columns indicate healthy controls; light and dark blue columns indicate transplanted subjects categorised, according to their clinical and laboratory profile, as belonging to the Stable or Unstable transplant recipient sub-group, respectively. See Patient and Methods section for details. (**A,B**) Indicate percentage and number of circulating T, $CD4^+$ and $CD8^+$ T lymphocytes; (**C**) Indicates CD4/CD8 ratio; (**D**) Refers CD54 expression level in $CD4^+$ and $CD8^+$ T lymphocytes; CD54 expression on the T cell effectors has been expressed as ratio of the mean intensity fluorescence (MIF) value for $CD4^+$ and $CD8^+$ T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb. (**E,F**) Show percentage and number of the circulating T_{R3-56} lymphocytes; (**G,H**) Show percentage and number of circulating Treg; (**I,L**) Indicate the growth ability, as represented by their expression of the ki67 molecule, of the circulating T_{R3-56} and Treg population, respectively; Statistical evaluation of data has

been performed by means of the Mann-Whitney test. Statistical significance values are indicated.

No differences were observed in the amount of B cells, iNKT and NK cells as well as in the growth capacity of adaptive and innate immune effectors (figure 27) comparing the subgroup of transplant recipients with stable disease versus their counterparts with unstable disease.

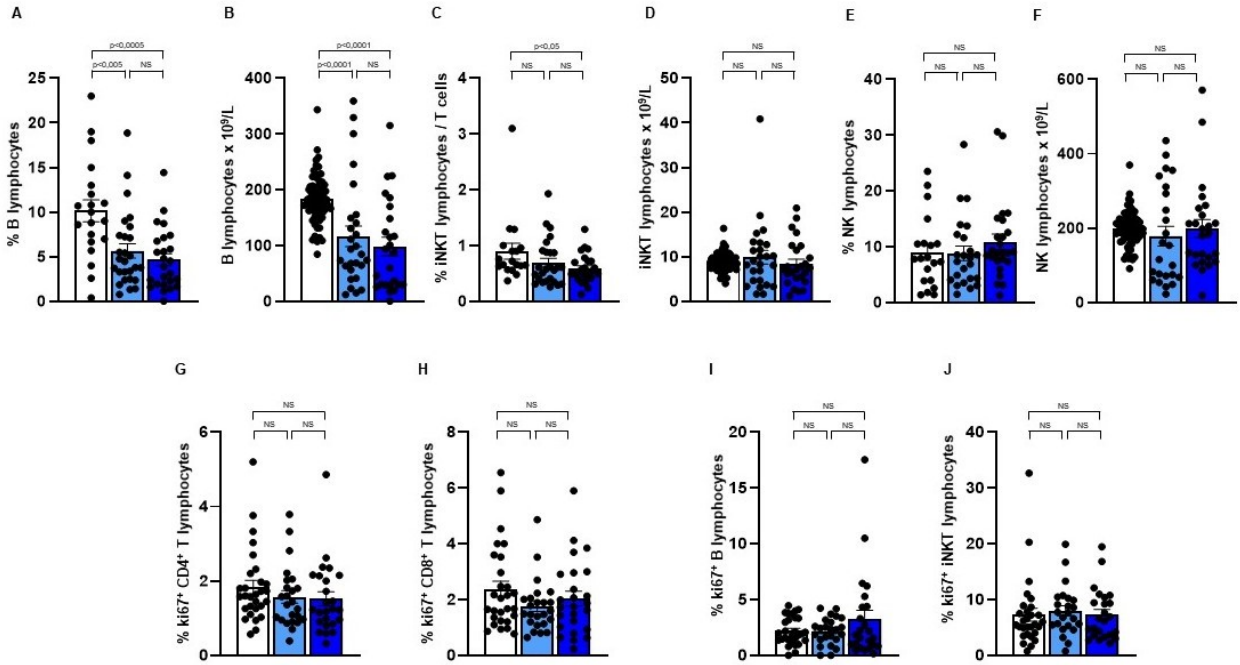

Figure 27. Analysis of the immune profile of kidney transplant recipients classified according to the Stable or Unstable disease status. White columns indicate healthy controls; light and dark blue columns indicate transplanted subjects categorised, according to their clinical and laboratory profile, as belonging to the Stable or Unstable transplant recipient sub-group, respectively. See Patient and Methods section for details. (**A**-**F**) indicate percentage and number of circulating B, iNKT and NK lymphocytes; (**G-J**) indicate growth ability, as represented by intracellular ki67 expression, of $CD4^+$, $CD8^+$, iNKT T cells and B lymphocytes, respectively; Statistical evaluation of data has been performed by means of the Mann-Whitney test. Statistical significance values are indicated.

To investigate whether high level of circulating $T_{R3\text{-}56}$ cells, as defined by a three-SEM increase in the median value obtained in healthy controls, could be significantly associated with unstable graft control, we analysed the presence of circulating $T_{R3\text{-}56}$ cells at a percentage >9.16% of T cells in kidney transplant recipients categorized in the unstable versus stable disease subgroup, as defined in the Patients and Methods Section. As shown (Table 6), 19 of 25 subjects with unstable disease showed a percentage of $T_{R3\text{-}56}$ cells >9.16% of circulating T lymphocytes (defined higher $T_{R3\text{-}56}$ level), as compared with 10 of 25 subjects with stable renal disease ($p<0.05$ by Fisher's exact test; Odds Ratio 4.75; 95% CI 1.384 to 14.37). These data suggest that the level of circulating $T_{R3\text{-}56}$ cells could represent a valuable criterion for identifying renal transplant recipients with early signs of immune-mediated graft damage, in the absence of any treat for clinical and/or laboratory rejection.

Table 6. Higher levels of the circulating $T_{R3\text{-}56}$ cells significantly associate with Unstable disease[1] condition in a cohort of kidney transplanted subjects showing no infections, no rejection episodes and no changes in immuno-suppression therapy in the previous six months.

	N	Age Mean (range)	Males / Females	$T_{R3\text{-}56}$/T cells <9.16 [2]	$T_{R3\text{-}56}$/T cells >9.16 [2]
	50	51.82	31/19	21	29
Stable Disease [1]	25	53.54 (38-68)	15/10	15 [3]	10 [4]
Unstable Disease [1]	25	50.32 (35-67)	16/9	6	19

[1] Subgroup categorisation criteria have been detailed in the Patients and Method Section. [2] The number has been obtained increasing of three SEM the median value found in the healthy controls for $T_{R3\text{-}56}$ percentage/ T lymphocytes (see Patient and Method Section for details). [3] Significant different from the Unstable disease Group ($p<0.05$ by Fisher exact test; Odd Ratio 0.21 (95% CI: 0.0695 to 0.722). [4] Significant different from the Unstable disease Group ($p<0.05$ by Fisher exact test; Odd Ratio 4.75 (95% CI 1.384 to 14.37)

6. Conclusions

6.1. General remarks

The immune response ultimately results from a balance between activation and inhibition. The success or failure of this relevant balance depends on a combination of genetic and epigenetic factors, as well as a variety of molecules, cells, and the tissue microenvironment involved in immune regulation mechanisms.

Our understanding of all the mechanisms involved in immune regulation is not yet definitive, and the overall picture is likely much broader than what is currently described in the scientific literature. Given the plasticity of the immune system and the diversity of organisms, it is highly probable that many additional cells and molecules yet to be identified play roles in immune regulation.

Therefore, it cannot be ruled out that other factors and cells, beyond those covered in this PhD thesis, need to be considered to fully grasp the complex balance between immune system activation and inhibition.

At the same time, it is equally probable that some of our current knowledge may need to be revised regarding the role of cells that have been specifically described as immunoregulatory. These cells might have different, broader, and more versatile roles in the intricate balance between activation and inhibition of both innate and adaptive immune responses.

In this complex context, it is highly worthwhile to propose to the scientific community the investigation of "new cells", such as $T_{R3\text{-}56}$, in the role of immunoregulatory cell populations. This would contribute to a deeper understanding of the immune system and its dynamic and plastic complexity.

From our studies, the $T_{R3\text{-}56}$ cell subset is emerging as a critical player in the regulation of immune responses, particularly in the context of immune-mediated diseases, like haematological disorders, transplantation conditions and infections.

$T_{R3\text{-}56}$ cells appeared to be involved in negatively regulating immune responses. They primarily influence CTLs by modulating their activity, potentially preventing excessive immune activation and tissue damage. This function is essential for maintaining immune homeostasis and preventing autoimmunity.

Through their activity, $T_{R3\text{-}56}$ cells may contribute to mitigating inflammation and tissue damage.

$T_{R3\text{-}56}$ cells are distinct from Tregs, although our studies suggest that both subsets play a role in immune regulation. While Tregs generally suppress immune responses to prevent autoimmunity, $T_{R3\text{-}56}$ cells may provide additional regulatory effects, especially in the context of specific infections or conditions.

The presence of $T_{R3\text{-}56}$ cells has been associated with changes in other immune cell populations, such as increased B cells and Th17 cells during severe infections. This interaction suggests that $T_{R3\text{-}56}$ cells may assist in a broader modular immune response, influencing both humoral and cellular immunity.

$T_{R3\text{-}56}$ cells can modulate the inflammatory response by regulating the activity of CTLs and other effector cells. In the context of severe infections like COVID-19, an increase in $T_{R3\text{-}56}$ cells may be an attempt to control inflammation and prevent an overactive immune response, which could lead to hyperinflammation and tissue damage.

Understanding the role of $T_{R3\text{-}56}$ cells in immune regulation could have significant implications for managing diseases characterized by dysregulated immune responses. For instance, targeting $T_{R3\text{-}56}$ cells or their interactions with other immune cells might provide new therapeutic strategies for controlling severe infections, autoimmune diseases, or other conditions involving immune dysregulation.

Further investigations are needed to fully understand the mechanisms by which $T_{R3\text{-}56}$ cells regulate immune responses and how these mechanisms vary across different diseases and conditions. Detailed studies could reveal new insights into their role in immune regulation and potential applications in clinical practice.

In summary, $T_{R3\text{-}56}$ cells appear to be part of immune regulation. Their ability to modulate the activity of immune effector cells, interact with other immune subsets, and influence inflammatory responses makes them a key focus for understanding and managing complex immune-related diseases.

6.2. Myelodysplastic Syndromes (MDS)

It has been extensively demonstrated that an altered pro-inflammatory immune response in the bone marrow characterizes the pathogenesis/progression of MDS [115,116,118,119,123]. Immune-mediated mechanisms have been extensively described as relevant to the pathogenesis of MDS in the early stages of the disease [115,117,119,120,123,202,209]. It has been observed that defective immune tolerance, which preferentially affects the CTL response, underlies bone marrow failure in MDS, while an immunosuppressive bone marrow environment is a hallmark of advanced stages of MDS [115,117,120,121].

To address the role of $T_{R3\text{-}56}$ in the pathogenesis/progression of MDS, we analysed their levels in the PB and BM of 58 MDS patients. A trend of increasing BM $T_{R3\text{-}56}$ in the MDS cohort was revealed from very low/low-risk stages to high/very high-risk stages of the disease. This scenario is accompanied by progressively reduced CTL activation in the BM (assessed by CD54 expression) from very low/low-risk MDS patients to high/very high-risk patients. In this context, the absence of statistical significance may be related to the small number of patients in the high/very high-risk stage compared to the very low/low-risk group. Notably, a significant positive correlation between $T_{R3\text{-}56}$ and bone marrow blasts was revealed.

These findings are consistent with the involvement of $T_{R3\text{-}56}$ cells in the pathogenesis/progression of MDS, as proposed for the Treg subset [117,202,209,221,222,223], and align with previous data obtained in AML [105,224]. Indeed, the potential role of $T_{R3\text{-}56}$ in promoting immune escape of dysplastic/leukemic clones could be hypothesized.

The significant inverse relationship between the amount of BM $T_{R3\text{-}56}$ and BM CTL activation and expansion, observed in very low/low-risk MDS patients, suggests the possible involvement of a defective control of CTL effectors by $T_{R3\text{-}56}$ cells to the immune mediated mechanisms involved in the emergence of dysplastic clones, as proposed for the Treg subset [202,209,221].

In addition, we describe a significant inverse correlation between BM $T_{R3\text{-}56}$ and the activation of BM CTL in very low/low-risk individuals. These data add $T_{R3\text{-}56}$ lymphocytes to the regulatory cell-mediated network controlling CTL activation,

as described in type 1 diabetes [112]. Additionally, we observed a strong inverse correlation between the presence of a BM skewed $CD8^+$ T cell repertoire and the amount and recruitment of $T_{R3\text{-}56}$ in the bone marrow.

Therefore, the involvement of $T_{R3\text{-}56}$ subset in controlling antigen-dependent activation and expansion of $CD8^+$ T cells in the bone marrow of very low/low-risk MDS patients could be hypothesized. The lack of significant correlation of $T_{R3\text{-}56}$ subset, observed in very low/low-risk MDS patients showing a skewed $CD4^+$ T cell repertoire, is conceivable with the preferential involvement of this regulatory cell subset in controlling CTL activity, as previously described [112].

The role of altered CTL in damaging physiological polyclonal haematopoiesis has been extensively described [115,120,121,122,123]. This study adds $T_{R3\text{-}56}$ subset to the complex cell mediated regulatory network involved in the control of adaptive immune responses in MDS.

$T_{R3\text{-}56}$/CTL cell-to-cell contact can mediate significant alteration in the redox balance in CTLs [112]. This effect has been observed to modulate antigen-dependent effector function of human CTLs, likely interfering with cytoskeleton rearrangement processes relevant for CTL activity. The strong correlation we observed between BM $T_{R3\text{-}56}$ cells and resident CTLs activation and expansion suggests the relevance of cell-to-cell contact for $T_{R3\text{-}56}$ mediated regulatory function in MDS.

The BM microenvironment plays a key role in regulating immune responses, and disruptions in these pathways are thought to contribute to MDS pathogenesis. $T_{R3\text{-}56}$, as an immune regulatory factor, may be involved in altering immune cell function, inflammatory signalling, or bone marrow stromal interactions. However, the specific pathways and molecular interactions through which $T_{R3\text{-}56}$ impacts immune dysregulation remain largely unclear.

Moreover, it is necessary to investigate how $T_{R3\text{-}56}$ interacts with immune cells such as T cells, macrophages, or dendritic cells in the BM, and whether it influences the production of cytokines, chemokines, or other signalling molecules involved in immune regulation. Identifying the precise role of $T_{R3\text{-}56}$ in these processes would not only enhance the knowledge of MDS biology but may also reveal new molecular targets for therapeutic intervention. By targeting specific elements of

immune dysregulation, it may be possible to design innovative therapies aimed at restoring immune homeostasis in MDS patients, potentially improving outcomes and slowing disease progression.

Therefore, further research into $T_{R3\text{-}56}$ and its interactions within the immune system could hold promise for novel treatment strategies in MDS: this PhD thesis aimed to provide some additional details to the complex scenario of immune dysregulation in MDS.

6.3. Chronic Lymphocytic Leukaemia (CLL)

In the previous section on MDS, it becomes evident how crucial it is to understand the mechanisms underlying the neoplastic progression of haematopoiesis. Equally important in this context is understanding the role of the immune response in regulating both normal and malignant haematopoiesis, and even more relevant, the extent to which immune regulatory dysfunction contributes to the leukemic progression.

In order to expand the understanding of the role of immune regulation in leukemic progression, this PhD research aimed to assess the role of $T_{R3\text{-}56}$ in CLL determinism. In this disease model, the study of Prof. Terrazzano Research Group identified multiple aspects not only related to $T_{R3\text{-}56}$ (*see the published manuscript: Rubino V, Carriero F, Palatucci AT, Giovazzino A, Leone S, Nicolella V, Calabrò M, Montanaro R, Brancaleone V, Pane F, Chiurazzi F, Ruggiero G, Terrazzano G. Adaptive and Innate Cytotoxic Effectors in Chronic Lymphocytic Leukemia (CLL) Subjects with Stable Disease. Int J Mol Sci. 2023 May 31;24(11):9596. doi: 10.3390/ijms24119596*). However, in this thesis, we focus exclusively on the characterization of the $T_{R3\text{-}56}$ T cell subset (despite the author of this PhD thesis having been involved in all aspects of the study indicated above), aiming to highlight its potential involvement as a regulatory cell population in leukemic progression.

As emphasized throughout this thesis, the functions of immune effectors are intricately connected to cell-mediated regulatory networks, involving both Treg cells and the TR3-56 subset. In the context of CLL, our findings reveal that while the percentage of Treg and $T_{R3\text{-}56}$ cells decreases when evaluated across the total lymphocyte population, their prevalence increases when assessed within the T-cell compartment specifically. This discrepancy highlights a key aspect of these regulatory subsets: although they may represent a smaller fraction of the total lymphocyte pool, their relative abundance within the T-cell subset becomes more pronounced and this shift suggests a targeted expansion of these regulatory cells, potentially in response to specific pathological conditions.

Additionally, the absolute number of circulating Treg and $T_{R3\text{-}56}$ cells is significantly higher in CLL patients compared to healthy controls. CLL is characterized by a predominance of B cells, which constitutes the majority of

lymphocytes in these patients. Despite the overall lymphocyte composition being skewed towards B cells, the T-cell compartment demonstrates a significant expansion of both Treg and $T_{R3\text{-}56}$ cell subsets. This expansion may represent an adaptive immune escape mechanism, where the increased presence of these regulatory cells could contribute to the disease's progression by dampening effective immune responses against the tumour.

Our data not only confirm the previously observed expansion of Treg cells in CLL [225,226,227] but also reveal a significant increase of $T_{R3\text{-}56}$ regulatory population. This finding underscores the relevance of these regulatory subsets in CLL and suggests that their expansion may play a crucial role in the disease pathophysiology, potentially offering new insights for potential approaches of targeted therapeutic strategies.

Finally, and no less importantly, these data provide an additional layer of understanding regarding the role of $T_{R3\text{-}56}$ in the complex landscape of immune regulation and its dysregulation.

6.4. COVID-19

Our retrospective study analysed a cohort of patients infected with SARS-CoV-2 during the first and second waves of the pandemic from 2020 to 2021 [207,208]. It specifically focused on individuals who had not received prior therapies or anti-SARS-CoV-2 vaccination before hospital admission. The study aimed to clarify the role of $T_{R3\text{-}56}$ cells and their interaction with the molecular and cellular effectors of the antiviral inflammatory immune response involved in COVID-19. Understanding these interactions could be crucial for identifying potential disease and prognostic biomarkers, as well as for pinpointing therapeutic targets and improving clinical management of COVID-19 patients and potentially patients with other viral or microbial infections in the future. The cohort was initially divided into three groups of increasing severity: Group 1 (WHO category 3), Group 2 (WHO category 4), and Group 3 (WHO categories 5, 6, 7), as previously described. Within these groups, we evaluated the profile of innate and adaptive immune responses.

Our analysis revealed that the overall lymphocyte population among white blood cells was significantly lower in Groups 2 and 3 compared to Group 1.

Additionally, Groups 2 and 3 exhibited reduced percentages of T cells. In contrast, a significant increase in CTLs [23,167-171] was observed in Group 3, highlighting an active effort by the immune system to eliminate SARS-CoV-2 infected cells. B lymphocytes progressively increased from Group 1 to Group 3, suggesting that an active humoral immune response against SARS-CoV-2 is aimed at neutralizing the virus [167-171,180]. In particular, the increase in both T cells and B cells was correlated with disease severity in our patient cohort. Moreover, Group 3 showed a reduced percentage of Th1 cells [23,167-171].

Conversely, the percentage of Th17 cells [88,167-171,179] significantly increased in Groups 2 and 3 compared to Group 1. This increase may indicate the onset of chronic inflammatory conditions in patients at the severe stage of COVID-19 [88,167-171,179]. Initially, this event may be helpful in counteracting virus spread. However, it could contribute to tissue damage and severe disease if the inflammatory response becomes dysregulated or excessive [157-163,167-171,183,184]. Although T lymphocytes decreased with the severity of clinical

conditions (from Group 1 to Group 3), they were accompanied by an increase in CTLs in Group 3.

In contrast, in Group 3, the percentage of activated T lymphocytes was lower than in Group 2 and similar to that in Group 1. This event indicates a progressive escalation in severity groups and may suggest a compensatory mechanism mediated by immunoregulatory processes to mitigate excessive immune activity in patients in Group 3 with more severe clinical conditions.

Therefore, we studied the presence of T lymphocytes involved in regulating immune responses, which could explain the observed reduction in T lymphocyte activation status. In this context, it is noteworthy that the percentage of Treg cells shows a significant decrease in Group 3, according to previous observation in the cohort comprising treated and untreated patients [184]. This observation indicates an intensified and favoured immune response during the severe phases of COVID-19 within this group. Conversely, it suggests that Tregs may not play a significant role in mitigating the exacerbation of the immune response in our cohort of patients with more severe clinical symptoms (Group 3). Consequently, our focus shifted to studying the $T_{R3\text{-}56}$ population. Specifically, the percentage of $T_{R3\text{-}56}$ cells was significantly increased in Group 3. The higher presence of $T_{R3\text{-}56}$ cells in Group 3 may indicate a compensatory response to increased inflammation and immune activation, as evidenced by the observed increases in CTLs, B cells, and Th17 cells during the severe phases in our patient cohort.

We suggest that $T_{R3\text{-}56}$ higher presence may serve to balance and mitigate the inflammatory effects of immune effector activity in COVID-19. The reduced activation of T cells observed in Group 3 may be related to this compensatory role of $T_{R3\text{-}56}$ cells within the same patient group.

We also analysed the serum cytokine concentrations in the three patient groups. It is interesting to note that TNF-α was significantly elevated in Groups 2 and 3. However, no statistical differences in the levels of other cytokines were observed between these patient groups. This finding suggests a potential role of TNF-α in influencing the severity of clinical conditions within these groups.

The concurrent increase in $T_{R3\text{-}56}$ cells along with increased CTLs, B cells, Th17 lymphocytes, and TNF-α suggests an overall activation of the immune

system. $T_{R3\text{-}56}$ cells may play a regulatory role in balancing the immune response to prevent excessive immune reactions, hyperinflammation, and tissue damage observed in severe cases of COVID-19. This coordinated immune response could reflect dynamic efforts to control viral infection and maintain tissue homeostasis, thus avoiding the exacerbation of immune responses.

The observed increase in $T_{R3\text{-}56}$ cell percentages in patients with more severe clinical conditions in our cohort underscores the importance of further clarifying the functional significance of these cells in the context of SARS-CoV-2 infection and other viral diseases.

Although our study provides preliminary evidence of a potential correlation between $T_{R3\text{-}56}$ cells and disease severity, further research is needed to fully outline the mechanisms underlying this association and determine its therapeutic implications.

Finally, we stratified patients into a group with a higher percentage of $T_{R3\text{-}56}$ cells (*$T_{R3\text{-}56}$High Group*). Within this group, we analysed the correlations between $T_{R3\text{-}56}$ and CTLs and NK cells.

In untreated patients belonging to the *$T_{R3\text{-}56}$High Group*, we observed that higher percentages of $T_{R3\text{-}56}$ were correlated with elevated levels of CTLs and NK cells. This evidence suggests a potential shift towards a robust antiviral response mediated by effector cells, likely associated with the severity of the patient clinical condition. Additionally, it may suggest that increased levels of $T_{R3\text{-}56}$ represent an attempt at homeostatic control to mitigate the exacerbation of the immune response during the more severe phases of COVID-19.

Furthermore, there is a positive correlation between the percentage of $T_{R3\text{-}56}$ cells and IL-17 levels in the *$T_{R3\text{-}56}$High group*. IL-17 is produced by various immune cells, including Th17 cells, γδ T cells, NKT cells, and innate lymphoid cells [88,167-171,179]. Th17 and IL-17 are known to play a role in promoting inflammatory processes by recruiting neutrophils and other immune cells and in COVID-19 [88,167-171,179]. No correlations were found between other cell types and cytokines in the *$T_{R3\text{-}56}$High group*. The positive correlation between IL-17 and $T_{R3\text{-}56}$ observed here suggests that $T_{R3\text{-}56}$ cells may play a role in attenuating the

chronic inflammatory response, similar to the concomitant increase observed in T_{R3-56} in Group 3.

The exacerbated immune response in COVID-19 necessarily involves uncontrolled engagement of immune effectors and the release of pro-inflammatory molecules [88,157-174,179,184], resulting in widespread tissue damage and potentially severe clinical outcomes [167-171]. This dysregulated immune reaction may significantly contribute to the progression of ARDS and the multiorgan failure observed in severe cases of the disease [167-171].

In this scenario, it is plausible to consider the observed increase in T_{R3-56} cells in our patient cohort as an attempt to mitigate the exacerbated immune response.

However, it is also reasonable to hypothesize that in certain contexts, such as infection or inflammation, the regulatory function of T_{R3-56} cells may adapt and they may also exhibit additional effector functions. This dual potential suggests that while T_{R3-56} cells are primarily immunoregulatory, they may also act as effectors, contributing to immune responses against pathogens when needed. Thus, despite their classification as immunoregulatory cells, the functional adaptability of T_{R3-56} cells and other immune cells allows them to switch to effector roles depending on the specific immune context encountered.

This intriguing hypothesis, which suggests the remarkable plasticity of the immune system requires support from studies demonstrating this mechanism.

Studies on Tregs have highlighted their versatile roles beyond traditional immune suppression. Tregs not only prevent autoimmune diseases and maintain immune balance but also exhibit dynamic functionalities [228,229]. They can directly kill tumour cells via granzyme-dependent mechanisms, a role that contrasts with their typical immunosuppressive function. Additionally, Tregs interact with non-immune cells and reside in non-lymphoid tissues, performing tissue repair and homeostasis functions [228,229]. Similarly, T_{R3-56} cells may exhibit immune plasticity adapting to peripheral tissue contexts by balancing suppressive and effector functions.

Our results suggest that T_{R3-56} cells could play a dual role in the immune response to SARS-CoV-2 infection. They might help mitigate excessive inflammation and tissue damage, aiding in immune homeostasis and tissue repair.

Conversely, their increased presence in severe COVID-19 cases may indicate an enhanced immune response against the virus.

In this study, no significant differences were found in several immune parameters (percentage of white blood cells, T cells, Th1 cells, and Treg cells) between the total cohort and the subset of patients who had not received any therapies prior to hospitalization. Despite the development of COVID-19 therapies aimed at modulating or supporting the immune response against the virus (e.g., directed antiviral drugs and corticosteroids to suppress inflammation), these treatments did not visibly affect the immune profiles examined in this retrospective analysis. Therefore, understanding and effectively managing the immune response remains critical in COVID-19 therapy to improve patient outcomes and mitigate disease-associated complications.

In summary, our findings shed light on the complex immunological dynamics of SARS-CoV-2 infection and underscore the potential role of $T_{R3\text{-}56}$ cells as key components of the immune response in COVID-19. We propose using $T_{R3\text{-}56}$ cells as biomarkers to identify severe COVID-19 cases and recommend further research on these cells in the context of other infections. Enhancing our understanding of $T_{R3\text{-}56}$ cells and their interactions with the host immune system could lead to the development of targeted therapies to combat viral infections and mitigate the global impact of COVID-19.

6.5. Kidney transplantation

The previous sections have suggested that $T_{R3\text{-}56}$ cells may play a significant role in the pathogenesis of haematological diseases and in anti-infective responses. Our data collectively highlight a potential immunoregulatory function of $T_{R3\text{-}56}$ cells, suggesting that when these cells are altered in number or function, they may become pathogenic and disrupt immune responses.

Therefore, we aimed to assess the role and involvement of $T_{R3\text{-}56}$ cells also in the context of physiological and pathological conditions such as organ transplantation. In such scenarios, the immune response plays a crucial role in organ rejection, and pharmacological control of the anti-graft response is essential. Investigating the impact of $T_{R3\text{-}56}$ cells in transplantation could provide valuable insights into how these cells contribute to graft acceptance or rejection, and may inform strategies to improve transplant outcomes by targeting these cells for therapeutic intervention.

It is noteworthy that this study reveals an intriguing finding: in a cohort of 53 kidney transplant recipients who do not exhibit any overt clinical or laboratory signs of renal rejection, higher levels of circulating $T_{R3\text{-}56}$ are associated with unstable control of the transplanted kidney. This observation suggests that elevated of these cells might be linked to subclinical immune dysregulation or instability in graft function, even in the absence of clear rejection markers.

The presence of higher $T_{R3\text{-}56}$ levels could indicate a potential role for these cells in modulating or affecting the immune response in a way that impacts graft stability. This finding underscores the importance of further investigating $T_{R3\text{-}56}$ cells as potential biomarkers or modulators of transplant outcomes. Understanding how these cells influence immune regulation and graft acceptance could provide new insights into transplant immunology and potentially lead to improved monitoring and therapeutic strategies for managing kidney transplant recipients.

These results suggest that the evaluation of the circulating $T_{R3\text{-}56}$ T cell subset might serve as a potential indicator of early immune-mediated processes that could potentially impact graft tolerance. Kidney transplantation represents an important therapeutic option to effectively treat end-stage renal disease [186]. In this context, despite advances in immunomodulatory approaches, immune-mediated damaging

processes continue to pose a significant challenge for allograft damage and loss. Furthermore, valuable criteria to promptly identify early immune-mediated damage in kidney transplant recipients are still lacking.

The key role of T lymphocytes, able to orchestrate the whole immune response by controlling both humoral and cytotoxic activities, has been widely demonstrated [190,191].

T cell-dependent processes have been described to depend on multiple immune regulatory networks involving different regulatory T cells subset characterized only in part by the expression of the Foxp3 transcription factor.

We investigated on the $T_{R3\text{-}56}$ subpopulation in a cohort of 53 kidney allograft recipients showing no signs of graft rejection, no infectious episodes and no changes in immunosuppressive regimen in the last 6 months. Our broad comparative immune profile of renal transplant recipients versus healthy controls revealed several peculiar features: i. increased expression of the CD54 molecule, largely associated with antigen-dependent T cell activation, by T cell effectors [217,218]; ii. increased number and percentage of cytotoxic T cells; iii. decreased amount and growth ability of the Tregs; iv. increased percentage and number of the $T_{R3\text{-}56}$ regulatory T cell subset.

The constant availability of allo-antigens, as represented by the graft, should be considered as a key feature underlying the immune scenario by us observed. Indeed, a low-grade pro-inflammatory microenvironment, characterises the transplanted organ even in the presence of an effective immune-modulatory therapy [185,186]. A significant association of $CD4^+$ and CTL activation with the reduction of the Treg subset has been widely found to underlie long-standing infections, transplantation and autoimmune diseases in human [230,231] and animal models [232,233]. In this context, the increased level of $T_{R3\text{-}56}$ in the transplanted subjects, when considering the immune-modulating role of this T cell subset and the concomitant increase in the CTL population, could underlie an attempt to restore/maintain graft immune tolerance control in the presence of defective Treg-mediated immune-suppression.

Compelling evidence indicate the growth capacity as a key feature of the Treg population [72]. Such treat has been widely associated with the needing of a

dynamic regulation of this T cell subset, specifically involved in the maintenance of a complex homeostatic balance. In this context, reduced growth ability of the Treg subset, could be likely related to early disturbance of immune tolerance control of the graft in kidney recipients. Accordingly, the presence of increasing level of activated helper T cells, the one responsible for immune orchestration [190,191] in the subjects showing highest amount of the $T_{R3\text{-}56}$ population, could represent an attempt, in subjects showing early signs of graft functional recognition, to restore transplant tolerance control, thus avoiding episodes of clinical rejection.

We found that categorization of kidney transplant recipients according to their stable graft control, evaluated by changes in creatinine levels and 24-h proteinuria over two consecutive bi-monthly analyses, revealed a significant association of higher $T_{R3\text{-}56}$ amounts with increasing CTL level and decreased growth ability of the Treg population. Furthermore, our study revealed a significant association of highest $T_{R3\text{-}56}$ levels with unstable graft control.

These observations, taken together, suggest a scenario in which early derangement of graft tolerance, in the presence of maintained kidney functional effectiveness, could be associated with increasing levels of the $T_{R3\text{-}56}$ regulatory T cell population. The concomitant immune-modulating therapy, that characterise the cohort of transplant recipients by us analysed, has to be probably related to the inability to observe inverse association of the $T_{R3\text{-}56}$ amount with the activation level of their CTL target. The observation that in kidney transplant recipients, high level of circulating $T_{R3\text{-}56}$ regulatory T cells significantly associates with decreased Treg growth ability, suggests $T_{R3\text{-}56}$ evaluation as a potential marker of early defective graft tolerance control. More extensive evaluations are needed to propose the potential use of the analysis of the circulating $T_{R3\text{-}56}$ T cell regulatory subset as a valuable indicator of graft stable control in kidney transplant recipients.

6.6. Concluding Remarks

Immune regulation involves a delicate balance between defending against pathogens and preventing excessive immune responses that could lead to autoimmune diseases or tissue damage. Key components of immune regulation mainly include Tregs, which are crucial for maintaining tolerance to self-antigens and preventing autoimmune reactions. Within this framework, subsets such as T_{R3-56} cells play specific roles under different conditions, contributing to immune regulation and homeostasis.

Immune tolerance is essential for avoiding autoimmune diseases, control the pathogen infection and ensuring successful graft acceptance. Some cells or pathogens, such as cancer cells, may evade immune surveillance by exploiting regulatory T cells to escape immune detection. Inflammation modulation is also critical for preventing chronic damage, and regulatory cells help maintain this balance. Effective immune responses require the activation of immune cells like CTLs, which are carefully regulated by various cell types to ensure appropriate responses. Regulatory cells, including Tregs and potentially T_{R3-56} cells, can serve as valuable biomarkers and therapeutic targets for conditions such as cancer, chronic infections, and transplant rejection.

This thesis presents research conducted during the doctoral period on the characterization of T_{R3-56} cells across four pathological models: MDS, CLL, COVID-19, and kidney transplantation. All these models share a common theme: the complex balance between immune activation and immune regulation.

The investigation into T_{R3-56} cells across these diverse conditions underscores their potential role in managing this balance.

Briefly, T_{R3-56} cells are implicated in the dysregulation of immune responses, contributing to disease progression in MDS. Unlike NKT and NK cells, T_{R3-56} cells are crucial in regulating CTL effector functions. Their increase with disease severity in MDS correlates with decreased CTL activation and progression, supporting their role in the immune escape of dysplastic clones.

In CLL, the expansion of T_{R3-56} cells correlates with immune evasion and disease advancement. Indeed, our findings confirm an expansion of both Treg and T_{R3-56} cells within the T-cell compartment. This suggests that these regulatory cells

contribute to immune evasion and disease progression, marking them as potential targets for new therapeutic strategies.

In the context of COVID-19, increased levels of $T_{R3\text{-}56}$ cells in severe cases correlate with higher levels of CTLs and pro-inflammatory cytokines, indicating their role in modulating excessive immune activation and inflammation.

In kidney transplant recipients, elevated $T_{R3\text{-}56}$ levels are associated with unstable graft control, suggesting that these cells could serve as early indicators of immune-mediated issues affecting graft tolerance.

These findings highlight the importance of $T_{R3\text{-}56}$ cells in regulating immune responses in various pathological contexts. By contributing to both immune activation and suppression, $T_{R3\text{-}56}$ cells appear to play a crucial role in maintaining immune homeostasis and influencing disease outcomes. Further research into their precise mechanisms and functions could provide valuable insights into developing targeted therapies for these conditions.

Our findings provide valuable insights into the multifaceted roles of $T_{R3\text{-}56}$ cells across various disease models. These cells exhibit potential regulatory abilities that extend beyond traditional immune modulation to include cytotoxic and antiviral functions. This dual functionality is particularly noteworthy, as it point to the $T_{R3\text{-}56}$ cells as versatile players within the immune system, capable of acting as effector cells under specific conditions. This concept is supported by studies on CD3+ CD56+ T cells, which also demonstrate such adaptability.

Given the complex and adaptive nature of the immune response, $T_{R3\text{-}56}$ cells are likely to exhibit remarkable plasticity, allowing them to switch between regulatory and effector roles depending on the context, such as during infections, inflammation, or within the tumour microenvironment. This potential adaptability aligns with the broader concept of immune plasticity, a critical feature of immune regulation and function.

The dynamic roles of other immune cells, such as Tregs, further illustrate this concept. Tregs, traditionally known for their suppressive functions, have also been shown to possess cytotoxic activity against tumour cells through mechanisms involving granzymes. Their interactions with non-immune cells and their roles in tissue repair and organ homeostasis highlight their multifaceted functions.

Similarly, it is plausible that $CD3^+$ $CD56^+$ T cell subtypes, including $T_{R3\text{-}56}$ cells, could adapt to different tissue environments, performing both suppressive and effector functions as needed. Understanding these dual roles could have significant implications for developing therapeutic strategies. By deepening our understanding of $T_{R3\text{-}56}$ cells and their interactions within the immune system, we may pave the way for novel approaches to treating a range of diseases.

Collectively, the findings reported in this PhD thesis reveal the complex roles of $T_{R3\text{-}56}$ cells across various diseases and their potential as biomarkers and therapeutic targets. Further research is warranted to optimize their clinical applications and enhance our ability to manage and treat these conditions effectively.

The research conducted during my PhD training was driven by a passionate and engaged ambition to contribute to the understanding of immunoregulation and its disruptions in disease mechanisms. The entire research presented in this thesis has been captivated by the way the immune system reveals its dual nature: defending us when functioning properly and attacking us when dysregulated. Understanding the "yin and yang" of immune responses has fed my research activities and enriched my spirit as an aspiring researcher.

7. Publications related to the data produced by the Doctoral candidate's research activities and included in the thesis

1. **Carriero F**, Rubino V, Leone S, Montanaro R, Brancaleone V, Ruggiero G, Terrazzano G. Regulatory TR3-56 Cells in the Complex Panorama of Immune Activation and Regulation. Cells. 2023 Dec 15;12(24):2841.
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3. Rubino V, Leone S, **Carriero F**, Pane F, Ruggiero G, Terrazzano G. "The potential etiopathogenetic role and diagnostic utility of CD3+ CD56+ regulatory T lymphocytes in Myelodysplastic Syndromes" Eur J Haematol. 2023 May;110(5):578-579.
4. Rubino V*, **Carriero F*,** Palatucci AT, Giovazzino A, Leone S, Nicolella V, Calabrò M, Montanaro R, Brancaleone V, Pane F, Chiurazzi F, Ruggiero G, Terrazzano G. "Adaptive and Innate Cytotoxic Effectors in Chronic Lymphocytic Leukaemia (CLL) Subjects with Stable Disease" Int J Mol Sci. 2023 May 31;24(11):9596. * <u>These Authors equally contributed</u>
5. **Carriero F**, Palatucci AT, Giovazzino A, Salemi F, Carrano R, Sabbatini M, Ruggiero G, Terrazzano G. Evaluation of the TR3-56 regulatory T cell subset in kidney transplant recipients. Valentina Rubino, (2024 SUBMITTED)
6. **Carriero F,** Rubino V, Gelzo M, Scalia G, Raia M, Ciccozzi M, Gentile I, Pinchera B, Castaldo G, Ruggiero G, Terrazzano G. Immune profile in COVID-19: unveiling TR3-56 cells in SARS-CoV-2 infection. (2024 SUBMITTED)

8. Other publications by the Doctoral candidate produced during the PhD course

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Review

Regulatory T_{R3-56} Cells in the Complex Panorama of Immune Activation and Regulation

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Abstract: The interplay between immune activation and immune regulation is a fundamental aspect of the functional harmony of the immune system. This delicate balance is essential to triggering correct and effective immune responses against pathogens while preventing excessive inflammation and the immunopathogenic mechanisms of autoimmunity. The knowledge of all the mechanisms involved in immune regulation is not yet definitive, and, probably, the overall picture is much broader than what has been described in the scientific literature so far. Given the plasticity of the immune system and the diversity of organisms, it is highly probable that numerous other cells and molecules are still to be ascribed to the immune regulation process. Here, we report a general overview of how immune activation and regulation interact, based on the involvement of molecules and cells specifically dedicated to these processes. In addition, we discuss the role of T_{R3-56} lymphocytes as a new cellular candidate in the immune regulation landscape.

Keywords: immune regulation; immune regulatory cell phenotypes; T_{R3-56}

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1. Introduction

The immune system protects the body from infections and maintains overall health [1–3]. This protection occurs through the activation of the immune system, which represents a complex process by which the immune response is stimulated in response to the presence of pathogens (such as viruses, bacteria, fungi, etc.), foreign substances, or abnormal cells (e.g., cancer cells) in the body [1–4]. This process is usually called "immune activation" and can involve various cells and immune molecules harmonically acting to create a defense against these threats [1–5].

Immune activation processes are expressed by the innate and adaptive immune responses of the immune system, each with distinct roles and mechanisms to defend the body against infections and other threats [1–5].

The innate immune response is rapidly acting and represents the first line of defense, thus providing immediate but relatively non-specific protection [1–6]. In fact, the innate response does not discriminate among specific pathogens but recognizes common characteristics shared by many pathogens, such as certain molecules on the surface of the pathogen [7–11].

Components of the innate immune system include physical barriers such as the skin and mucous membranes, as well as cellular and biochemical elements such as phagocytes (white blood cells that engulf and digest pathogens) [7–11] and natural killer (NK) cells (infected host or anomalous cells) [12–14].

The innate response often promotes pro-inflammatory phases and is itself triggered by inflammation, which helps in the recruitment of immune cells to the site of infection and improves the body's overall defense [15–17].

The adaptive immune response develops more slowly but is highly specific and targets particular pathogens precisely, adapting to the microenvironmental conditions during the immune response itself [1–3]. Such a response is characterized by "immunological memory", which is a fundamental feature of the adaptive immune system offering an effector advantage upon subsequent encounters with the pathogen [1–3,18,19]. In this regard, after encountering a specific pathogen, the adaptive immune system "remembers" previous encounters and responds more effectively upon subsequent exposures to the same pathogen [1–3,18,19].

The adaptive response involves specialized white blood cells called B and T lymphocytes [1–3]. Briefly, B cells are immune cells specialized in the production and secretion of antibodies, proteins specifically capable of recognizing and binding to specific antigens, expressed by pathogens such as bacteria or viruses [1–3,20,21]. This binding can either directly neutralize pathogens or flag them for elimination by other immune cells, thereby contributing to the battle against infections and the maintenance of immune balance in the body [1–3,20,21]. Moreover, T cells perform various functions [1–3,22], including assisting B cell functions (T helper, Th) [1–3,23], directly killing infected cells (cytotoxic T lymphocytes, CTL) [1–3,24], and regulating the immune response [25]. The functions of T cells are expressed in a marked versatility (or plasticity), which takes on considerable value in coordinating immune responses, adapting to different challenges, and guaranteeing an effective but controlled defense against infections and other threats linked to the control exercised by the immune system [1–3,22–25].

In a perspective, immune cells have several highly specialized roles in the body, including the identification and neutralization of threats (the effector functions of immune activation), as well as the ability to activate or inhibit the response itself (the regulatory mechanisms) [1–3,15–26].

2. The Immune Regulation

The orchestration of the immune response is a sophisticated and intricately managed process that guarantees the immune system's efficiency while preventing exaggerated or detrimental reactions [25–30]. Regulation involves an ample network of immune cells, signaling molecules, and regulatory mechanisms that work together to maintain immune balance and prevent immune-related diseases [25–30].

In this regard, both the inappropriate initiation and incorrect termination of the immune response can lead to various serious health issues, including chronic conditions, autoimmune diseases, and even cancer [31–35].

On the basis of the "danger model", originally postulated by P. Matzinger, the initiation of immune cell responses when there is no actual threat or presence of harmful pathogens (such as viruses, bacteria, fungi, etc.) in the body represents a risk for the emergence of several immune-mediated diseases [36–38]. Physiologically, once the immune system has eliminated the pathogen, it should return to its basal state without expressing functional residues that are dangerous for the health of the host organism's own components [1–9]. The continuation of an active immune response in the absence of a threat can seriously damage the molecular and cellular components of body tissues (the self) [1–9].

The inappropriate initiation and non-termination of immune effector functions, dependent on an immune regulatory failure, represents the basis for immune cells to act in an autoaggressive way in the absence of the pathogen, generating damage to healthy tissues [38–41].

2.1. The Interplay between Immune Activation and Regulation

Taking a broader perspective on the functions and organization of the immune system, the prevailing hypothesis suggests that immune responses are remarkably flexible and

adaptable [42–44]. Individual immune cells therefore possess the ability to adapt their functional capabilities over time, responding to the specific demands of their microenvironment, whether it is to trigger an active response (the effector or activation phase) [1–24] or to maintain control through immune regulation [25–30].

This intricate balancing act within the immune response evokes the concept of "immune plasticity" [45,46]. Consequently, it is reasonable to consider that disruptions in immune plasticity could serve as a major factor in the failure of both immune activation and regulation, resulting in immune system-related disorders such as immunodeficiencies [47] and autoimmune diseases [38–41].

Current research is dedicated to gaining insight into the mechanisms governing immune regulation and exploring new therapies tailored to address conditions related to the immune system [48].

2.2. The Main Features of Immune Regulation: Aspects, Molecules, and Cells

The regulation of immune responses is a complex and finely orchestrated process that involves several aspects and key mechanisms crucial to maintaining the delicate balance between an effective defense and the restoration of the state of health, avoiding harmful excessive reactions [25–48].

A peculiar feature of the immune system is the ability to distinguish between the body's own cells and tissues (self) and foreign invaders (non-self) [1–9]. Discrimination between self and non-self is critical to prevent the immune system from mistakenly attacking the body cells, which can lead to autoimmune diseases [31–33]. Self-recognition [42–44] is largely based on tolerance mechanisms [42–44,49].

The immune system has mechanisms to recognize and tolerate self-antigens, preventing the immune response from targeting and attacking the body's own cells and tissues [49]. Central tolerance occurs during the development of immune cells in the thymus (for T cells) and bone marrow (for B cells), where self-reactive cells are eliminated or rendered non-functional [30,49–54]. Peripheral tolerance mechanisms further suppress or regulate self-reactive immune cells in the periphery to prevent autoimmune reactions in tissue [49,55].

Cytokines are signaling molecules produced by immune cells that regulate the immune response [56]. They can have pro-inflammatory or anti-inflammatory properties. For example, pro-inflammatory cytokines like interleukin (IL)-1, IL-6, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) promote inflammation and immune activation [56], while anti-inflammatory cytokines like IL-10 and transforming growth factor beta (TGF-β) dampen immune responses and promote tolerance [57].

Checkpoint molecules, such as programmed cell death protein 1 (PD-1) [58] and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [59], are involved in regulating immune responses and preventing excessive immune activation [60]. They act as "brakes" on immune cells and can inhibit their activation and effector functions [60]. Targeting these checkpoint molecules has been successful in immunotherapy approaches, particularly in autoimmunity and cancer treatment [60].

The immune system employs feedback mechanisms to regulate its own activity. Various immune cells and molecules can produce inhibitory or activating signals that modulate the immune response [23–30,56–60]. These feedback mechanisms help maintain immune balance and prevent excessive or prolonged immune activation [44–46].

The local tissue environment can profoundly influence immune responses. The presence of specific molecules or cells in tissue can boost or dampen immune reactions [8–11,36–38,41–44].

All these aspects can account for the enormous value of environmental factors in determining immune plasticity and, therefore, positively or negatively influencing immune regulation [42–46].

The scientific literature has highlighted the role of numerous cells with regulatory functions of the immune response. In this sense, the aforementioned characteristics of

immune plasticity make it highly probable that immune regulation is mediated by a large and non-definitive number of cells functionally capable of being involved in immune regulation [42–60].

In this review, we will address the synthetic description of the main cells described as possessing immune regulation ability.

Regulatory T cells (Tregs) are a specialized subset of CD4+ T lymphocytes (T cells) that play a crucial role in immune regulation and maintaining immune tolerance [26,29,30,55,61,62]. They are essential in preventing excessive immune responses and controlling immune-related diseases, including autoimmune disorders, allergies, and graft rejection in transplantation [61–63]. Tregs are characterized by the expression of a transcription factor called FoxP3 (Forkhead box P3), which is considered a master regulator of their development and function [62,63]. Mutations or deficiencies in FoxP3 lead to severe autoimmune diseases [61–63], highlighting the critical role of Tregs in immune homeostasis.

Two subtypes of Tregs have been described: the natural constitutive (nTreg) [29,61–63] and the inducible (iTreg) cells [61–64]. nTregs develop in the thymus and derive from some progenitor T cells that undergo a selection process conferring them regulatory properties [29,61–63]. nTregs are characterized by specific surface markers, such as CD4 and CD25 (interleukin-2 receptor alpha chain) [29,61–63]. They have a natural ability to suppress the activation and proliferation of other immune cells, including effector T cells, which helps maintain immune homeostasis and prevent autoimmune reactions [29,61–63]. iTregs are generated in peripheral tissues, such as the gut or sites of inflammation, in response to specific environmental cues [61–64]. The iTreg subtype arises from the differentiation of conventional CD4+ T cells (non-regulatory T cells) in response to signals from the local tissue microenvironment and the presence of certain cytokines, such as transforming growth factor-beta (TGF-β) [61–64]. iTregs can tailor their regulatory functions to specific tissues [61–64].

Tregs use various mechanisms to suppress immune responses: They secrete immunosuppressive cytokines like interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β). These cytokines can suppress the activity and proliferation of other immune cells, such as T cells, B cells, and antigen-presenting cells, thereby limiting immune activation [29,61–64]; Tregs can directly interact with and suppress the function of other immune cells through cell-to-cell contact [29,61–64]. This interaction involves molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3) on the surface of Tregs, which interact with ligands on target cells, leading to the inhibition of immune responses [63–65]; Tregs can also modulate the metabolic environment to suppress immune responses. They use metabolic pathways, such as increased adenosine production or the consumption of IL-2, to create an immunosuppressive milieu that dampens immune activation [61].

Tregs are crucial for maintaining self-tolerance and preventing autoimmune diseases [29,61–65]. They recognize self-antigens and suppress the activation and function of autoreactive T cells that could potentially cause harm to the body's own tissues [29,61–65]. However, the balance between Tregs and effector T cells can be disrupted in certain conditions, leading to immune dysregulation [29,41,42,44,55,61–65]. The deficiency or dysfunction of Tregs can result in uncontrolled immune activation and the development of autoimmune diseases [29,41,42,44,55,61–65]. On the other hand, an excessive or overactive Treg response can contribute to immune suppression and hinder effective immune responses against infections or cancer [29,41,42,44,55,61–65].

Research on Tregs and their role in immune regulation is a rapidly evolving field. Several approaches to harnessing the therapeutic potential of Tregs in treating autoimmune diseases, allergies, transplant rejection, and other immune-related disorders have been evaluated [66,67]. Strategies include Treg-based cellular therapies and the modulation of Treg function and stability for therapeutic interventions [66,67].

CD8+ suppressor T cells represent a subtype of Tregs and have been described as having a unique ability to suppress immune responses, which may be useful in preventing

autoimmune reactions [68–70]. CD8+ Tregs appear to be a specialized subset of cytotoxic T cells, whose functions and mechanisms of action are still not entirely clear but play several crucial roles in immune regulation [68–70].

In the context of T lymphocytes with immunoregulatory abilities, Type 1 (Tr1) and Type 2 (Tr2) regulatory T cells are certainly worth mentioning. The Tr1 subset plays a crucial role in regulating the immune response and maintaining immune tolerance [71]. The mechanisms and functions of Tr1 cells are not fully understood [71–73]. However, the scientific literature has described that Tr1 cells predominantly produce the anti-inflammatory cytokines IL-10 [71–73]. Such cytokines suppress the activity of other immune cells, including T cells and macrophages, dampening inflammation [71–73]. The ability of Tr1 cells to regulate immune responses makes them an interesting target for potential therapeutic interventions in conditions involving immune dysregulation, such as autoimmune diseases and allergies [72,73]. In this regard, it is worth noting that Tr1 cells have been described as contributing to the immune evasion of tumors by suppressing the anti-tumor immune response [72,73]. This can be a promising challenge in cancer immunotherapy. Moreover, Tr1 cells are involved in preventing excessive allergic responses by inhibiting the activation of immune cells responsible for allergy-related inflammation [73,74]. The Tr2 subset has also been described as Th3 cells and is involved in immune regulation and suppressing inflammatory responses [75,76]. They play a crucial role in maintaining immune homeostasis by dampening excessive immune activation and preventing immune-mediated tissue damage [75,76]. Tr2 cells exert their immunosuppressive effects through the secretion of TGF-β, which has anti-inflammatory properties and can inhibit the activity of various immune cells, including T cells, B cells, and APCs [75,76]. Tr2 cells have been implicated in the regulation of immune responses in a variety of contexts, including allergic reactions, autoimmune diseases, and tissue inflammation [75,76]. The differentiation and development of Tr1 and Tr2 cells are influenced by various factors, including the cytokine environment and interactions with other immune cells [77]. They can arise from different sources, including conventional CD4+ T cells that have been exposed to specific signals, as well as from the conversion of other regulatory T cell subsets [77].

Natural killer T (NKT) cells are a unique subset of immune cells that possess both T cell and natural killer cell characteristics [78,79]. These cells express both the T cell receptor (the CD3 molecule) and the natural killer cell marker (the CD56 molecule) on their surface [78,79]. NKT cells play a critical role in the immune response by bridging the innate and adaptive immune systems [80]. They recognize a variety of lipid and glycolipid antigens presented by the non-classical major histocompatibility complex (MHC) molecule, CD1d [80,81]. Upon activation, NKT cells rapidly produce large amounts of cytokines, such as IFN-γ and IL-4, which can modulate the immune response and suppress the activation and proliferation of other immune cells, such as T cells and NKs [79,80]. Moreover, NKT cells have been found to play a role in various immune-related diseases and conditions, including infectious diseases, cancer, and autoimmune disorders [82–85]. Their functional plasticity and ability to modulate immune responses render them a promising target for immunotherapy approaches [84].

Some other cell types, with various mechanisms, have been described as capable of regulating immune responses.

In this regard, the anti-inflammatory role of regulatory B cells (Bregs) has been described [86]. Bregs represent a subset of B lymphocytes with immunosuppressive functions, mainly mediated by the production of anti-inflammatory cytokines such as IL-10, IL-35, and TGF-β [86,87]. Bregs are characterized by differential expression of CD5 and CD1d in the mouse immune system and CD24 and CD38 in the human immune system [86–88]. Some evidence suggests that Bregs are involved in infections, inflammation, and autoimmunity [86].

NK cells [1–5,12–14,89] are a vital component of the innate immune system, and although their primary role is to recognize and eliminate infected or abnormal cells, a large body of literature suggests that they also play a role in immune regulation [90–92]. NK cells

recognize and kill tissue cells that display abnormal characteristics, such as infected cells, tumor cells, or cells lacking major histocompatibility complex class I (MHC-I) molecules, based on the missing-self hypothesis [93]. NK cytotoxic function helps prevent the spread of infections and the development of tumors [89,93]. NK cells can also produce numerous cytokines that have both pro-inflammatory and immunosuppressive effects, thus contributing to immune regulation [90–93]. In addition, they also contribute to immune tolerance by shedding potentially harmful autoreactive or infected cells and sparing healthy ones [94–97]. NK cells and Tregs can interact, influencing the balance between the activation and inhibition of immune responses [98,99]. Furthermore, NK cells play a crucial role in establishing immune tolerance during pregnancy, facilitating the development of a semi-allogeneic fetus (with different genetic material) within the maternal environment and preventing its rejection [92].

Gamma delta (γδ) T cells are a subset of T lymphocytes that possess a T cell receptor (TCR) composed of γ and δ chains, in contrast to the more common α and β chains of conventional T cells [100,101]. γδ T cells are a relatively small population of T cells in the peripheral blood and have more limited diversity than αβ-TCRs, which allows them to recognize a distinct set of antigens, including non-peptide molecules [101,102]. γδ T cells are often found in tissues such as the skin, the intestinal mucosa, and the respiratory epithelium [102]. γδ T cells contribute to immune surveillance by recognizing and responding to a wide range of stress-induced or non-peptide antigens, such as those produced by infected or transformed cells [101,102]. They can also produce numerous cytokines, such as IFN-γ and TNF-α, which influence the immune response [101]. For their production and roles, they have been implicated in some autoimmune diseases, where they can contribute to inflammation and tissue damage [101].

Dendritic cells (DCs) and macrophages are key players in the immune system, and their versatility extends beyond their role as immune sentinels and scavengers and their known ability to present antigens to T lymphocytes [1–10]. Indeed, DCs and macrophages are specialized antigen-presenting cells (APCs) that have attracted attention for their intriguing immunomodulatory properties, which allow them to fine-tune immune responses based on the unique signals they encounter and the specific context of the immune challenge [1–10]. DCs can also interact with Tregs and other immune modulators to further optimize the immune response [103]. Therefore, DCs serve as central coordinators in the immune response, ensuring the body's defenses are alert against threats (immunogenic DCs) and the immune responses are contained to prevent damage to one's own tissues (tolerogenic DCs) [104]. This immunomodulation testifies to the complexity of our immune system and its ability to maintain balance in the face of different challenges. Macrophages can assume distinct functional states based on the signals they receive. They can be "classically activated" (M1) to promote inflammation and defense against pathogens or "alternatively activated" (M2) to resolve inflammation, promote tissue repair, and suppress excessive immune responses, reflecting their ability to influence immune modulation [105,106].

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous group of leukocytes with the ability to suppress immune responses [107,108]. MDSCs originate from myeloid progenitor cells [107,108]. Under certain pathological conditions, such as chronic inflammation or cancer, MDSCs can undergo expansion and become an important component of the immune cell population [107,108].

Finally, the literature highlights a pathogenetic role for some clusters of circulating cells (CIC cells) [109]. CICs express different genetic markers (see previous reference), and there is evidence that the loss of function of specific CIC populations is a contributing factor in T1D [109,110].

3. A New Cell Candidate for Immune Regulation: The $T_{R3\text{-}56}$

In 2020, we investigated the role of CD3+CD56+ regulatory T cells in the progression of type 1 diabetes (T1D) [111]. We found that individuals with T1D had a significant reduction in the number of CD3+CD56+ regulatory T cells compared to healthy individuals [111].

Such an occurrence was associated with an increase in the activation and effector functions of CD8+ T cells, which are known to contribute to the destruction of insulin-producing beta cells in the pancreas [111]. The study also demonstrated that the reduced numbers of CD3+CD56+ regulatory T cells correlated with disease progression in T1D patients. The decline in these regulatory T cells was associated with increased insulin requirements, indicating a worsening of the disease [111]. Overall, the study suggested that the loss of CD3+CD56+ regulatory T cells contributes to the progression of T1D by allowing for the activation and effector functions of CD8+ T cells. The findings highlight the importance of these regulatory T cells in maintaining immune tolerance and controlling autoimmune responses in T1D.

In this study, we also demonstrated that this CD3+CD56+ T regulatory subset [111] is different from the NKT subset [78–85]. Specifically, CD3+CD56+ regulatory cells (i) are not CD1d-restricted; (ii) do not express Valpha24/Vbeta11 chains but display a heterogeneous V-beta repertoire; and (iii) are unable to kill K562 cells in vitro. In addition, (iv) only 1–5% of CD1d-restricted T cells are positive for the CD56 molecule. We also demonstrated that this CD3+CD56+ regulatory subset is genetically, metabolically, and functionally distinct from the NKT subset [111].

We called this subset $T_{R3\text{-}56}$ [111].

In addition, we investigated the role of bone marrow $T_{R3\text{-}56}$ cells in patients with very-low-risk/low-risk myelodysplastic syndrome (MDS) [112,113] according to the Revised International Prognostic Scoring System (IPSS-R) [112,113]. MDS comprises a group of blood disorders characterized by ineffective hematopoiesis and a consistent risk of leukemia evolution [112]. We found that in patients with very-low-risk/low-risk MDS, there was an inverse association between the number of $T_{R3\text{-}56}$ cells and the activation and expansion of bone marrow cytotoxic T cells [112,113]. Such evidence suggests that $T_{R3\text{-}56}$ cells may play a role in regulating the activity of cytotoxic T cells in the bone marrow. Furthermore, the study showed that $T_{R3\text{-}56}$ cells from MDS patients exhibited a regulatory phenotype and were capable of suppressing the proliferation and activation of cytotoxic T cells [112,113]. This indicates that $T_{R3\text{-}56}$ cells may have immunosuppressive functions in the bone marrow microenvironment, as we previously described for Tregs [114]. Indeed, the imbalance between $T_{R3\text{-}56}$ cells and cytotoxic T cells in the bone marrow of very-low-risk/low-risk MDS patients may contribute to the immune-mediated elimination of healthy hematopoiesis, affecting MDS pathogenesis. On the other hand, an increased number and activity of $T_{R3\text{-}56}$ cells could contribute to the generation of an immune-suppressed microenvironment in high-risk MDS, which may contribute to the progression of acute leukemia [112,113].

Moreover, we also described the role of $T_{R3\text{-}56}$ in chronic lymphocytic leukemia (CLL) with stable disease [115]. We observed that the Treg and $T_{R3\text{-}56}$ percentages decreased when evaluated in the context of total lymphocytes. However, when specifically analyzed in the T cell compartment alone, the Treg and $T_{R3\text{-}56}$ percentages decreased in CLL subjects. Furthermore, the absolute number of circulating Treg and $T_{R3\text{-}56}$ cells is significantly higher in CLL patients than in healthy controls. Since lymphocytes are mainly composed of B cells in CLL patients, the small percentage of T cells within the lymphocyte compartment appears to exhibit a preferential expansion of the Treg and $T_{R3\text{-}56}$ regulatory cell subsets as a possible immune escape mechanism [115].

The role of $T_{R3\text{-}56}$ cells in the regulation of immune response in specific contexts such as diabetes, cancer, or MDS opens a new scenario towards the possibility of individuating possible molecular targets on these cells to tune the control that this cell subset exerts over the immune system (Figure 1).

For instance, diabetes represents a typical disease for which an effective therapy has not been precisely identified, considering that insulin administration per se does not preserve organs and tissues from the pathological consequences of a hyperglycemic environment [116–118]. Indeed, focusing on the role of $T_{R3\text{-}56}$ cells in their modulatory action over CD8+ cytotoxic lymphocytes could represent a favorable target to keep the self-destruction of pancreatic cells releasing insulin under control. The identification

of specific targets/pathways on these cells could lead to the generation of monoclonal antibodies or small synthetic molecules able to intervene in the treatment of diabetes, better controlling the disease progression and allowing for second-organ preservation. Similarly, this approach could be pursued in the fields of cancer and MDS.

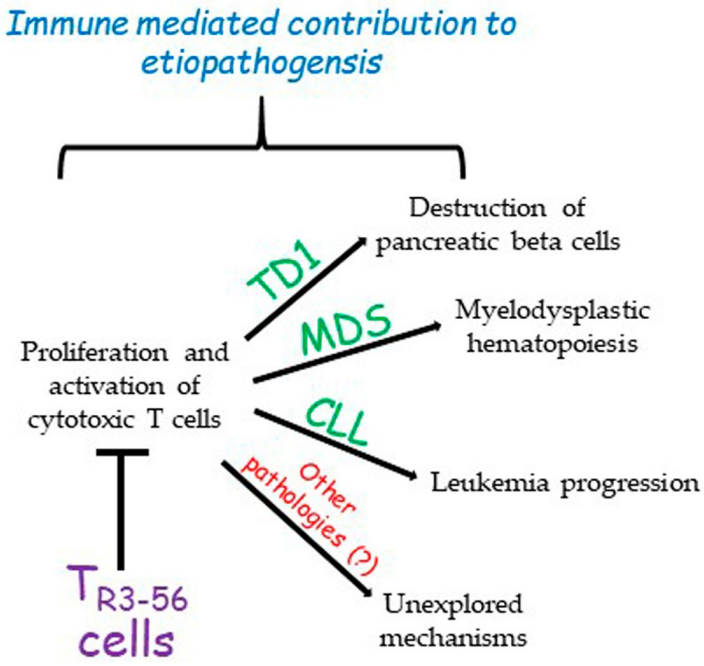

Figure 1. The described role of $T_{R3\text{-}56}$ cells. So far, $T_{R3\text{-}56}$ cells have been described as playing a role in the determinism of type 1 diabetes (TD1), myelodysplastic syndromes (MDSs), and chronic lymphocytic leukemia (CLL). However, it is possible that this regulatory cell population could be involved in other pathologies. This contribution remains to be explored.

4. Previous Observations on CD3+CD56+ Co-Expressing T Cells in Cancer Immune Surveillance

Several studies in recent decades have detected a T lymphocyte population co-expressing CD3+CD56+ molecules, often defining it as NKT-like cells, giving a confusing and non-definitive characterization of the phenotype and role of these cells. CD3+CD56+ T cells are increased in the peripheral blood of patients with solid tumors [119,120]. Such immune cells have been observed in women undergoing in vitro fertilization treatments [121]. A role for CD3+CD56+ T cells has been reported in the pathogenesis of non-alcoholic fatty liver disease [122] and in the development of allergic and autoimmune disorders [123]. Several studies have evaluated the contribution of the CD3+CD56+ T cell population in the pathophysiology and evolution of hematological malignancies: CD3+CD56+ T cell dysfunction has been hypothesized to contribute to the failure of the host immune response against leukemic blasts in acute myeloid and acute lymphocytic leukemia patients [124]; CD3+CD56+ T cells are expanded in the bone marrow of patients with chronic myeloid leukemia (CML) [125] and are decreased in CML patients treated with tyrosine kinase inhibitors [126]; and a higher proportion of CD3+CD56+ lymphocytes has been revealed in lymph nodes affected by large B cell lymphoma [127].

Overall, all these data reveal a general increase in the number of CD3+CD56+ T lymphocytes in cancer patients without addressing a possible explanation for this phenomenon.

Therefore, it is legitimate to argue that the current knowledge does not allow a definitive understanding of these cells. However, a more extensive phenotypic and functional characterization of all the lymphocyte subtypes co-expressing CD3 and CD56 represents the only approach to determining their role and possible involvement in effector and/or immune regulation mechanisms. In this regard, our original and pioneering research on $T_{R3\text{-}56}$ cells in the TD1, MDS, and CLL models revealed the phenotypic and functional characteris-

tics of this distinct subpopulation of CD3+ CD56+ T cells, highlighting its distinctiveness in immunoregulation [111–114].

Nonetheless, it is currently not possible to exclude that CD3+CD56+ cell phenotypes are more numerous or that plastic elements may influence their functions.

5. Conclusions

The immune response is ultimately the result of a balance between activation and inhibition; the success and/or failure of the immune response depends on a set of genetic/epigenetic factors and an array of molecules, cells, and tissue microenvironments involved in both activating and inhibitory mechanisms of immune regulation (Figure 2).

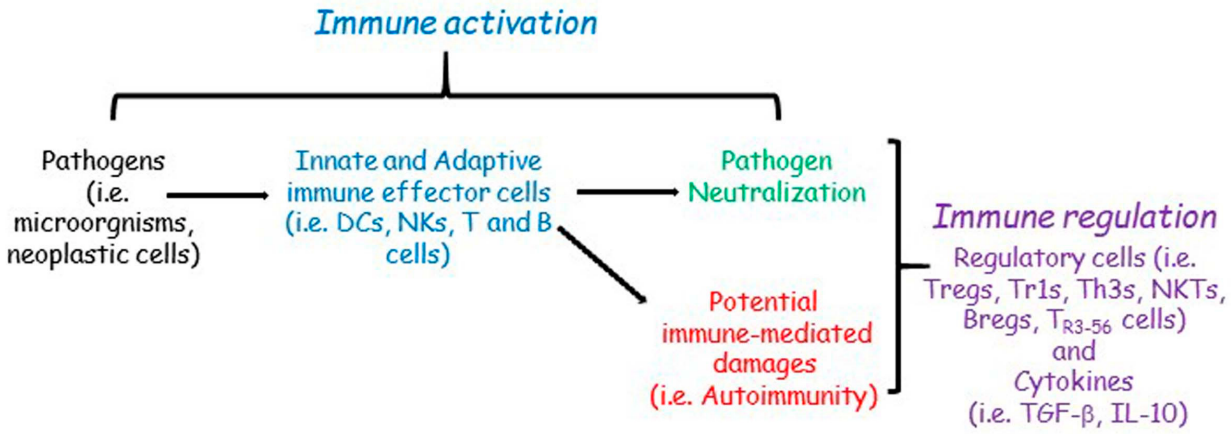

Figure 2. Simplified immune plasticity network. Pathogens activate the innate and adaptive immune effector cells (dendritic cells, DCs; natural killers, NKs; T and B cells) that induce pathogen neutralization during the immune activation phase. However, immune activation could also exert potential immune-mediated damage as a sort of side effect. The immune regulation phase (T regulatory cells, Tregs; Type 1 regulatory cells, Tr1s; T helper 3 cells, Th3s; natural killer T cells, NKTs; B regulatory cells, Bregs; T CD3+ CD56+ regulatory cells, $T_{R3\text{-}56}$ cells; transforming growth factor beta, TGF-β; interleukin 10, IL-10) modulates immune activation and avoids immune-mediated damages.

The knowledge of all the mechanisms involved in immune regulation is not yet definitive, and, probably, the overall picture is much broader than what has been described in the scientific literature so far. Given the plasticity of the immune system and the diversity of organisms, it is highly probable that numerous other cells and molecules are still to be ascribed to the immune regulation process.

Therefore, it cannot be excluded that other factors and cells other than those reported in this review should be taken into consideration to fully understand the complex harmony between the activation and inhibition of the immune system.

At the same time, it is equally probable that some current knowledge about the role of cells that have hitherto been specifically described as immunoregulatory might need to be revised. Such cells might have different, broader, and more plastic roles in the complex balance between the activation and inhibition of innate and adaptive immune responses.

In this complex framework, it appears highly compelling to propose to the scientific community the investigation of some "new cells", such as $T_{R3\text{-}56}$, in their role as immunoregulatory cell populations, contributing to deepening our knowledge of the immune system and its plastic and dynamic complexity.

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ORIGINAL ARTICLE

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Bone marrow CD3+CD56+ regulatory T lymphocytes ($T_{R3—56}$ cells) are inversely associated with activation and expansion of bone marrow cytotoxic T cells in IPSS-R very-low/low risk MDS patients

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Abstract

Background: Emergence of dysplastic haematopoietic precursor/s, cytopenia and variable leukaemia risk characterise myelodysplastic syndromes (MDS). Impaired immune-regulation, preferentially affecting cytotoxic T cells (CTL), has been largely observed in MDS. Recently, we described the $T_{R3—56}$ T cell subset, characterised by the co-expression of CD3 and CD56, as a novel immune-regulatory population, able to modulate cytotoxic functions. Here, we address the involvement of $T_{R3—56}$ cells in MDS pathogenesis/progression.

Objectives: To analyse the relationship between $T_{R3—56}$ and CTL activation/expansion in bone marrow (BM) of very-low/low-risk MDS subjects.

Methods: Peripheral blood and BM specimens, obtained at disease onset in a cohort of 58 subjects, were analysed by immune-fluorescence and flow cytometry, to preserve the complexity of the biological sample.

Results: We observed that a trend-increase of BM $T_{R3—56}$ in high/very-high MDS stage, as compared with very-low/low group, associates with a decreased activation of BM resident CTL; significant correlation of $T_{R3—56}$ with BM blasts has been also revealed. In addition, in very-low/low-risk subjects the $T_{R3—56}$ amount in BM inversely correlates with the presence of activated BM CTL showing a skewed Vβ T-cell repertoire.

Conclusions: These data add $T_{R3—56}$ to the immune-regulatory network involved in MDS pathogenesis/progression. Better knowledge of the immune-mediated processes associated with the disease might improve MDS clinical management.

KEYWORDS
bone marrow, cytotoxic T-lymphocytes, myelodysplastic syndrome, T lymphocytes regulatory

Stefania Leone and Valentina Rubino contributed equally to this study.

1 | INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of acquired haematopoietic clonal disorders characterised by ineffective haematopoiesis with peripheral cytopenia and a variable risk of leukaemia evolution.[1] Compelling evidence indicates that a mutual, dynamic interaction between the genetic alterations of the haematopoietic stem cell, an aberrant pro-inflammatory microenvironment and the immune dysregulation might underlie MDS pathogenesis.[2,3]

The involvement of autoimmune mechanisms and the activity of bystander T cells, potentially recruited by recognised dysplastic antigens and likely related to immunogenic acquired somatic mutations, have been hypothesised to be relevant for the selection/progression of dysplastic clone/s able to escape immune-mediated damaging.[4,5]

Deranged activation and clonal expansion of $CD8^+$ cytotoxic T cells (CTL) in bone marrow (BM) have been described as a key element in MDS pathogenesis.[4,6] In early MDS stages (very-low and low risk),[7] activated CTL and a pro-inflammatory milieu contribute to damage polyclonal haematopoiesis, favouring the selection of dysplastic clone/s able to escape the immune attack. At variance, an immune-suppressive environment might 'turn-off' the CTL functions and foster the dysplastic clone/s expansion/progression in late MDS stages.[8]

The occurrence of haematopoietic stem cell pyroptosis,[3] a deranged inflammatory cytokine profile,[9] an oligoclonal $CD8^+$ and $CD4^+$ T cell repertoire,[10] an ineffective tolerance control[8,11] and the association with autoimmune disorders[12,13] have been largely described in MDS subjects.

Basing on these observations, several immune-modulating strategies have been proposed in MDS clinical management. Immunosuppressive therapies (IST) have been employed in several clinical trials, resulting in a wide variable response rate (0%-66%).[14-17] The efficacy of these treatments, only in a subgroup of MDS subjects, has pointed out the need of valuable selection criteria, that, until now, are lacking.

Innovative therapeutic approaches, targeting molecules involved in immune-mediated pathways, are currently in study in several clinical trials.[18] Encouraging preliminary results are confirming that a better comprehension of the mechanisms underlying immune-dysregulation in MDS might improve not only our knowledge of MDS pathogenesis but also patient clinical management. Moreover, understanding how immune-mediated pathways participate to MDS pathogenesis/progression, will be likely relevant to identify new therapeutic targets.

Fine-tuning of immune response is usually obtained by multiple regulatory processes, all belonging to the immune tolerance network, that are in place to prevent potentially deleterious immune responses against *self*-tissues.[19,20] The key role of regulatory populations in the prevention of autoimmunity and of immune mediated diseases has been largely described.[21]

Regulatory cells represent a heterogeneous group of differentiated T cell subsets including the interleukin-10 producing T_R1,[22] the transforming growth factor-β producing T_H3[23] and the regulatory T (Treg) cells, constitutively expressing the Foxp3 transcription factor.[24]

T cells, characterised by the co-expression of CD3 and CD56, have been described in acute myeloid leukaemia (AML).[25,26] In both studies this T cell population, distinct from NKT subset[27] and expressing a αβ T cell receptor (TCR), was observed to be expanded in patients' peripheral blood (PB), compared to controls. Significant decrease of these cells has been also described in AML remission.[26]

Recently, we found[28] that co-expression of CD3 and CD56 molecules identifies the T_{R3-56} lymphocytes, as a novel human regulatory T cell subset. This cell population preferentially exerts suppressive activity on proliferation, cytotoxicity and IFN-γ production by activated human CTL. Regulatory functions of human T_{R3-56} require cell-to-cell contact and occur in both autologous and allogeneic conditions. T_{R3-56} suppressive activity involves reduction of intracellular reactive oxygen species in CTL. Perturbation in number and function of T_{R3-56} cells has been by us related to the deranged CTL function observed in type 1 diabetes.[28] The involvement of T_{R3-56} in the

TABLE 1 Patient clinical characteristics

Total number of MDS patients	58
Age	
years, median (range)	77 (40-89)
Sex, *n* (%)	
Male	36 (62)
Female	22 (38)
Blood routine, median (range; interquartile range)	
Haemoglobin, g/dl	10.25 (7.8-13.2; 9.1-12.25)
Platelet count, $\times 10^9$/L	140 (30-383; 104-197)
Neutrophil count, $\times 10^6$/L	2061 (320-7588; 1008-3190)
Bone marrow blasts, median (range; interquartile range)	
Bone marrow blasts, %	0.4 (0-18; 0-2)
Cytogenetic by IPSS-R criteria, *n* (%)	
Very good	0 (0)
Good	55 (95)
Intermediate	3 (5)
Poor	0 (0)
Very poor	0 (0)
WHO 2016 classification, *n* (%)	
MDS-SLD	26 (45)
MDS-MLD	14 (24)
MDS-RS-SLD	3 (5)
MDS-RS-MLD	6 (10)
MDS-del(5q)	3 (5)
MDS-EB1	2 (3)
MDS-EB2	4 (7)
IPSS-R classification, *n* (%)	
Very-low risk	14 (24)
Low risk	32 (55)
Intermediate risk	5 (9)
High	3 (5)
Very-high risk	4 (7)

pathophysiology of other immune-mediated disorders needs to be investigated.

Defective cell-mediated immune-regulatory pathways contribute to MDS pathogenesis.[4,10] Indeed, the increase in Treg in advanced stages of MDS, as well as the occurrence of functional defects and altered migration of this cell subset in the first phases of the disease, have been described by us and others.[29-33] Moreover, we previously showed that Treg level in BM identifies a subgroup of low-risk MDS patients characterised by lower Treg percentage and significant BM recruitment of CD8 T lymphocytes with a skewed αβ TCR repertoire.[30]

No data are available on T_{R3-56} involvement in MDS pathogenesis/progression.

This study aims to analyse T_{R3-56} cells in BM of MDS subjects. The possibility that defective control of cytotoxic effectors by T_{R3-56} subset might participate in MDS pathogenesis/evolution has been also addressed.

2 | METHODS AND MATERIALS

2.1 | Patients and controls

Fifty-eight consecutive newly diagnosed MDS patients were enrolled in the study. BM and PB sample collection, haematological investigation, cytogenetic characterisation was performed according to the World Health Organisation (WHO) recommendations. Patients were categorised according to WHO 2016 and Revised-IPSS (IPSS-R) score.[7] Fourteen were classified as very-low risk, 32 as low risk, 5 as intermediate risk, 3 as high risk, 4 as very-high risk. Detailed description of clinical characteristic of our MDS cohort is reported in Table 1. BM and PB samples were obtained during routine diagnostic procedures. For TCR repertoire analysis 10 healthy donors, sex/age matched with MDS subjects, were enrolled in the study.[31] Moreover, to identify the occurrence of BM preferential T cell expansions, BM and PB Vβ TCR repertoire analysis was performed in 5 very-low and 16 low risk MDS patients, as previously described.[31]

Informed consent was obtained from each individual before PB and BM sample collection. Study was approved by the local Ethical Committee. None of the recruited patients was receiving medical treatments that could have an impact on their immune condition. Enrolled patients were not affected by immune-mediated diseases and acute or chronic viral infections.

2.2 | mAb, immunofluorescence and flow cytometry

FITC, PE, PEcy5, PEcy7 and APC labelled mAb against CD3, CD4, CD8, CD56, CD25, CD45, CD54, PE anti-γδ TCR and isotype-matched controls were purchased from BD PharMingen. Anti-Vβ14, anti-Vβ12, anti-Vβ7.2, anti-Vβ20, anti-Vβ18, anti-Vβ7.1, anti-Vβ22, anti-Vβ13.2, anti-Vβ1, anti-Vβ17, anti-Vβ5.3, anti-Vβ5.1, anti-Vβ23, anti-Vβ4, anti-Vβ2, anti-Vβ13.1, anti-Vβ5.2, anti-Vβ8, anti-Vβ9, anti-Vβ11, anti-Vβ3, anti-Vβ13.6, anti-Vβ21.3F, anti-Vβ16, anti-TCR mAbs were from Beckman-Coulter. PE-labelled CD1d tetramer loaded with alpha-galactosyl-ceramide and PE-labelled CD1d negative control tetramer were from ProImmune. T_{R3-56} lymphocytes have been identified by co-staining with anti-human CD3 and anti-human CD56 mAb as described[28]; this T cell subset is distinct from NKT cells and preferentially expresses a heterogeneous TCR αβ repertoire (Figure S1), as previously described.[28] Moreover, in our cohort we observed that T_{R3-56} cells preferentially expressed CD8 co-receptor. Indeed, less than 30% of the T_{R3-56} lymphocytes showed CD4 co-receptor, while less than 3% were negative for both CD4 and CD8 molecules (not shown). All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using FSC and SSC parameters, as well as CD45 labelling. Flow cytometry and data analysis were performed by a two-laser equipped FACScalibur apparatus and the CellQuest analysis software (Becton Dickinson). For the comparative analysis of CD54 expression on BM CTL, immune-fluorescence data were expressed as ratio of mean intensity fluorescence (MIF) value for the CD8 population and the control MIF value obtained after staining the same cell population with the isotype control mAb, as described.[31] To define CD4 and CD8 TCR skewing, we considered the occurrence of a percentage of expression exceeding of three standard deviation that observed, for each Vβ family analysed, in 10 healthy controls sex/age matched with the MSD cohort, as described.[6,31] Occurrence of a skewed BM CD4 and CD8 repertoire with an expression frequency higher that 20% respect to PB was considered as BM preferential skewing, as described.[31] This approach might be useful in order to identify T cell clone expansions potentially associated with the recognition of BM antigens likely relevant for MDS pathogenesis/progression.

2.3 | Statistical analysis

Statistical evaluation of data, by *InStat 3.0* software (GraphPad Software Inc.), was performed by *Mann-Whitney* or *Spearman's* correlation test. Two-sided *p* values less than .05 were considered significant.

3 | RESULTS

3.1 | T_{R3-56} lymphocytes, activated CTL and blasts in BM of MDS subjects

To address the role of T_{R3-56} subset in MDS pathogenesis/progression, we first analysed their BM level. In addition, we evaluated the activation of BM CTL by their CD54 expression[30]; the correlation of BM T_{R3-56} lymphocytes with BM blast number has been also analysed. As shown in Figure 1 A, an increasing-trend of BM T_{R3-56} percentage was observed from very-low/low risk to high/very-high risk group (5.1 ± 0.39; median 4.65; interquartile range [IQR] 3.43-6.69 in

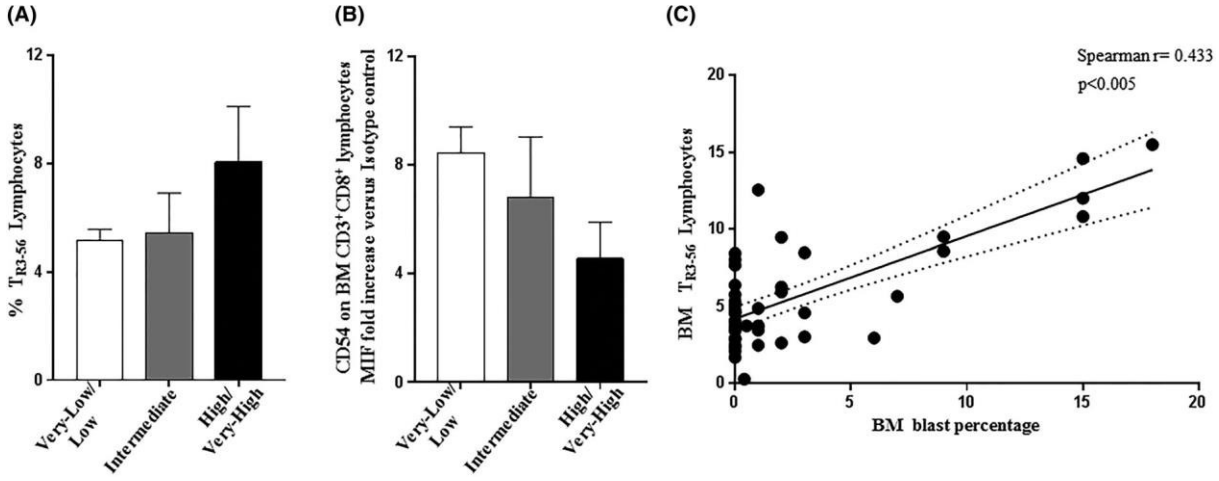
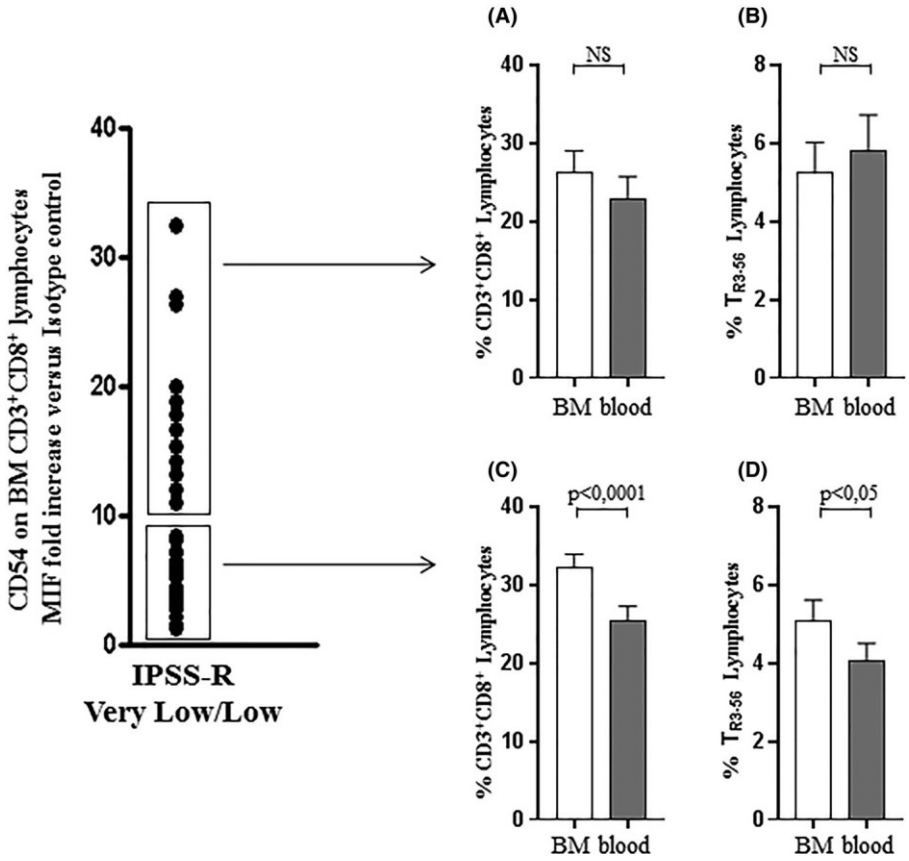


FIGURE 1 BM T_{R3-56} lymphocyte percentage positively correlates with BM blast number and seems to be inversely associated with BM CD54 expression on BM CTL in MDS subjects. (A) White, grey and black columns indicate BM percentage of T_{R3-56} cell subset in very-low/low risk (N 46), intermediate risk (N 5) and high/very-high risk (N 7) MDS patients, respectively. (B) White, grey and black columns indicate CD54 expression on BM CTL in very-low/low risk (N 46), intermediate risk (N 5) and high/very-high risk (N 7) MDS patients, respectively. As shown, an increasing trend of BM T_{R3-56} percentage accompanied by a decreasing trend for CD54 expression on CTL is observed from very-low/low risk to high/very-high risk group. (C) Spearman's evaluation of correlation between BM T_{R3-56} cell subset percentage and BM blast in MDS subjects. As shown, a significant ($p < .005$) positive correlation ($r = .433$) has been revealed

FIGURE 2 T_{R3-56} lymphocyte percentage in BM of very-low/low risk MDS patients inversely correlates with CD54 expression on BM CTL. Left part of the figure shows very-low/low risk MDS patients grouping according to CD54 expression on BM CTL, as previously described.[30] (A–D) Analysis of BM versus blood $CD3^+CD8^+$ and T_{R3-56} in the very-low/low risk MDS patients, grouped according to their CD54 expression on BM CTL. White and grey columns indicate BM and PB percentage, respectively. (A and B) Analysis of BM versus blood CTL distribution in the subjects categorised according their CD54 expression on BM CTL; (C and D) analysis of BM versus blood T_{R3-56} distribution in the subjects categorised according their CD54 expression on BM CTL. As shown, significant BM recruitment of CTL ($p < .0001$) and T_{R3-56} ($p < .05$) lymphocytes has been observed only in the subjects with lower CD54 expression on BM CTL



very-low/low risk; 5.44 ± 1.4; median 3.67; IQR 3.56–9.46 in intermediate risk; 8.22 ± 1.8; median 4.58; IQR 3.69–14.58 in high/very-high risk). In addition, a decreasing-trend has been shown for CD54 expression from very-low/low risk to high/very-high risk group (8.45 ± 0.9 in very-low/low risk; 6.81 ± 2.2 in intermediate risk; 4.65 ± 1.3 in high/very-high risk) (Figure 1B).

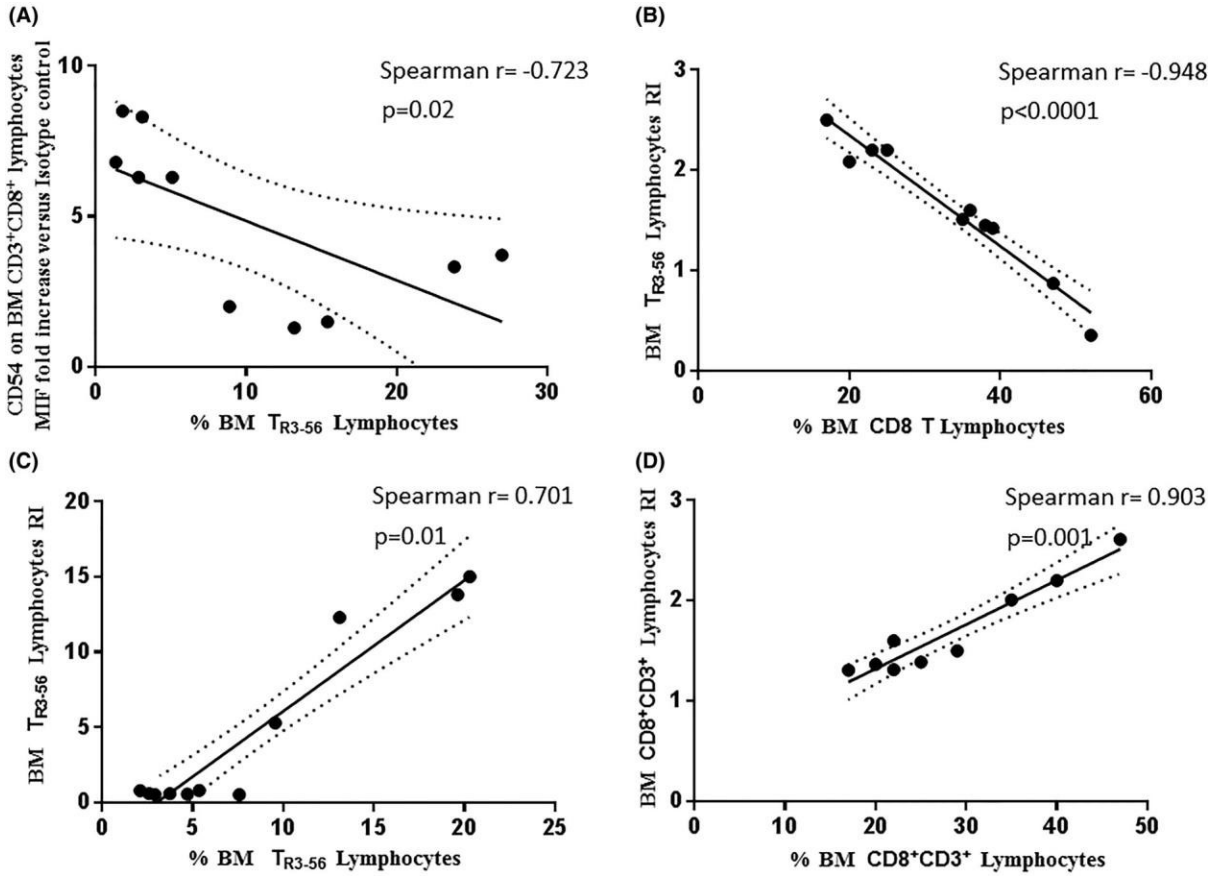


FIGURE 3 Spearman's correlation analysis of BM $T_{R3—56}$ lymphocytes, BM CTL percentage and activation in very-low/low risk MDS patients categorised according to the presence of a preferential BM T cell skewed Vβ repertoire. (A) Spearman correlation analysis of CD54 expression on BM CTL versus BM $T_{R3—56}$ percentage in MDS subjects with ≥2 CTL Vβ skewed clones in BM; significant negative correlation (Spearman $r = —.723$; $p < .05$) is shown. (B) Spearman correlation analysis of BM $T_{R3—56}$ RI (ratio between the percentage of T_{R3-56} lymphocytes in BM vs. their percentage in PB) versus BM CTL percentage in MDS subjects with ≥2 CTL Vβ skewed clones in BM; significant negative correlation (Spearman $r = —.948$; $p < .0001$) is revealed. (C) Spearman correlation analysis of BM $T_{R3—56}$ vs BM T_{R3-56} percentage in MDS subjects with <2 CTL Vβ skewed clones in BM; significant positive correlation (Spearman $r = .701$; $p < .05$) is observed. (D) Spearman correlation analysis of BM CTL RI versus BM CTL percentage in MDS subjects with ≥2 $CD4^+$ Vβ skewed clones in BM; significant positive correlation (Spearman $r = .903$; $p < .005$) is shown

Expression of CD54 on CTL is thinly regulated through the TCR complex and has been largely considered to be directly involved in antigen-dependent CTL activation processes.[32,33] Thus, might represent a valuable marker of antigen dependent CTL activation in BM of MDS patients.

Late stages of MDS have been associated with the occurrence of immune-suppression mechanisms. In order to analyse $T_{R3—56}$ potential role in mediating immune-modulation in BM microenvironment we analysed, by Spearman's assay, the relationship of $T_{R3—56}$ with BM blast number in our cohort. As shown in Figure 1C, a significant positive correlation ($r = .433$; $p < .005$) has been revealed.

Since immune-mediated mechanisms are relevant to the pathogenesis of MDS in the early stages of the disease,[4,6-10] we focused on very-low/low risk MDS patients. We previously described that CD54 expression level on BM CTL may identify two MDS subgroups showing high (≥10 MIF fold increase versus isotype control) or low (<10 MIF fold increase versus isotype control) CD54 expression on BM CTL.[30] Therefore, we analysed BM immune profile in very-low/low risk MDS subjects categorised according to CD54 expression on BM CTL. As shown in Panels B and D of Figure 2, lower CD54 expression in BM CTL associates with increased BM CTL (33.32 ± 2.03% in BM vs. 25.38 ± 1.88% in PB; $p < .0001$) and with higher BM $T_{R3—56}$ (5.07 ± 0.54% in BM vs. 4.05 ± 0.46% in PB; $p < .05$). No significant differences were observed in the BM CTL and $T_{R3—56}$, nor in PB distribution in subjects showing higher CD54 expression on BM CTL (Panels A and C of Figure 2).

These data add $T_{R3—56}$ subset to the immune-regulatory network potentially involved in MDS pathogenesis/progression mechanisms. Indeed, a $T_{R3—56}$-dependent control of CTL activation in BM of MDS patients might be hypothesised.

3.2 | $T_{R3—56}$ lymphocytes and CTL skewed T cell repertoire in BM of very-low/low risk MDS subjects

The involvement of Treg subset in the control of T cell expansion in BM of low risk MDS subjects has been by us described.[31] To evaluate

whether T_{R3-56} subset might also participate in the control of BM T cell clonal expansion, we analysed PB and BM TCR repertoire in very-low/low risk MDS patients. This approach allowed the comparative evaluation of TCR repertoire in BM microsite versus PB and the identification of T cell clonal expansions, potentially related to the recognition of BM antigens.

Very-low/low risk MDS individuals were divided in two groups: those with <2 TCR preferential BM Vβ skewing versus those showing ≥2 TCR preferential BM Vβ skewing in CTL repertoire, as previously described.[31] As shown in Figure 3A, a significant inverse correlation (Spearman $r = -.723$; $p < .05$) between BM CTL CD54 expression and BM T_{R3-56} percentage has been observed.

Immune response is a microsite process involving the recognition of specific antigens by resident and/or microsite recruited immune effectors. Thus, the frequency of immune cells, specifically localised in BM respect to PB, might represent a valuable tool to analyse the mechanisms underlying the immune-activation events occurring in BM. With this aim, we analysed the ratio between the percentage of lymphocytes, belonging to the CTL and/or the T_{R3-56} subset, and the percentage of the same cell population in the PB (recruitment index [RI]). In this context, a ratio >1 indicates the preferential BM recruitment from PB of each immune subset.

We show (Figure 3B) a strong negative correlation between BM T_{R3-56} RI and BM CTL percentage (Spearman $r = -.948$; $p < .0001$) in the very-low/low risk MDS subjects showing ≥2 preferential TCR Vβ expansions in BM $CD8^+$ T cells. At variance, (Figure 3C) a significant correlation (Spearman $r = .701$; $p < .05$) between BM T_{R3-56} RI and their BM percentage was observed in the very-low/low risk individuals with <2 preferential BM TCR Vβ skewing in $CD8^+$ T cells.

Subjects with ≥2 preferential BM TCR Vβ expansions in $CD4^+$ T cells (Figure 3D) showed a positive correlation between BM CTL RI and their BM percentage (Spearman $r = .903$; $p < .005$). No significant correlations were observed in the very-low/low risk subjects characterised by the presence of <2 skewed TCR Vβ families in BM $CD4^+$ T cells (not shown).

These data inversely associate BM T_{R3-56} level with BM CTL expansion and activation in our cohort. In addition, a correlation between $CD4^+$ T cell skewing and $CD8^+$ BM percentage, has been also revealed in very-low/low risk MDS subjects with ≥2 TCR Vβ expansions in BM $CD4^+$ T cells.

4 | DISCUSSION

Deranged pro-inflammatory immune response in BM has been largely demonstrated to characterise MDS pathogenesis/progression.[2-5,9,13] The role of a defective immune-tolerance, that preferentially affect CTL response, has been observed to underlie BM failure in MDS, while a BM immunosuppressive milieu is a hallmark of MDS late stages.[4,6,8,10,11,19,20,34]

We recently described T_{R3-56} lymphocytes as a regulatory T cell subset, distinct from NKT and NK population, expressing a αβ TCR and specifically involved in the regulation of CTL effector function.[28] The increase of a T cell population, characterised by the co-expression of CD3 and CD56, has been previously described in AML.[25,26] Notably, significant modulation of these cells has been observed in AML remission.[26]

To address the role of T_{R3-56} in MDS pathogenesis/progression, we analysed their level in PB and BM of 58 MDS patients. An increase-trend for T_{R3-56} was revealed in MDS cohort from very-low/low to high/very-high risk stage of the disease. This scenario is accompanied by a progressively reduced BM CTL activation (analysed by CD54 expression) from very-low/low risk to high/very-high MDS patients. In this context, the absence of statistical significance could be related to the low number of patients in the high/very-high risk stage, compared to the very-low/low risk group. Notably, a significant positive correlation of T_{R3-56} with BM blasts has been revealed.

These findings are conceivable with the involvement of T_{R3-56} cells in MDS pathogenesis/progression, as proposed for the Treg subset[8,29-31,34,35] and it is in line with previous data obtained in AML.[25,26] Indeed, the possible role of T_{R3-56} in favouring dysplastic/leukemic clone immune-escape, might be hypothesised.

Immune-mediated mechanisms have been extensively described as relevant for MDS pathogenesis in the first stages of the disease.[4,6-10,13,29-31] The significant inverse relationship between BM T_{R3-56} amount and BM CTL activation and expansion, observed in very-low/low risk MDS subjects, suggests the possible participation of a defective control of CTL effectors by T_{R3-56} subset to the immune-mediated mechanisms involved in the emergence of dysplastic clones, as proposed for the Treg subset.[29-31]

We previously observed that BM Treg inversely correlates with BM recruitment of CTL showing a skewed TCR Vβ repertoire in low risk MDS subjects.[31] Here, we describe a significant inverse correlation of BM T_{R3-56} and the activation of BM CTL in very-low/low risk individuals. These data add T_{R3-56} lymphocytes to the regulatory cell-mediated network controlling CTL activation, as we recently described in type 1 diabetes.[28] Moreover, we observed a strong inverse correlation between the presence of a BM skewed CD8 T cell repertoire and the amount and BM T_{R3-56} recruitment. Thus, the participation of T_{R3-56} subset to the control of activation and antigen-dependent expansion of CD8 T cells in BM of very-low/low risk MDS subjects might be hypothesised. The lack of significant correlation of T_{R3-56} subset, observed in MDS very-low/low risk subjects showing a skewed $CD4^+$ T cell repertoire, is conceivable with the preferential involvement of this regulatory cell subset in the control of CTL activity, as by us previously described.[28]

The role of deranged CTL in damaging physiological polyclonal haematopoiesis has been largely described.[4,6,10-13] This study adds T_{R3-56} subset to the complex cell-mediated regulatory network involved in the control of adaptive immune response in MDS.

We found[28] that T_{R3-56}/CTL direct contact is able to mediate significant alteration of redox balance in CTL. This effect has been observed to modulate antigen-dependent effector function of human CTL, probably interfering with cytoskeleton rearrangement processes relevant for CTL activity. The strong correlation by us observed between BM T_{R3-56} cells and resident CTL activation and expansion

suggests the relevance of cell to cell contact for T_{R3-56} mediated regulatory function in MDS. These observations support the possibility that the mechanisms, by us previously observed to mediate T_{R3-56} dependent modulation of CTL function, might be also relevant in MDS model.

Further studies are necessary to elucidate the mechanisms below T_{R3-56} contribution to immune dysregulation in MDS. Better knowledge of immune-regulatory pathways in BM microsite may provide new insights into the complex scenario underlying MDS pathogenesis and/or progression, also allowing the proposal of additional molecular targets for innovative therapeutic approaches.

AUTHOR CONTRIBUTIONS

Stefania Leone and Valentina Rubino performed most of the experiments and data analyses and contributed to write the paper. Anna Teresa Palatucci, Angela Giovazzino and Flavia Carriero performed the experiments and data analyses. Stefania Leone, Giuseppe Cerciello and Fabrizio Pane participated in the clinical management of the patients. Giuseppina Ruggiero and Giuseppe Terrazzano designed the research study, analysed the data and wrote the paper.

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CONFLICT OF INTEREST

The author declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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RES PONSE

European Journal of Haematology WILEY

The potential etiopathogenetic role and diagnostic utility of $CD3^+CD56^+$ regulatory T lymphocytes in Myelodysplastic Syndromes

Novelty statements

What is the new aspect of your work?

The confirmation, by an independent study, of the key role of $CD3^+CD56^+$ regulatory T cells in MDS pathogenesis/progression.

What is the central finding of your work?

The relevance of a deranged CTL control in BM, by defective $CD3^+CD56^+$ regulatory T cells, for MDS pathogenesis/progression.

What is (or could be) the specific clinical relevance of your work?

Possibility to consider $T_{R3\text{-}56}$ cells as an additional diagnostic/prognostic marker of MDS.

To the Editor,

Serio et al.[1] show a significant reduction of $CD3^+CD56^+$ regulatory T cells ($T_{R3\text{-}56}$) in bone marrow (BM) of low-risk myelodysplastic subjects, as compared with the high-risk and the AML group; in addition, the BM frequency of mature granulocytes, a recognised marker of residual effective haematopoiesis, was observed to inversely correlate with $T_{R3\text{-}56}$ in the MDS cohort. Such data are of great interest and confirm and extend, in an independent MDS cohort, the trend-increase of BM $T_{R3\text{-}56}$ from very low/low risk to high/very high risk MDS and the inverse correlation with the cytotoxic T-cell (CTL) activity, likely fostering the escape of leukaemic blasts to immune-surveillance, by us recently described.[2] Authors also observed BM $T_{R3\text{-}56}$ frequency as negatively correlated with WT1 expression in AML, but not in MDS patients. Moreover, analysis of $T_{R3\text{-}56}$ frequency after treatments in MDS patients, showed a persistent increase of this cell subset, regardless of therapy. Serio et al. conclude that BM $T_{R3\text{-}56}$ frequency could be a flow-cytometry marker for MDS diagnosis, since consistently increased in high-risk MDS and AML, highlighting the role for this cell subset in promoting expansion of the dysplastic precursor/s with consequent ineffective myelopoiesis.[1]

Serio et al. data support the etiopathogenetic role of $T_{R3\text{-}56}$ in MDS and the possible use of this population as diagnostic/prognostic marker of the disease. We previously suggested that type 1 diabetes (T1D) progression is associated with the loss of $T_{R3\text{-}56}$-dependent control of CTL effector functions[3] and, as commented by Serio et al., we highlighted the trend-increase of BM $T_{R3\text{-}56}$ cells, with concomitant reduction of cytotoxic T-cell activity in MDS.[2] The cytofluorimetric approach allows, in MDS, the evaluation of immune profile, in BM, the microsite in which deranged precursor/s maturation takes place; thus, the possibility that the analysis of $T_{R3\text{-}56}$ might contribute to improve homogeneity of the diagnostic framing for MDS patients in whom an immune-mediated etiopathogenesis is conceivable, has to be also considered.[4-7]

Deranged activation and clonal expansion of the BM CTL represent a key element in the MDS pathogenesis.[2,4-7] In very-low/low-risk MDS patients, activated CTL and a pro-inflammatory environment contribute to impairment of polyclonal haematopoiesis, fostering the selection of dysplastic clones and their escape from immune control. Conversely, an immunosuppressive environment could disable CTL functions and favour the expansion/progression of dysplastic clones in the advanced stages of MDS.[2,4-7] In very-low/low-risk MDS subjects, the inverse correlation between BM $T_{R3\text{-}56}$ amount and BM CTL activation and expansion[2] suggest the possible participation of defective control of CTL effectors by the $T_{R3\text{-}56}$ subset in immune-mediated mechanisms involved in the emergence of dysplastic clones, as proposed for the Treg subset.[5] Mature granulocytes are significantly reduced in high-risk compared to low-risk MDS. Thus, the observation that the BM mature granulocytes frequency is inversely correlated with BM $T_{R3\text{-}56}$,[1] intriguingly draws attention to the role of this regulatory subset in the etiopathogenesis and diagnosis of MDS.

AUTHOR CONTRIBUTIONS

All the authors wrote the manuscript and provided substantial contribution to the manuscript review and final approval.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Article

Adaptive and Innate Cytotoxic Effectors in Chronic Lymphocytic Leukaemia (CLL) Subjects with Stable Disease

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Abstract: Chronic lymphocytic leukaemia (CLL) is characterised by the expansion of a neoplastic mature B cell clone. CLL clinical outcome is very heterogeneous, with some subjects never requiring therapy and some showing an aggressive disease. Genetic and epigenetic alterations and pro-inflammatory microenvironment influence CLL progression and prognosis. The involvement of immune-mediated mechanisms in CLL control needs to be investigated. We analyse the activation profile of innate and adaptive cytotoxic immune effectors in a cohort of 26 CLL patients with stable disease, as key elements for immune-mediated control of cancer progression. We observed an increase in CD54 expression and interferon (IFN)-γ production by cytotoxic T cells (CTL). CTL ability to recognise tumour-targets depends on human leukocyte antigens (HLA)-class I expression. We observed a decreased expression of HLA-A and HLA-BC on B cells of CLL subjects, associated with a significant reduction in intracellular calnexin that is relevant for HLA surface expression. Natural killer (NK) cells and CTL from CLL subjects show an increased expression of the activating receptor KIR2DS2 and a reduction of 3DL1 and NKG2A inhibiting molecules. Therefore, an activation profile characterises CTL and NK cells of CLL subjects with stable disease. This profile is conceivable with the functional involvement of cytotoxic effectors in CLL control.

Keywords: chronic lymphocytic leukaemia; HLA class I molecules; cytotoxic T cells; NK cells; immune activation profile

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1. Introduction

Chronic lymphocytic leukaemia (CLL), the most common leukaemia in adulthood, is characterised by the expansion of mature B cells expressing CD5, CD19 and CD23 [1–3]. The clinical outcome of CLL is very heterogeneous. In this context, early stage asymptomatic patients show partial long-term benefits or no therapeutic effect from early drug treatment, while patients with advanced disease necessarily require first-line therapy to limit disease progression [1–3]. Several alterations, such as p53 mutation, immunoglobulin heavy-chain variable gene (IGHV) mutational status, cytogenetic and epigenetic modification [4–6], as well as micro-environmental stimulation [7], have been proposed as predictive and/or diagnostic biomarkers of the disease. However, the need for additional disease markers is recognised as

essential to improve the diagnostic and predictive criteria of CLL disease. Regarding therapies, the recent success of immunotherapy in refractory/relapsed CLL [8] highlights the possibility of effectively manipulating immune effectors to target leukaemia clones.

In this context, the study of the immune profile of subjects characterised by clinically stable CLL could provide interesting data on the possible balance between the immune response and disease progression. Although the mechanisms underlying the immune-mediated control of tumour cells in human models are still largely undefined, the involvement of natural killer cells (NK), cytotoxic $CD8^+$ T lymphocytes (CTL) and Interferon (IFN)-γ dependent pathways in the control of tumour progression have been widely recognised [9–18].

The presence of a functional T-cell compartment and the occurrence of multiple defects, such as impaired proliferation, cytotoxicity and inability to form effective immune-synapses, have been reported in subjects with CLL [19–22]. Moreover, the analysis of NK cell activity revealed defects in lysis, including antibody-dependent cellular cytotoxicity (ADCC), but also unaffected NK functions in CLL [23–27]. Therefore, the role of these innate cytotoxic effectors and the involvement of their receptor repertoire need to be further characterised in CLL.

In this respect, the ability of NK effectors to recognise and kill susceptible cell targets depends on a complex signalling network involving a recognition repertoire, which physiologically includes both activating and inhibiting of NK receptors [28–30]. T-cell activation requires the recognition of antigenic peptides within human leukocyte antigen (HLA)-I molecules expressed on the membrane of target cells [31,32]. The existence of an association between the tumour progression and the selection of HLA-I molecules defective clones has been described in several cancers [33–35]. In this context, the possibility that altered HLA-I expression might affect single allele/loci of the HLA complex has been largely proposed [33–36]. Antigen presentation via HLA-I molecules depends on a complex intracellular molecular network of the antigen processing machinery (APM), physiologically involved in the correct assembly of the HLA-I molecular complex and in the loading of the peptide into the HLA-I groove [36–38]. The involvement of HLA-I molecules in the immune escape mechanisms of CLL appears to correlate with a prevalent downregulation of HLA-C molecules, as well as a defective expression of HLA-A and HLA-B antigens [39]. Furthermore, in very advanced CLL requiring transplantation, individuals homozygous for one or more HLA-I loci have a worse prognosis than heterozygotes [40]. Therefore, a more complete evaluation of both the expression of HLA-I molecules and the correct functional architecture of the APM need to be further investigated in the CCL, as key elements for immune-mediated control of leukaemia progression.

Immune response is a complex phenomenon aimed to ensure protection against pathogens, also maintaining tissue homeostasis [41,42]. Cell-mediated control of immune effectors are usually dependent on a heterogeneous group of differentiated cell subsets including the $FoxP3^+$ $CD4^+$ $CD25^+$ regulatory T cells (Treg) [43–46]), the interleukin (IL)-10 producing T_{R1} [47,48], the transforming growth factor (TGF)-β producing T_{H3} [49] lymphocytes.

We recently described that $T_{R3\text{-}56}$, co-expressing CD3 and CD56 molecules, represents a peculiar human regulatory T-cell subset, phenotypically, metabolically and genetically distinct from NKT subset [50,51]. $T_{R3\text{-}56}$ subset is preferentially involved in the control of cytotoxic activity and in the production of IFN-γ by cytotoxic T cells [5,51]. $T_{R3\text{-}56}$ cells are significantly reduced in type 1 diabetes (T1D) at disease onset [51] and are inversely correlated with the presence of activated cytotoxic T lymphocytes with a skewed Vβ T-cell repertoire in the bone marrow (BM) of myelodysplastic subjects [52].

Here, we address the analysis of cytotoxic effectors, belonging to both innate and adaptive compartment, as well as of HLA-I expression by B cell clones, in CLL subjects with stable disease.

2. Results

2.1. Circulating T Cells, NK, Treg and T_{R3-56} in CLL Patients

We analysed the number and percentage of circulating T and NK lymphocytes in CLL subjects showing stable disease, as compared with the sex-age matched healthy subject group.

According to the literature [19–27], we observed that the reduction in T (18.54 ± 2.45 vs. 74.64 ± 0.67 in controls; $p < 0.0001$) and NK (3.90 ± 0.58 vs. 9.48 ± 0.45 in controls; $p < 0.0001$) lymphocyte percentages (Figure 1A,C, respectively) is accompanied by an increase in the absolute number of T (2268 ± 225.4 vs. 1456 ± 38.58 in controls; $p < 0.0001$) and NK cells (379.7 ± 44.94 vs. 197.5 ± 5.23 in controls; $p < 0.0001$) in CLL subjects (Figure 1B,D, respectively). Furthermore, we observed a significant decrease in the CD4/CD8 T-cell ratio in CLL patients when compared to healthy subjects (2.21 ± 0.42 vs. 2.53 ± 0.09 in controls; $p < 0.001$) (Figure 1E).

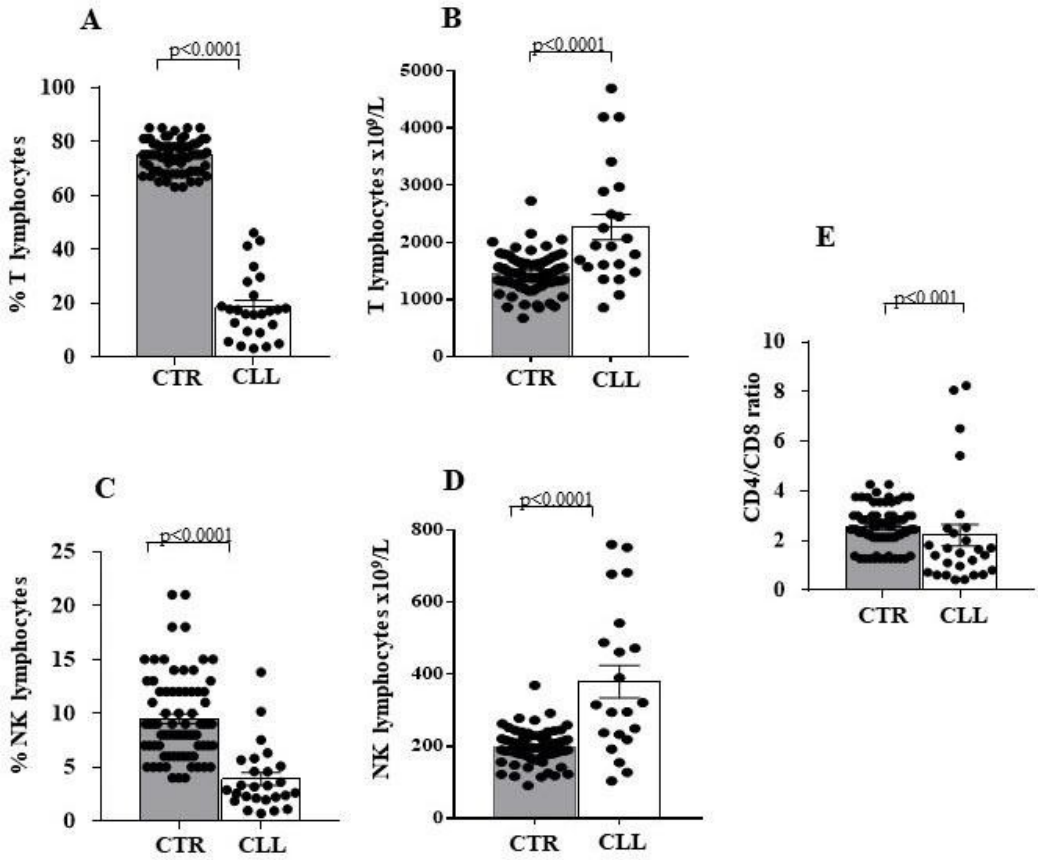

Figure 1. Significant decrease in the percentage, associated with increased number characterises circulating T and natural killer (NK) cells of chronic lymphocytic leukaemia (CLL) subjects with stable disease. Panels (**A**,**C**) indicate the percentage of circulating T and NK lymphocytes; panels (**B**,**D**) indicate the number of T and NK cells in peripheral blood; panel (**E**) shows the CD4/CD8 T-cell ratio of circulating lymphocytes. Comparative analysis of LLC and healthy controls shows decreased percentage of circulating T and NK effectors associated with a significant increase in their number. Grey and white columns indicate data obtained in healthy controls (CTR in *x* axis) and CLL individuals (CLL in *x* axis), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Statistical significance values are indicated. The applied flow cytometry gating strategy is reported in Section 4.2.

In addition, we evaluated the presence of both Treg [43–45] and $T_{R3\text{-}56}$ [51,52] in the cohort of CCL subjects. As shown, Treg percentage is reduced in CLL subjects when compared to healthy controls (1.31 ± 0.17 vs. 2.43 ± 0.18 in healthy controls; $p < 0.0001$) (Figure 2A,B), while the Treg absolute number (Figure 2B) is increased (177.2 ± 25.8 vs. 21.17 ± 1.01 in healthy controls; $p < 0.0001$). In contrast, the percentage of the $T_{R3\text{-}56}$ population shows no significant difference in the two groups (3.11 ± 0.47 vs. 3.91 ± 0.39 in healthy controls) (Figure 2C). In addition, a greater number of circulating $T_{R3\text{-}56}$ cells was observed in subjects with CLL (341.5 ± 61.99 vs. 91.31 ± 11.58 in healthy controls; $p < 0.001$) (Figure 2D). Moreover, the level of circulating Treg and $T_{R3\text{-}56}$ cells directly correlates to the CLL cohort (Spearman r = 0.625; $p < 0.005$) (Figure 2E).

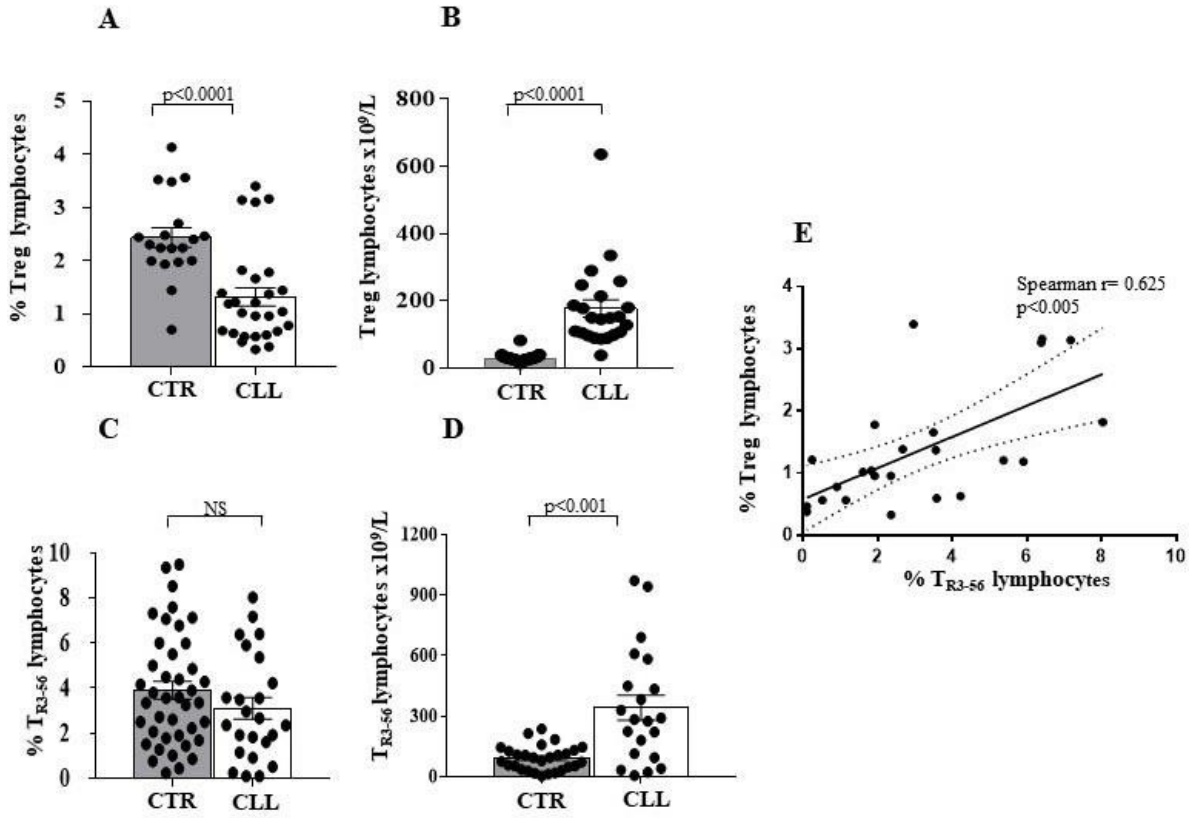

Figure 2. Analysis of circulating $CD4^+CD25^+$ (Treg) and $CD3^+CD56^+$ ($T_{R3\text{-}56}$) regulatory T-cell subsets in CLL subjects with stable disease. Panel A to D show comparative analysis of the percentage and the number of circulating Treg and $T_{R3\text{-}56}$, in CLL subjects and healthy controls. Decreased percentage (**A**) and increased number (**B**) of the Treg cells has been shown to be associated with not significant difference in percentage of circulating $T_{R3\text{-}56}$ lymphocytes (**C**) and increased number of this T-cell subset (**D**) in the CLL cohort. Grey and white columns indicate data obtained in healthy controls (CTR in *x* axis) and CLL individuals (CLL in *x* axis), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Panel (**E**) shows the significant correlation, as evaluated by *Spearman's* test, between percentage of circulating Treg and $T_{R3\text{-}56}$ lymphocytes in CLL subjects. Statistical significance values are indicated. NS indicates the not statistically significant value. The applied flow cytometry gating strategy is reported in in Section 4.2.

To describe the T-cell profile of our CLL cohort, we analysed the percentage of Treg and $T_{R3\text{-}56}$ subset in the T-cell population. In this regard, an increased percentage of Tregs (13.81 ± 1.07 vs. 7.68 ± 0.53 in healthy controls; $p < 0.0001$) and $T_{R3\text{-}56}$ (15.48 ± 2.34 vs. 6.01 ± 0.63 in healthy controls; $p < 0.0001$) was observed in the T-cell subset from CLL subjects (Figure 3A,B, respectively).

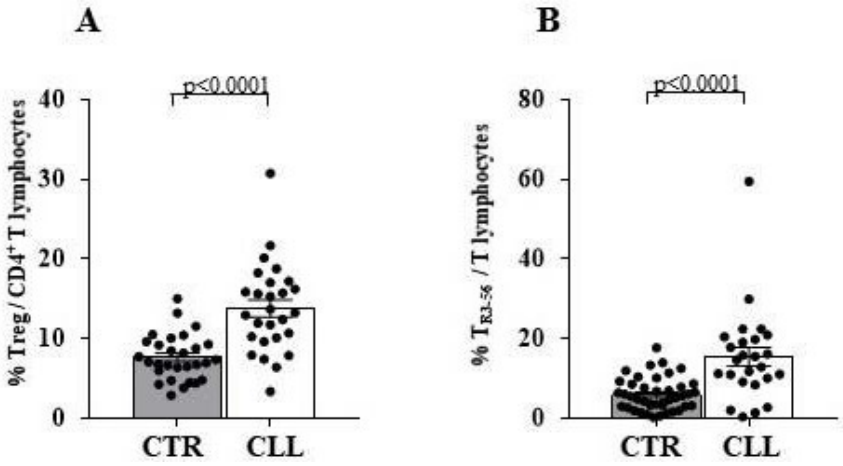

Figure 3. Percentage of Treg and $T_{R3\text{-}56}$ lymphocytes are significantly increased in the T-cell compartment of CLL subjects. Panel (**A**) shows comparative analysis of the percentage of the Treg subset in the $CD4^+$ T-cell population in the CLL subjects, as compared with controls. As shown, significant increase in Treg cells has been revealed in the CLL cohort; panel (**B**) shows percentage of $T_{R3\text{-}56}$ lymphocytes in the T cells of CLL subjects, as compared with healthy individuals. As shown, significant increase in $T_{R3\text{-}56}$ cells has been revealed in the CLL cohort. Grey and white columns indicate data obtained in healthy controls (CTR in *x* axis) and CLL individuals (CLL in *x* axis), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Statistical significance values are reported. The applied flow cytometry gating strategy is reported in Section 4.2.

Therefore, an increase in regulatory T-cell subsets appears to characterise the T-cell compartment in CLL patients, as already highlighted [53,54]. This evidence suggests that an increased rate of Treg and $T_{R3\text{-}56}$ differentiation within the T-cell compartment characterises the CLL individuals with stable disease.

2.2. Circulating Cytotoxic T Cells Are Characterised by an Increased Expression of CD54 and High Production of Interferon-γ in CLL Subjects with Stable Disease

We analysed the CD54 expression, as a marker of antigen-dependent CTL activation [52,55–57], and the production of IFN-γ by T cells and NK effectors in CLL subjects.

An increased expression of CD54 (13.26 ± 0.80 vs. 6.72 ± 1.39 in healthy controls; $p < 0.0001$) and a higher IFN-γ production (62.94 ± 5.97 vs. 34.84± 4.81 in healthy controls; $p < 0.005$) by CTL characterise CLL subjects (Figure 4A,C, respectively).

Furthermore, $CD3^+$ $CD4^+$ T lymphocytes present a higher CD54 expression (7.04 ± 0.25 vs. 2.51 ± 0.5 in controls; $p < 0.0001$) (Figure 4B,D), while the IFN-γ production by $CD3^+$ $CD4^+$ cells are substantially comparable between CLL and healthy controls (26.06 ± 4.50 vs. 20.65 ± 2.97, respectively). Similar results were observed when IFN-γ was analysed in the NK population (19.95 ± 4.21 vs. 24.02 ± 4.34 in healthy controls) (Figure 4E).

Taken in all, CLL subjects with stable disease appear to be characterised by an activation profile of circulating CTL.

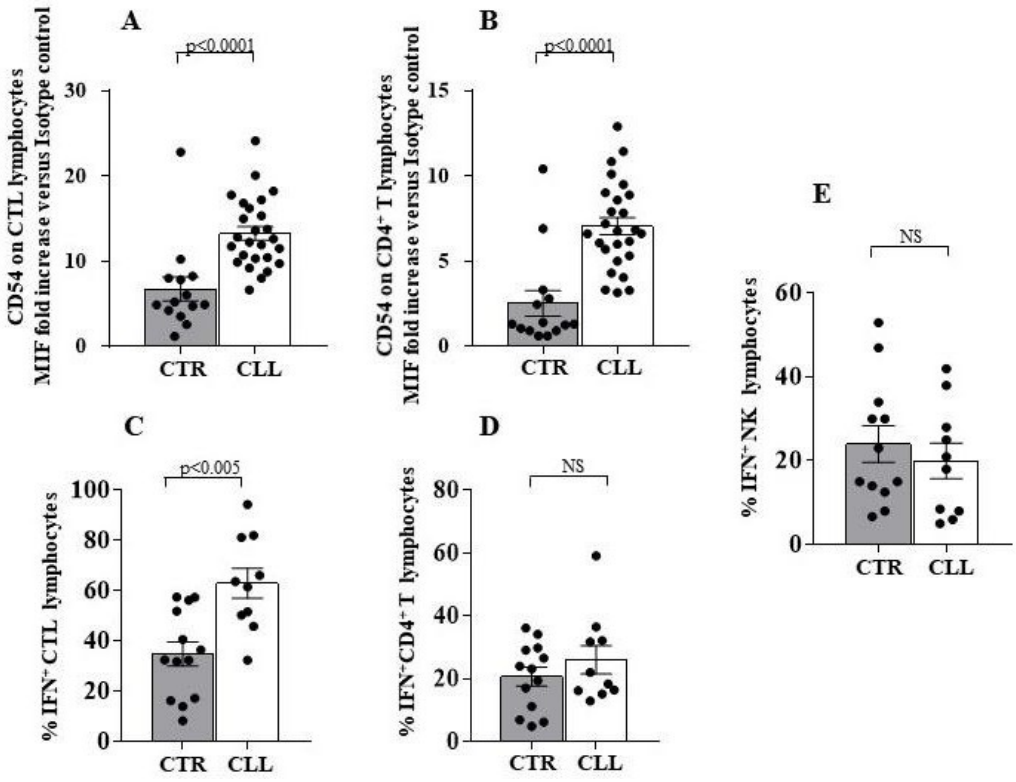

Figure 4. Significant increase in surface CD54 expression and higher interferon (IFN)-γ production characterises cytotoxic T cells of CLL subjects with stable disease. Panel (**A**,**B**) refer CD54 expression level on CD8 and CD4 T cells, respectively. As detailed in the Materials and Methods Section, immune-fluorescence data were expressed as ratio of the mean intensity fluorescence (MIF) value for the CD4 and CD8 cells and the control MIF value obtained after staining the same cell population with the isotype control mAb. As shown, CD4 and CD8 T-cell effectors are characterised by significant increase in surface CD54 molecule expression in the CLL cohort. Panels (**C**–**E**) show IFN-γ production by CD8, CD4 T lymphocytes and NK cells, respectively. Cytokine production analysis of has been performed by immune-fluorescence and flow cytometry detection after an over-night (ON) culture in the presence of PMA and ionomycin (see Materials and Methods Section for details). Grey and white columns indicate data obtained in healthy controls (CTR in *x* axis) and CLL individuals (CLL in *x* axis), respectively. For the comparative analysis of CD54 expression on T-cell effectors, immune-fluorescence data were expressed as ratio of mean intensity fluorescence (MIF) value for the CD4 and CD8 T-cell subset and the control MIF value obtained after staining the same cell population with the isotype control mAb. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Statistical significance values are indicated. NS indicates the not statistically significant value. The applied flow cytometry gating strategy is reported in Section 4.2.

2.3. A Lower Expression of HLA-A and HLA-BC Molecules and a Reduction of Intracellular Calnexin Characterise Circulating B Cells, but Not T Lymphocytes from CLL Subjects with Stable Disease

To evaluate the expression of HLA-I molecules, we used specific monoclonal antibodies (mAbs) able to recognise specific HLA-I molecular structures [58–62] (see Materials and Methods section). Analysis of HLA-I expression on circulating B lymphocytes of CLL subjects with stable disease revealed a significant reduction in HLA-A (37.49 ± 5.77 vs. 104.9 ± 27.81 in healthy controls; $p < 0.005$), HLA-BC (47.58 ± 7.25 vs. 128.4 ± 38.57 in controls; $p < 0.05$) and β2-microglobulin surface level (54.29 ± 8.96 vs. 156.81 ± 41.91 in healthy controls; $p < 0.005$) (Figure 5A). The expression of the HLA-ABC molecules, evaluated as a whole, on the membrane of the leukaemia B-cell clones was not significantly different from the B lymphocytes of the control population (61.32 ± 9.31 vs. 110.7 ± 32.78

in healthy controls) (Figure 5A). At variance, HLA-I expression on T lymphocytes showed no significant difference between CLL subjects and healthy controls (Figure 5B).

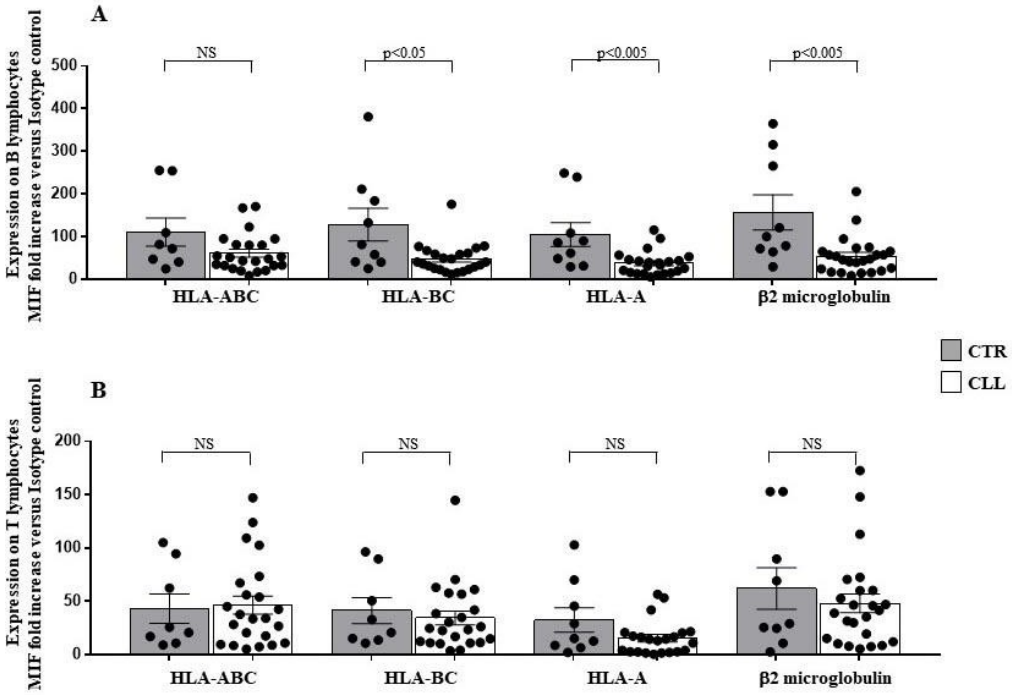

Figure 5. Significant decrease in human leukocyte antigen (HLA)-A and HLA-BC expression level characterises B lymphocytes of CLL subjects with stable disease. Panel (**A**,**B**) show HLA-ABC, HLA-A and HLA-BC surface expression level on B and T lymphocytes, as indicated. As shown, significant decrease in surface expression of HLA-A and HLA-BC molecules level has been observed in the B-cell compartment of the CLL subjects, as compared with healthy controls. No significant changes were revealed, for all the HLA-I molecules analysed, in the T lymphocytes of the CLL cohort. As detailed in the Materials and Methods Section, immune-fluorescence data were expressed as ratio of the mean intensity fluorescence (MIF) value for B and T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb, as described [52,55]. Grey and white columns indicate data obtained in healthy controls (CTR caption of (**A**,**B**) Panels) and CLL individuals (CLL caption of (**A**,**B**) Panels), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Statistical significance values are indicated NS indicates the not statistically significant value. The applied flow cytometry gating strategy is reported in Section 4.2.

To investigate the molecular basis of the observed altered expression of HLA-I on leukaemia clones, we analysed the expression of the APM molecules in circulating B and T lymphocytes from CLL subjects. A reduced level of calnexin (10.25 ± 1.63 vs. 20.42 ± 3.97 in healthy controls) was found to characterise the B cell compartment from CLL subjects (Figure 6A). In addition, TAP-1 [36–38,61] (2.47 ± 0.32 vs. 0.71 ± 0.29 in controls), Tapasin [36–38,59] (2.27 ± 0.25 vs. 0.99 ± 0.32 in healthy controls), LPM7 (3.81 ± 0.72 vs. 1.5 ± 0.44 in healthy controls) and LMP10 (2.4 ± 0.27 vs. 0.73 ± 0.31 in healthy controls) molecules increased in the T-cell compartment (Figure 6B).

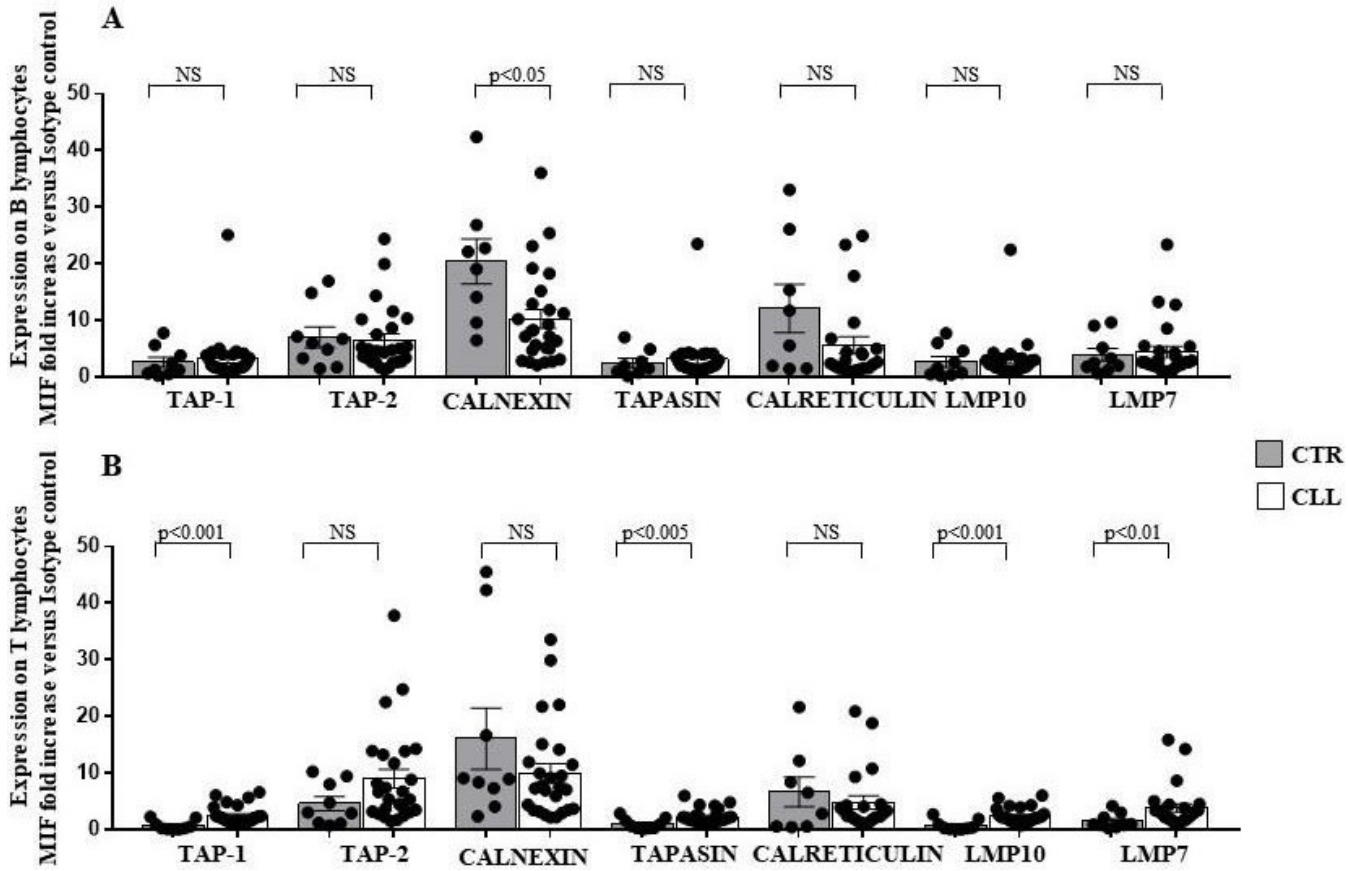

Figure 6. Significant decrease in intracellular calnexin expression in B lymphocytes, associated with up-regulation of TAP-1, Tapasin, LMP7 and LMP10 in the T-cell compartment, characterises CLL subjects with stable disease. Panel (**A**,**B**) show TAP-1, TAP-2, calnexin, tapasin, calreticulin, LMP10 and LMP7 intracellular expression in B- and T-cell compartment, as indicated. As shown, significant decrease in intracellular calnexin has been observed in the B cells of the CLL subjects, as compared with controls; at variance significant increase in TAP-1, Tapasin, LMP10 and LMP7 was revealed in the T lymphocytes of the CLL cohort. As detailed in the Materials and Methods Section, intracellular immune-fluorescence data were expressed as ratio of the mean intensity fluorescence (MIF) value for B and T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb. Grey and white columns indicate data obtained in healthy controls (CTR caption of (**A**,**B**) Panels) and CLL individuals (CLL caption of (**A**,**B**) Panels), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Statistical significance values are indicated. NS indicates the not statistically significant value. The applied flow cytometry gating strategy is reported in Section 4.2.

Extracellular expression of calreticulin represents a marker of endoplasmic reticulum (ER) stress, usually associated with intracellular protein misfolding and immunogenic cell death processes [63]. The extracellular calreticulin expression increased in both circulating B (30.67 ± 4.40 vs. 13.74 ± 3.76 in healthy controls) and T lymphocytes (1.5 ± 0.22 vs. 0.71 ± 0.35 in healthy controls) of the CLL subjects (Figure 7).

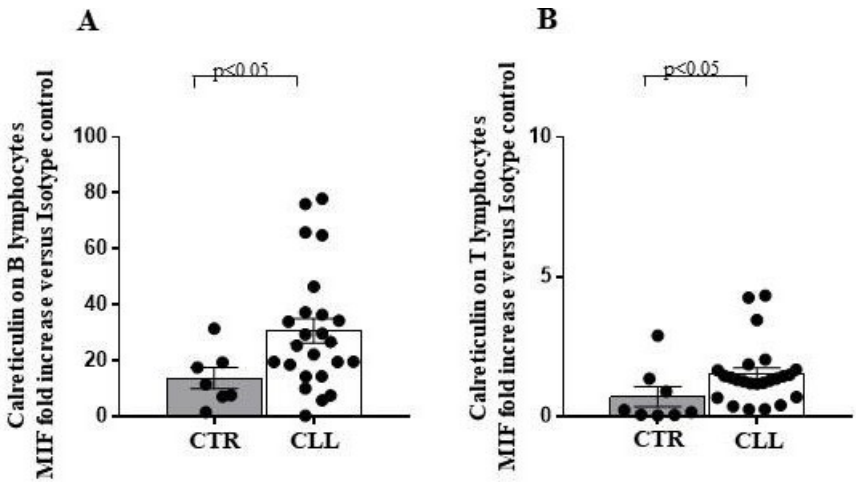

Figure 7. Higher surface calreticulin expression, as a measure of the overloading of the intracellular protein folding capacity, characterises circulating B and T lymphocytes of CLL subjects, as compared with healthy controls. Panel (**A**,**B**) show evaluation of surface calreticulin expression on circulating B and T lymphocytes of CLL individuals and healthy controls, as indicated. As shown, significant increase in the surface calreticulin expression has been revealed in both B and T cells from the CLL subjects. As detailed in the Materials and Methods Section, immune-fluorescence data were expressed as ratio of the mean intensity fluorescence (MIF) value for B and T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb. Grey and white columns indicate data obtained in healthy controls (CTR in *x* axis) and CLL individuals (CLL in *x* axis), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Statistical significance values are indicated. The applied flow cytometry gating strategy is reported in in Section 4.2.

In addition, the binding of the specific anti HLA-ABC mAb revealed an increase in HLA-I surface expression when B cells and T cells from CLL subjects were cultured with IFN α-2b (Figure 8A,B). This finding suggests that the defective expression of HLA-I molecules in CLL B cell clones might be susceptible to cytokine-mediated in vitro restoration.

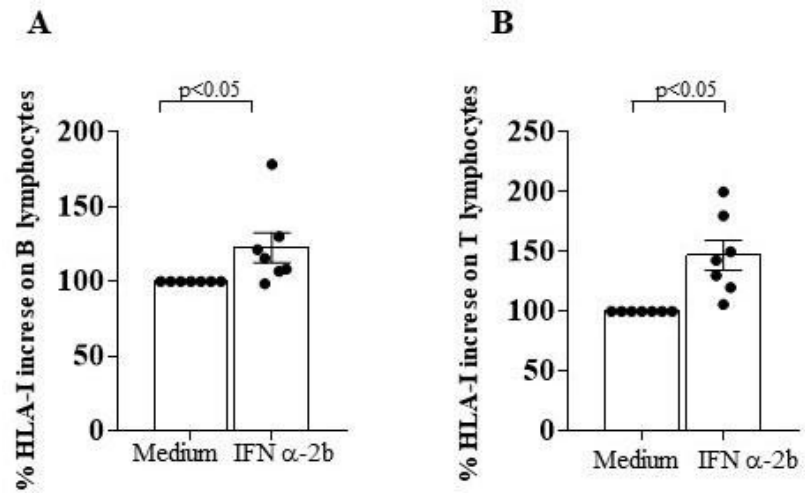

Figure 8. In vitro treatment with interferon (IFN) α-2b significantly increases HLA-I expression on B and T lymphocytes of CLL subjects with stable disease. Panel (**A**,**B**) indicate % increase in HLA-I surface expression level on B and T cells of CLL subjects after an overnight culture in the presence of medium alone or saturating concentration of IFN α-2b, as indicated in *x* axis. Statistical evaluation of data has been performed by means of the *Wilcoxon matched-pairs signed rank* test. Statistical significance values are indicated. The applied flow cytometry gating strategy is reported in Section 4.2.

2.4. Circulating NK Lymphocytes from CLL Subjects with Stable Disease Show Higher Expression of KIR2DS2 Activating Receptor and Significant Reduction of 3DL1 and NKG2A Inhibiting Molecules

We analysed the expression of both activating and inhibiting receptors on NK effectors of CLL subjects with stable disease. Increased expression of the activating receptor KIR2S2 (CD158j) [64] in CLL patients compared to healthy donors was observed (6.10 ± 2.01 vs. 0.10 ± 0.12, respectively; $p < 0.001$) (Figure 9A). No differences were found in the expression of the activating molecules Nkp46 (CD335), Nkp30 (CD337), Nkp44 (CD336) and NKG2D [27,29,30] (Figure 9A).The expression of the activating receptor 2B4 (CD244) [65,66] is decreased in CLL subjects (86.63 ± 4.82 vs. 99.77 ± 0.11 in healthy controls; $p < 0.0005$) (Figure 9A).

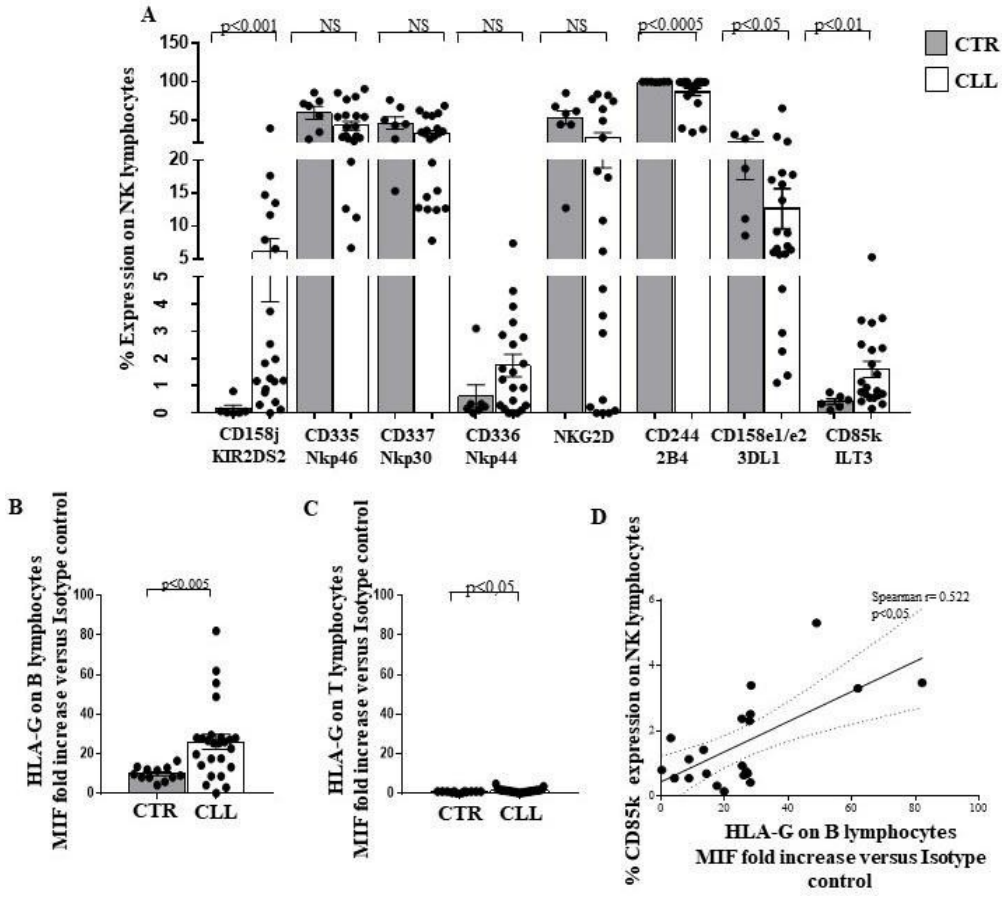

Figure 9. Analysis of activating and inhibiting receptor repertoire profile of circulating NK effectors of CLL subjects with stable disease, as compared with healthy controls. Panel (**A**) shows the expression of KIR2DS2, CD335, CD336, CD337, NKG2D, CD244, 3DL1 and CD85k on the surface of circulating NK effectors of CLL subjects, as compared with controls. As shown, significant increase in the activating KIR2DS2 receptor, associated with reduced level of the inhibiting structure 3DL1 has been observed together with a decrease in the activating CD244 molecule and an increase in the inhibiting CD85k receptor, able to bind the HLA-G molecule. Panel (**B**,**C**) show HLA-G expression level on the surface of B and T lymphocytes of the CLL cohort, as compared with controls. Significant increase of HLA-G expression has been revealed on both cell subsets in the CLL subjects. Grey and white columns indicate data obtained in healthy controls (CTR caption of Panel A and in *x*-axis of (**B**,**C**) Panels) and CLL individuals (CLL caption of Panel (**A**) and in *x*-axis of (**B**,**C**) Panels), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Panel (**D**) shows the correlation analysis, as evaluated by the *Spearman's* test, between the percentage of circulating NK cells expressing CD85k receptor and the HLA-G level on the B lymphocytes of the CLL subjects. Statistical significance values are indicated. NS indicates the not statistically significant value. The applied flow cytometry gating strategy is reported in Section 4.2.

Analysis of the inhibitory receptors 3DL1 (CD158e1/e2) and CD85k (ILT3), which binding to HLA-G molecules mediates NK inhibition [67–69], revealed reduced levels of the 3DL1 (12.63 ± 3.05 vs. 21.12 ± 4.10 in healthy controls; $p < 0.05$) associated with increased expression of CD85k (1.60 ± 0.29 vs. 0.41 ± 0.09 in healthy controls; $p < 0.01$) (Figure 9A).

Intriguingly, an increase in HLA-G was detected in circulating B cells (26.07 ± 3.78 vs 10.03 ± 0.99 in healthy controls; $p < 0.005$) and T cells (1.41 ± 0.2 vs. 0.8 ± 0.09 in healthy controls; $p < 0.05$) from subjects with CLL (Figure 9B,C).

An inverse correlation was observed between the expression of HLA-G on B lymphocytes and the level of CD85k, the specific HLA-G receptor, on NK effectors of CLL individuals (Spearman r = 0.522; $p < 0.05$) (Figure 9D).

Expression of the NK receptors on the CTL has been extensively described [70–72]. In CLL subjects, analysis of the CTL receptor repertoire revealed an increased KIR2S2 (CD158j) expression (3.66 ± 1.12 vs. 0.65 ± 0.29 in healthy controls), while no difference was observed for the other receptors (CD335, CD336, CD337, NKG2D, CD244, CD158e1/e2 and CD85k) (Figure 10A). No significant correlation was revealed between HLA-G expression on circulating B cells from the CLL subjects and CD85k expression on peripheral CTL, although a negative correlation trend has been observed (Spearman r= −0.265) (Figure 10B).

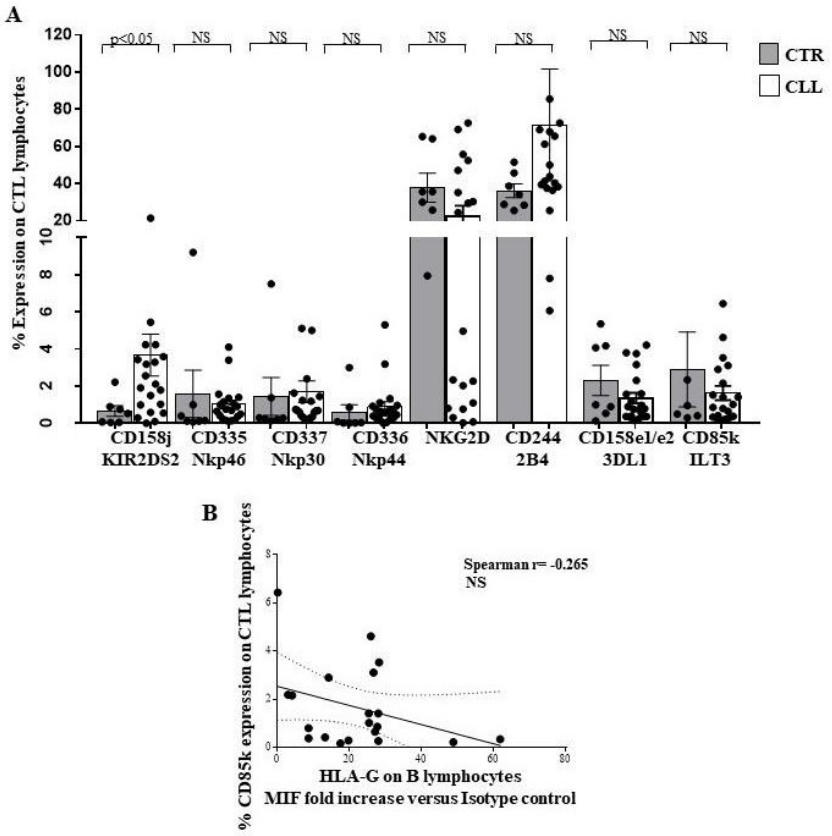

Figure 10. Analysis of receptor repertoire profile of circulating CTL effectors of CLL subjects with stable disease, as compared with healthy controls. Panel (**A**) shows the expression of KIR2DS2, CD335, CD336, CD337, NKG2D, CD244, 3DL1 and CD85k on the surface of circulating $CD8^+$ T-cell effectors of CLL subjects, as compared with controls. As shown, significant increase in the activating KIR2DS2 receptor has been observed. Grey and white columns indicate data obtained in healthy controls (CTR caption of Panel (**A**)) and CLL individuals (CLL caption of Panel (**A**)), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Panels (**B**) shows the correlation analysis, as evaluated by the *Spearman's* test, between the percentage of circulating $CD8^+$ T cells expressing CD85k receptor and the HLA-G level on the B lymphocytes of the CLL subjects with stable disease. Statistical significance values are indicated. NS indicates the not statistically significant value. The applied flow cytometry gating strategy is reported in Section 4.2.

Altered HLA-E molecule expression by B lymphocytes has been described in CLL [73,74]. Our data confirm such observation in CLL circulating B cells (15.24 ± 1.80 vs. 3.31 ± 1.27 in healthy controls), while no significant difference was revealed in the HLA-E expression by T cells of the CLL subjects with respect to controls (1.36 ± 0.19 vs. 0.79 ± 0.18 in healthy controls) (Figure 11A,B, respectively).

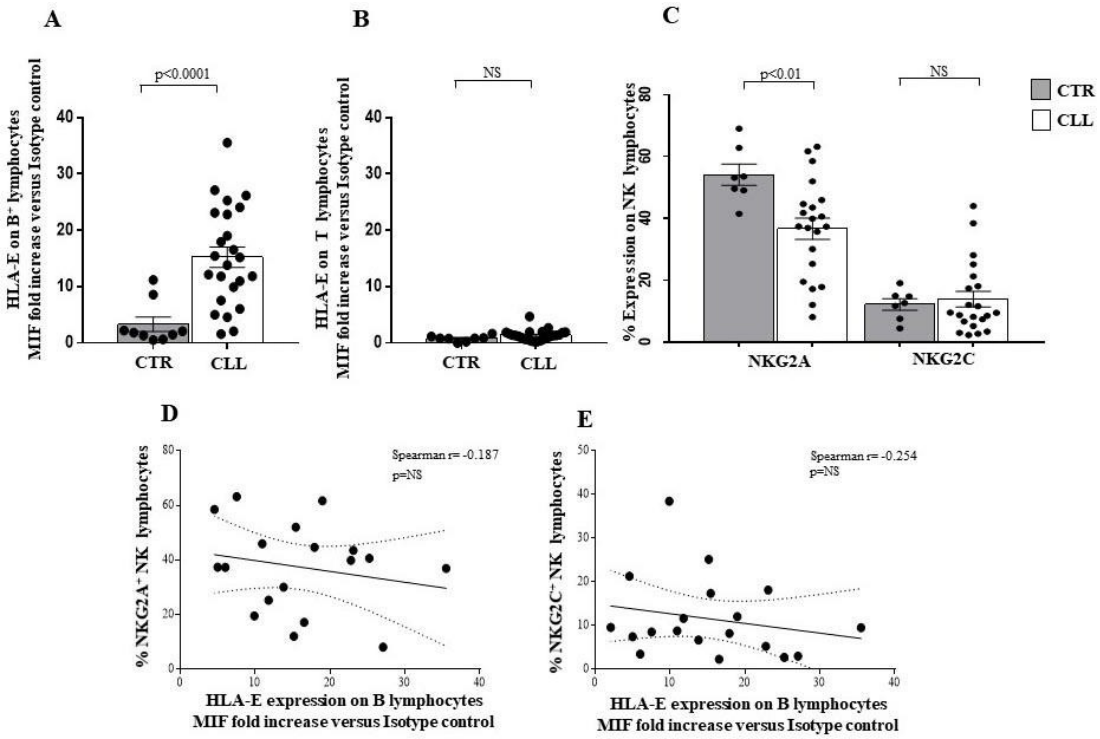

Figure 11. Analysis of HLA-E on B and T lymphocytes, and of NKG2A and NKG2C expression on circulating NK effectors of CLL subjects with stable disease. Panel (**A**,**B**) show the expression level of the HLA-E molecule on the surface of B and T lymphocytes, respectively. Significant increase in the HLA-E molecule on the surface of CLL B lymphocytes has been observed. Panel C shows the percentage of the HLA-E binding receptors NKG2A and NKG2C on circulating NK lymphocytes of the CLL cohort, as compared with controls. As shown, significant reduction of the inhibiting NKG2A receptor has been observed in the LLC cohort. Grey and white columns indicate data obtained in healthy controls (CTR in *x-axis* of A and B Panels and CTR caption in (**C**) Panel) and CLL individuals (CLL in *x*-axis of (**A**,**B**) Panels and CLL caption of C Panel) respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Panels (**D**,**E**) show the correlation analysis, as evaluated by the *Spearman's* test, between the percentage of circulating NK cells expressing NKG2A or NKG2C receptors and the HLA-E expression level on the B lymphocytes of the CLL subjects with stable disease. Statistical significance values are indicated. NS indicates the not statistically significant value. The applied flow cytometry gating strategy is reported in Section 4.2.

HLA-E molecule binding with NK effectors has been described to generate key inhibitory signals, mediated by the NKG2A receptor as well as activation of the NK cells after binding with the NKG2C molecule [75–77]. We observed a reduced NKG2A expression on NK cells of CLL subjects (36.63 ± 3.41 vs. 54.12 ± 3.45 in healthy controls; $p < 0.01$), while no difference was revealed in the level of NKG2C expression between CLL subjects and healthy individuals (Figure 11C). No correlation was observed between NKG2A and NKG2C expression on NK cells and HLA-E expression on CLL B lymphocytes (Figure 11D,E, respectively). In this context, a trend of negative association was revealed (Spearman r = 0.187 for NKG2A/HLA-E; Spearman r = −0.254 for NKG2C/HLA-E) (Figure 11D,E, respectively).

A decrease in NKG2A was observed in the CTL from CLL subjects (2.42 ± 0.35 vs. 7.63 ± 2.11 in healthy controls; $p < 0.005$), while no difference was revealed in NKG2C expression (Figure 12A).

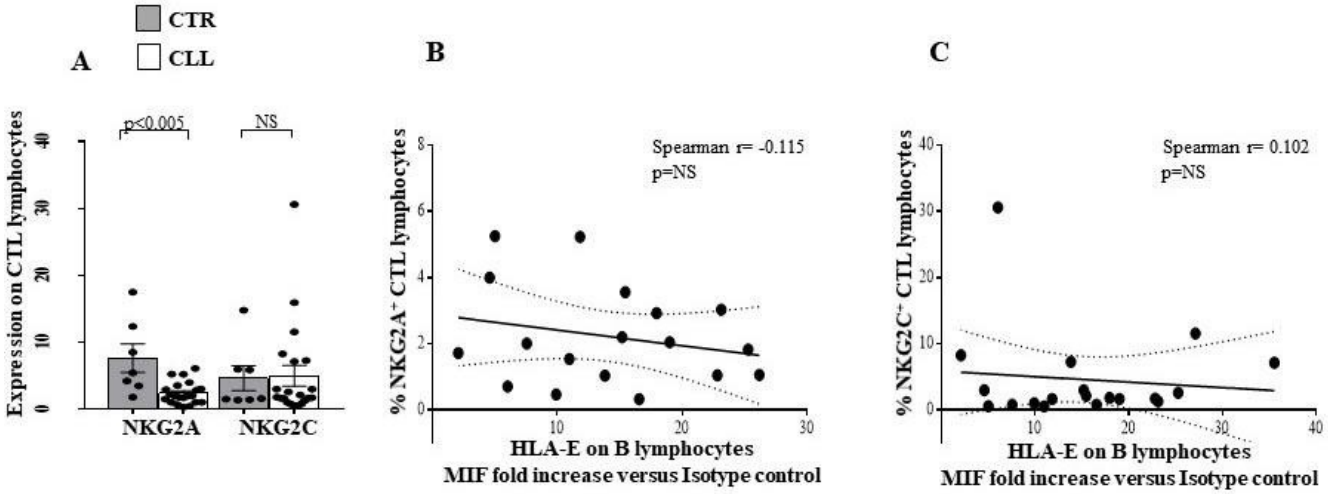

Figure 12. Comparative analysis of NKG2A and NKG2C receptor expression on circulating CTL effectors of CLL subjects with stable disease, as compared with healthy controls. Panel (**A**) shows the percentage of the HLA-E binding receptors NKG2A and NKG2C on circulating $CD8^+$ T lymphocytes of the CLL subjects, as compared with controls. As shown, significant reduction in the inhibiting NKG2A receptor has been observed in the LLC cohort. Grey and white columns indicate data obtained in healthy controls (CTR caption of Panel (**A**)) and CLL individuals (CLL caption of Panel (**A**)), respectively. Statistical evaluation of data has been performed by means of the *Mann–Whitney* test. Panels (**B**,**C**) show the correlation analysis, as evaluated by the *Spearman's* test, between the percentage of circulating CTL expressing NKG2A or NKG2C receptors and the HLA-E expression level on the B lymphocytes of the CLL subjects. Statistical significance values are indicated. NS indicates the not statistically significant value. The applied flow cytometry gating strategy is reported in Section 4.2. No differences were revealed between the level of NKG2A/NKG2C on CTL and the expression of HLA-E on B lymphocytes of CLL subjects (Panel (**B**,**C**), respectively). Taken together, these data indicate that an activation profile appears to characterise the NK effector recognition repertoire of CLL subjects with stable disease.

3. Discussion

Here, we describe that the reduced expression of HLA-A and HLA-BC Class-I molecules on B lymphocytes is accompanied by the expression of an activation profile by both adaptive and innate cytotoxic effectors in CLL subjects with stable disease. Indeed, we observed an increased CD54 expression, a greater IFN-γ production by CTL and the presence of a NK receptor repertoire with preferential expression of activating molecules in our cohort of CLL patients.

To investigate on the role of immune-mediated pathways in the control of CLL progression, we analysed the immune profile of the CLL subjects with stable disease, as compared with a cohort of 20 sex/age matched healthy controls. In this model, we focused on cytotoxic effectors as well as IFN-γ dependent pathways, largely described to play a key role in the control of cancer progression [9–18].

We confirmed the already described reduction of T and NK percentage, with numerical increase in both cell types in patients affected by CLL [19–24,78–80]. In addition, we also observed the decreased CD4/CD8 T-cell ratio [19–22,78–80] in our CLL cohort. These data suggest that a higher proportion of circulating CTL characterises the T-cell compartment in CLL subjects with stable disease. The functions of immune effectors have been described to depend on cell-mediated regulatory networks involving Treg [41–45] and $T_{R3\text{-}56}$-cell subset that we recently described to preferentially modulate CTL effectiveness in autoimmune [51] and in tumour disease [52,81,82].

Our data indicate that the Treg and $T_{R3\text{-}56}$ percentages decreased when evaluated on total lymphocytes, but increased when they are specifically analysed in the T-cells compartment alone. Furthermore, the absolute number of circulating Treg and $T_{R3\text{-}56}$ is significantly higher in CLL patients when compared to healthy controls. Since lymphocytes are mainly composed of B cells in CLL patients, the small percentage of T cells within the lymphocyte compartment appears to exhibit a preferential expansion of Treg and $T_{R3\text{-}56}$ regulatory cell subsets, as a possible immune escape mechanism. Our data confirm the Treg expansion in CLL [78–80] and highlight an expansion also of the recently characterised regulatory $T_{R3\text{-}56}$ population [51,52,81,82].

In our CLL cohort, we observed the increased CD54 expression, largely associated with antigen-dependent T-cell activation [52,55–57], together with increased IFN-γ production by CTL. Such a profile is conceivable with the involvement of adaptive cytotoxic effectors in the control of leukemic B cells, potentially fostering the establishment of a stable CLL disease [16,17,19,20,78–80].

The expression of HLA-I, required for CTL-dependent recognition of antigens, has been extensively related to the immune-mediated control of cancer initiation/progression, as well as to the efficacy of immunotherapy in mouse and human models [32–38]. In this regard, the altered expression of HLA-I has been described to have a relevant role in the immuno-editing processes of the tumour context [34–38]. Modulation of the expression of individual HLA-I loci/alleles has been implicated in determining defective CTL activation [34–39].

In this article, we describe a reduced expression of HLA-A and HLA-BC molecules on B cells from CLL subjects with stable disease, without changes in the T-cell compartment. To investigate the mechanisms underlying HLA-I altered expression, we analysed the APM molecules [36–38], essential for the correct assembly of the HLA-I. We observed a reduced surface expression of HLA-A and -BC molecules, associated with defective intracellular calnexin levels in B lymphocytes from CLL individuals. Conversely, we found that the unaffected surface HLA-I expression is accompanied by an increase in intracellular TAP-1, Tapasin, LMP7 and LMP10 APM molecules in the T-cell compartment. Such features are conceivable with the hypothesis of an ongoing selection process mediated by activated CTL, able to preferentially target tumoral B-cell compartment, as largely proposed [37,38]. Based on our data, we can speculate that the increased IFN-γ production by CTL may induce APM molecules in circulating T lymphocytes of CLL individuals, also affecting surface calreticulin expression, usually associated with the occurrence of an overloading of the intracellular protein folding by B and T cells, as well as with immunogenic cell death processes [63]. The lack of HLA-I expression by cancer cells has been associated with the inability to respond to the immune therapeutic approaches [36–38]. In this context, multiple attempts to restore HLA-I expression in clinical settings have been proposed [36–38]. Here, we demonstrated that in vitro IFN α-2b treatment of B and T cells, obtained from individuals with stable CLL, increased their HLA-I surface expression. Therefore, it is conceivable that leukemic B cells in our cohort of CLL subjects with stable disease are potentially susceptible to cytokine-mediated HLA-I upregulation, as described [83].

Overall, our data suggest that the presence of activated CTL might be involved in the control of the expansion of transformed B-cell clones, as well as in the modulation of HLA-I expression on B lymphocytes in CLL subjects with stable disease. However, in order to demonstrate the relevance of this hypothesis in CLL subjects, further studies evidencing similarities and/or differences between subjects with stable and advanced-stage CLL regarding the functional role of both the CD8 or CD4 expressing $T_{R5\text{-}56}$ lymphocytes will be needed [50,51] (see also Study Limitations).

Moreover, we analysed the NK receptor repertoire expressed by circulating NK lymphocytes [27–30] and by CTL of CLL subjects [70–72]. In this regard, we observed a significant up-regulation of the activating receptor KIR2S2 [64] in NK cells from CLL subjects, without significant changes in the expression of other activating molecules CD335, CD336, CD337, NKG2D and NKG2C [27–30,77]. We highlighted a reduced expression of CD244, already demonstrated to participate in activation processes in human NK models [65,66]. Concerning the inhibitory receptor repertoire of NK effectors in the CLL cohort, a significant down-modulation of 3DL1 and NKG2A [75–77] inhibitory molecules was revealed, while an increased level of the HLA-G binding CD85k receptor was observed [67,68]. Furthermore, we described that NK-dependent IFN-γ production was not significantly different from healthy controls. In addition, we found an increase in the activating KIR2S2 [64], accompanied by a reduction of the inhibitory NKG2A molecule [75–77] on CTL, in our cohort of CLL patients.

The role of altered HLA-G [78,80,81] expression in CLL is still controversial and of unclear prognostic significance [81]. Here, we demonstrated a preferentially increased expression of HLA-G on B cells, but not on T cells, from CLL patients with stable disease. Furthermore, we described a correlation between HLA-G expression by B cells and the percentage of the HLA-G binding inhibitory receptor CD85k on NK effectors in CLL subjects, while no significant correlation between HLA-G on B cells and CD85k expression by T lymphocytes was observed. The ability of HLA-G to induce CD85k expression on lymphoid human cell lines has been also observed [69]. Altered HLA-E molecule expression by B lymphocytes has been described in CLL [26] and is confirmed in our cohort. In this regard, no significant correlation was observed between B lymphocyte HLA-E expression and the HLA-E binding molecules NKG2A and NKG2C in the NK compartment or CTL compartment of subjects with CLL.

Taken together, our data suggest that circulating CTLs exhibit a potential activation profile involving TCR and NK-receptor-dependent pathways in CLL individuals with stable disease. In this regard, a more complex profile was observed in NK cells. Indeed, a significant up-regulation of the activating receptor KIRDS2, with a decrease in the level of the 3DL1 and NKG2A inhibitory molecules, is accompanied by both a reduced expression of the activating molecule CD244 and an increasing amount of the inhibitory receptor CD85k. Alterations of HLA class I molecules, as well as HLA-G and HLA-E, on B cells are of potential interest, as they could represent a putative escape mechanism of neoplastic clone B from CTL and NK recognition in CLL patients with stable disease. However, since the balance between the control of CTL and NK effectors may be crucial for disease determinism and outcomes, further studies of the functional mechanisms of the immune response and its regulation in CLL disease are needed (see Study Limitations).

4. Materials and Methods

4.1. Patients and Controls

Twenty-six patients, diagnosed as stage 0–1 LLC, according to *Rai* system, and as stage A, according to *Binet* system [84], all belonging to the Low Risk category, according to the *CLL-IPI* score [85], were enrolled in the study. Detailed description of clinical characteristic of our cohort is reported in Table 1.

Table 1. Patient clinical and haematological characteristics.

Parameters	Mean ± SD	Range
Male/Female ratio 15:11		
Age (years)	68 ± 7.23	45–74
Duration of illness (years)	8.91 ± 4.46	3–18
Haemoglobin (g/dL)	13.79 ± 1.26	11.9–16.5
Platelet count ($\times 10^9$/L)	188.2 ± 65.85	107–384
White blood cell count ($\times 10^9$/L)	24.82 ± 22.92	5.86–109.2
Neutrophil count ($\times 10^9$/L)	9.97 ± 8.59	0.61–34.94
Lymphocyte count ($\times 10^9$/L)	15.97 ± 15.59	3.96–74.26

Patients did not show lymphadenopathy greater than 2 cm in all the superficial lymph-node stations; the splenic dimensions are stable over time, with a splenic volume ranging between 250 and 400 mL.

Twenthy healthy donors, sex/age matched with CLL subjects, were also enrolled in the study.

Informed consent was obtained from each individual before each sample collection. Study was approved by the local Ethical Committee (protocol n. 347/19). None of the patients recruited was receiving immune-modifying medical treatments. Enrolled patients were not affected by immune-mediated diseases and acute or chronic viral infections.

4.2. Immune-Fluorescence and Flow Cytometry

FITC, PE, PEcy5, PEcy7 and APC labelled anti-CD3, -CD4, -CD8, -CD56, -CD25, -CD45, -CD54, -NKG2D, -HLA-E, -HLA-G and control isotype-matched monoclonal antibodies (mAbs) were purchased from BD PharMingen (San Jose, CA). FITC and PE-labelled anti-CD158j, -CD158e1/e2, -CD85, -nkp44, -CD244, -CD335, -CD337 mAbs were purchased from Beckman-Coulter, Paris, France. PE-labelled anti-NKG2A, -NKG2C mAbs were from R&D Systems, Inc. (Minneapolis, MN, USA). The TP 25.99.8.4 (anti-HLA-A, -HLA-B and -HLA-C associated with β2m) [58], LGIII-147.4.1 (anti-HLA-A associated with β2m) [59], B1.23.1 (anti-HLA-B and -C associated with β2m) [60], L368 (anti- β2m) [61], NOB-1 (anti-TAP1) [58] NOB-2 (anti- TAP2) [61], TO-5 (anti-calnexin) [62], TO-11 (anti-calreticulin) [62], TO-3 (anti-tapasin) [62], TO-7 (anti-LMP10) [61], Sy-3 (anti-LMP7) [63] mAbs were donated by Prof. Soldano Ferrone from Massachusetts General Hospital USA, and have been largely described for the characterisation of HLA and APM molecules in human tumours [36–38]. To analyse Foxp3 expression, intracellular staining was performed by using the anti-human Foxp3 kit (eBioscience, San Diego, CA, USA), following the manufacturer's instructions. Anti-human interferon (IFN)-γ and isotype-matched mAbs were purchased from Becton Dickinson PharMingen, San Jose, CA, USA. The production of IFN-γ has been analysed by culturing purified peripheral blood mononuclear cells (PBMC) overnight in the presence of PMA and Ionomycin (Sigma-Aldrich, St. Louis, MO, USA). To avoid extracellular cytokine export, the cultures were incubated in the presence of 5 μg/mL of Brefeldin-A (Sigma-Aldrich), as described [86]. Intracellular staining with the specific mAbs was performed by a fixing/permeabilisation kit (Caltag, Burlingame, CA, USA), following the manufacturer's instructions. INF α-2b was from Sigma-Aldrich.

$T_{R3\text{-}56}$ lymphocytes have been identified by the co-staining with anti-human CD3 and anti-human CD56 mAb, as described [51,52]. All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using *Forward Scatter* (FSC) and *Side Scatter* (SSC) parameters, as well as CD45 labelling.

Flow cytometry and data analysis were performed by a two-laser equipped FACScalibur apparatus and the CellQuest analysis software (Becton Dickinson). Flow cytometry gating strategy is reported in Figure 13.

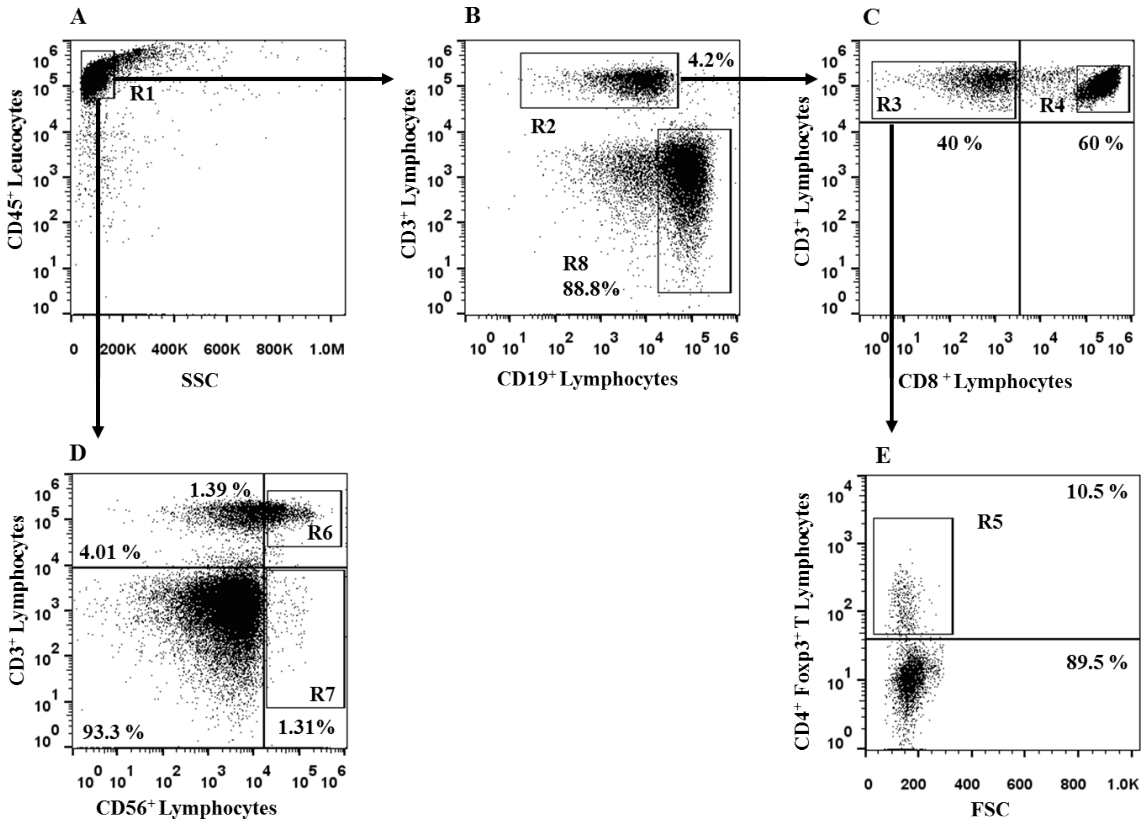

Figure 13. Flow cytometry gating strategy for analysis of CLL subjects with stable disease. Panel (**A**) shows the R1 gating on circulating $CD45^+$ cells of the CLL subjects. From R1 region, we identified $CD3^+$ T cells (R2, Panel (**B**)). From R2, we gated the $CD3^+$ $CD8^-$ (R3, Panel (**C**)) and $CD3^+$ $CD8^+$ T cells (R4, Panel (**C**)). From R3 region, we observed $CD4^+$ $Foxp3^+$ Treg (R5, Panel (**E**)). From R1 region (Panel (**A**)), we identified $CD3^+$ $CD56^+$ $T_{R3\text{-}56}$ cells (R6, Panel (**D**)) and $CD3^-$ $CD56^+$ NK lymphocytes (R7, Panel (**D**)). From R1 gate, the B cells were identified by the CD19 expression (R8, Panel (**B**)). The figure reports one representative experiment performed in CCL subjects.

For the comparative analysis of CD54 expression on T lymphocytes as well as of HLA-I and APM molecule expression in B and T cells, immune-fluorescence data were expressed as ratio of mean intensity fluorescence (MIF) value for each lymphocyte subset and the control MIF value obtained after staining the same cell population with the isotype control mAb, as described [5,52].

4.3. Statistical Analysis

Statistical evaluation of data, by *InStat 3.0* software (GraphPad Software Inc., San Diego, CA, USA), was performed by *Mann–Whitney*, *Wilcoxon matched-pairs signed rank test* or *Spearman's correlation test*, as indicated. Two-sided *p* values less than 0.05 were considered significant.

5. Conclusions

Our observations are conceivable with the hypothesis that the functional efficacy of innate and adaptive cytotoxic immune effectors may participate in the immunological scenario underlying disease stability in chronic lymphocytic leukaemia (CLL). Indeed, we described an overactivated profile of adaptive cytotoxic T lymphocytes (CTL), while the receptor-activating natural killer (NK) lymphocyte repertoire was substantially maintained in a cohort of CLL subjects with stable disease.

Stable CLL could be considered as a peculiar case of *a long-term equilibrium* between the clonally transformed B-cell population and immune-mediated antineoplastic activity.

Our data suggest the potential role of some of the immune mechanisms underlying this unique condition. However, further functional studies are needed to understand whether these mechanisms are actually present in CLL and to explore on the complex scenario underlying CLL pathogenesis/progression (see Study Limitations). Characterisation of the complex interaction between CLL leukaemia cells and immune effectors is extremely useful not only for a better understanding of CLL pathophysiology, but could add key information for designing innovative immunotherapeutic strategies.

Study Limitations

This research is based on the immunophenotypic characterisation of immune effectors and molecules involved in antigen recognition and not on functional analysis. The present study only includes observations on patients with stable CLL and not patients with unstable and/or advanced CLL, as the latter enrolled cohorts that were too small and did not allow for robust statistical analysis. Therefore, future studies are needed to demonstrate functional correlates between immune response regulation and its impact on CLL disease determinism, including stable and unstable CLL patients.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: Data supporting reported results can be obtained by corresponding authors (G.R. and G.T.).

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Immune Profile in COVID-19: Unveiling T_{R3-56} Cells in SARS-CoV-2 Infection

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Abstract: The emergence of COronaVIrus Disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), presented a global health challenge since its identification in December 2019. With clinical manifestations ranging from mild respiratory symptoms to severe multi-organ dysfunction, COVID-19 continues to affect populations worldwide. The complex interactions between SARS-CoV-2 variants and the human immune system are crucial for developing effective therapies, vaccines, and preventive measures. Understanding these immune responses highlights the intricate nature of COVID-19 pathogenesis. This retrospective study analyzed, by flow cytometry approach, a cohort of patients infected with SARS-CoV-2 during the initial pandemic waves from 2020 to 2021. It focused on untreated individuals at the time of hospital admission and examined the presence of T_{R3-56} cells in their immune profiles during the anti-viral immune response. Our findings provide additional insights into the complex immunological dynamics of SARS-CoV-2 infection and highlight the potential role of T_{R3-56} cells as crucial components of the immune response. We suggest that T_{R3-56} cells could serve as valuable biomarkers for identifying more severe cases of COVID-19, aiding in the assessment and management of the disease.

Keywords: SARS-CoV-2; Severe acute respiratory syndrome coronavirus 2; COVID-19; COronaVIrus Disease 2019; immune regulation; immune regulatory cell phenotypes; T_{R3-56}

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1. Introduction

The emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) presented a global health challenge [1,2], exhibiting a spectrum of clinical manifestations ranging from mild respiratory symptoms to severe pneumonia and multi-organ dysfunction, COronaVIrus Disease 2019 (COVID-19) [3–5]. The intricate interplay between the new SARS-CoV-2 variants and the human immune system remains crucial for developing effective diagnostic biomarkers, therapeutic interventions, vaccines, and preventive strategies [5–8].

Subsequent to viral entry [1–5], a series of immunological events are set, activating innate and adaptive immune responses aimed at controlling viral replication and eliminating infected cells [9–17]. As of mid-2024, COVID-19 persists as a global health challenge, and

advancements in vaccines and ongoing public health efforts are essential to managing its impact in the future.

Literature highlighted various immune responses during SARS-CoV-2 infection and described a broad involvement of the immune system in contrasting this infection, offering new insights for a comprehensive understanding of the processes and mechanisms of susceptibility and resistance to the infection. Of particular relevance is the phenomenon known as "cytokine storm," characterized by an excessive release of pro-inflammatory cytokines, which can lead to endothelial, acute respiratory distress syndrome (ARDS), and multi-organ failure in COVID-19 [9–15].

Natural killer (NK) cells play a crucial role in the innate immune response against SARS-CoV-2 contributing to early antiviral defence [9–12,16]. CD8+ T cells, also called Cytotoxic T lymphocytes (CTLs), directly eliminate virus-infected cells [11–14,17]. CD4+ T cells, also known as T helper (Th) cells, play a crucial role in coordinating immune responses [17,18]. Th1 cells are pivotal in the adaptive immune response against SARS-CoV-2, since they activate the immune response by producing cytokines such as interferon-gamma (IFN-γ), which boosts macrophage phagocytic activity and antigen presentation and supports the differentiation in CTLs [9–13,18].

Several cytokines are described to be mainly involved in COVID-19 [11,12,19,20]. In this regard, tumor necrosis factor-alpha (TNF-α) contributes to the cytokine storm [9–13,15,21]. Interleukin (IL)-6 drives the inflammatory response, with elevated levels linked to severe COVID-19 and complications such as ARDS [9–13,22,23]. While IL-10 helps to mitigate excessive inflammation [24], its role in the immune response to COVID-19 is still controversial [9–13]. Th17 cells may foster the immune response against SARS-CoV-2 by producing IL-17 and other cytokines that recruit neutrophils and help in combating viral infection to preserve mucosal integrity [9–13,25,26]. IL-17 can also amplify inflammatory responses by inducing the production of other pro-inflammatory cytokines (e.g., IL-6, TNF-α) [25,26].

B lymphocytes (B cells) and antibodies are critical components of the immune response against COVID-19. When B cells encounter the SARS-CoV-2 virus, they produce virus-specific antibodies that neutralize the virus and prevent it from infecting cells. No significant association was found between mortality and IgG or IgM seroconversion or antibody concentrations. Patients with severe COVID-19 tend to develop an early and robust humoral immune response, characterized by the production of SARS-CoV-2-specific IgG antibodies [9–12,27–29].

Dysregulated immune responses, marked by hyperinflammation and cytokine storm, were implicated in the pathogenesis of severe COVID-19, leading to tissue damage, vascular dysfunction, and multi-organ failure. Conversely, an effective and coordinated immune response involving both innate and adaptive immune mechanisms is crucial for viral clearance and resolution of infection [1–7,30–32]. In this regard, the immune system comprises a complex network of cells and molecules that safeguard the host against pathogens, including viruses. Within this intricate system, several subsets of immune cells play a pivotal role in orchestrating immune responses [33–36].

Among lymphocytes, regulatory T cells (Tregs) garnered significant attention for their ability to modulate immune function and maintain tolerance to self-antigens while preventing excessive immune responses to foreign invaders [34–36]. Dysregulation of Tregs can lead to autoimmune diseases or immune suppression, affecting overall immune function [34–36].

Several T lymphocyte subpopulations co-expressing CD3 and CD56 molecules were identified [37–61]. The CD3+ CD56+ T cell subtype is a distinct group displaying both T cell (i.e., CD3) and NK (i.e., CD56) characteristics [33,37–56]. Within this group, natural killer T (NKT) cells are notable for bridging innate and adaptive immunity by recognizing lipid antigens presented by CD1d molecules and producing cytokines such as IFN-γ and IL-4 [37–45]. While NKT cells are well-studied, the roles of other CD3+ CD56+ T cell subtypes are unclear, although they are involved in cytotoxic activity, cytokine production, and

possibly in immune regulation and disease mechanisms [45–54]. These cells are elevated in conditions such as solid tumors, non-alcoholic fatty liver disease, autoimmune disorders, and haematological malignancies, where they may contribute to disease pathology [45–53] and are often cytotoxic effectors [54–56].

Recently, we described a regulatory role for a subtype of CD3+ CD56+ T cells, defined as T_{R3-56} [57–61]. These cells exhibit a unique metabolic phenotype, primarily relying on oxidative phosphorylation, and possess a distinct transcriptomic profile compared to NK, NKT, CD3+CD56-, and CD8+ T cells. Our original studies focused on type 1 diabetes (T1D), revealing that T1D patients had significantly reduced T_{R3-56} cells, correlating with increased CTL activation and disease severity [57]. Lower frequencies of T_{R3-56} cells were associated with decreased β-cell function and diabetic ketoacidosis, and in our T1D cohorts, T_{R3-56} cells were shown to suppress CTL functions in vitro by reducing intracellular reactive oxygen species, with their suppressive function and phenotype altered in T1D children. Our findings suggest T_{R3-56} cells play a regulatory role in modulating CTLs and could serve as a biomarker for monitoring immunological self-tolerance in T1D. In myelodysplastic syndromes (MDS), T_{R3-56} cells inversely correlated with cytotoxic T cell activation, suggesting a regulatory role also in bone marrow [58,59]. Similarly, increased TR3-56 cells, proportional to Tregs, may contribute to immune escape in chronic lymphocytic leukaemia (CLL) [61].

Therefore, we proposed the role of T_{R3-56} lymphocytes as a new cellular candidate in the immune regulation landscape [33].

Given the growing interest in the role of all CD3+ CD56+ T cell subtypes, it is relevant to further investigate their involvement in various disease models, particularly in viral infections, as recently highlighted in COVID-19 [62,63].

This retrospective study aims to explore the role of T_{R3-56} cells in SARS-CoV-2 infection during the first and second waves of the pandemic (March 2020–April 2021). Based on prior analyses of an established patient cohort [20,64], classified according to World Health Organization (WHO) criteria [65], the study specifically focused on individuals who never received any treatment before hospitalization. While SARS-CoV-2 serves as an initial model for this investigation, the ultimate goal is to further advance the study of T_{R3-56} cells in viral infections.

2. Results

2.1. The Immune Asset in the Patients Stratified on the Severity of COVID Disease

We categorized COVID-19 patients into three groups: Group 1 (WHO 3), Group 2 (WHO 4), and Group 3 (WHO 5, 6, and 7), following our previously described stratification [20] based on WHO criteria [65]. However, in the current study, we analyzed data exclusively from hospitalized patients who never received therapy or anti-SARS-CoV-2 vaccination prior to admission (see Section 4).

Based on the evaluation of the percentage of whole white blood cells (WBCs), the lymphocytes were significantly reduced in the Groups 2 and 3 with more severe clinical conditions (Figure 1a). No statistically significant differences are observed in the monocyte population (Figure 1b). Finally, neutrophils slightly increased in Group 2 compared to Group 1 (Figure 1c).

Focusing on the lymphocyte population among the WBCs, we observed a reduction in the percentage of T cells in Groups 2 and 3 compared to Group 1 (Figure 2a). CTLs were significantly lower in Group 2 compared to Group 1 and Group 3 (Figure 2a). Th lymphocytes decreased following the severity of COVID disease (Figure 2c). B lymphocytes progressively increased from Group 1 to Group 3 (Figure 2d). A significant increment in Group 3 was exhibited by NK lymphocytes (Figure 2e).

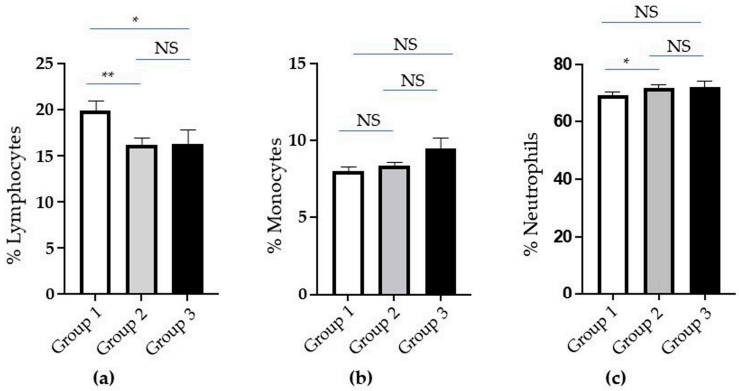

Figure 1. Analysis of white blood cells (WBCs) in groups of COronaVIrus Disease 2019 (COVID-19) patients based on increasing severity. (**a**) The percentage of whole lymphocytes, (**b**) monocytes, and (**c**) neutrophils in Groups 1 (white columns), 2 (grey columns), and 3 (black columns) of patients. Standard error (SE) bars are reported at the top of the columns. Statistical analysis (*Mann–Whitney test*) is reported: $p \leq 0.05$ (*); $p \leq 0.005$ (**); not significant (NS).

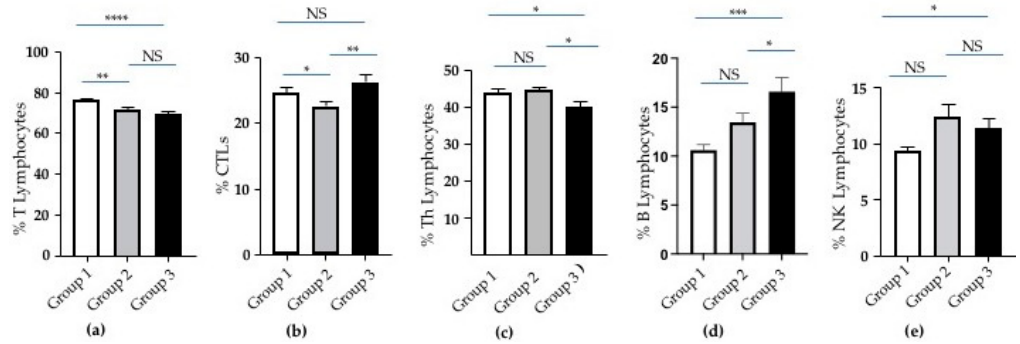

Figure 2. Analysis of lymphocyte subtypes in groups of COVID-19 patients based on increasing severity. (**a**) The percentage of T, (**b**) cytotoxic T cells (CTLs), (**c**) T helper (Th), (**d**) B, and (**e**) Natural Killer (NK) lymphocytes in Groups 1 (white columns), 2 (grey columns), and 3 (black columns) of patients. Standard error (SE) bars are reported at the top of the columns. Statistical analysis (*Mann–Whitney test*) is reported: $p \leq 0.05$ (*); $p \leq 0.005$ (**); $p \leq 0.0005$ (***); $p < 0.0001$ (****); and not significant (NS).

2.2. The Activated T Lymphocytes and the Treg and $T_{R3\text{-}56}$ Cells in the Patients Stratified on the Severity of COVID Disease

Since T lymphocytes appeared to decrease with the severity of clinical conditions (from Group 1 to Group 3), with an increase in CTLs in Group 3, we assessed the activation state of T lymphocytes by evaluating the Human Leukocyte Antigen (HLA)-DR expression on their cell surface (see Section 4) in the three groups. Intriguingly, the percentage of activated T lymphocytes significantly increased in Group 2 when compared to Group 1 (Figure 3a). Conversely, the percentage of the same cells in Group 3 was lower than in Group 2 and appeared similar to that in Group 1 (Figure 3a).

In addition, the percentage of Th1 cells was reduced in Group 3, when compared to Groups 1 and 2 (Figure 3b). Interestingly, we observed an increase in B cells in the same group (Figure 2d).

Th17 cell percentage significantly increased in Groups 2 and 3 when compared to Group 1 (Figure 3c).

Notably, a significant reduction in the percentage of Treg cells is evident in Group 3 (Figure 4a). Conversely, the percentage of $T_{R3\text{-}56}$ was significantly increased in Group 3, which expressed more severe clinical conditions (Figure 4b).

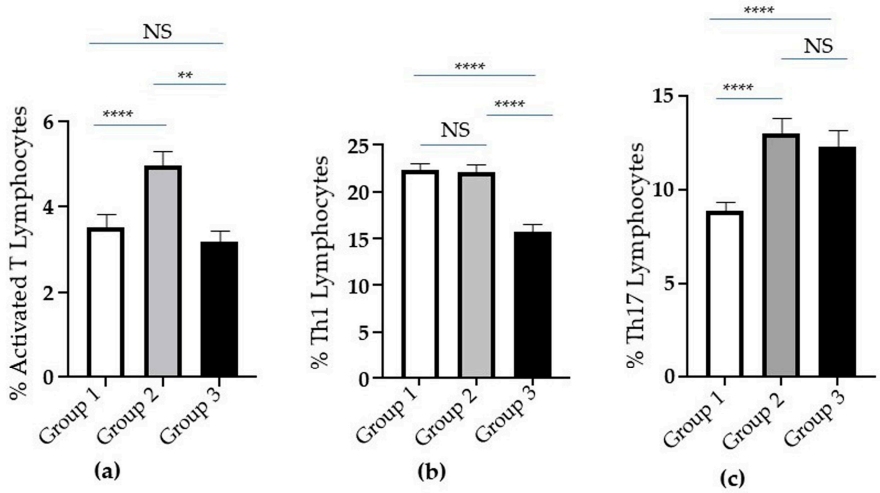

Figure 3. Analysis of activated T, Th1 and Th17 cells in groups of COVID-19 patients based on increasing severity. (**a**) The percentage of activated T, (**b**) Th1, and (**c**) Th17 lymphocytes in Groups 1 (white columns), 2 (grey columns), and 3 (black columns) of patients. Standard error (SE) bars are reported at the top of the columns. Statistical analysis (*Mann–Whitney test*) is reported: $p \leq 0.005$ (**); $p < 0.0001$ (****); and not significant (NS).

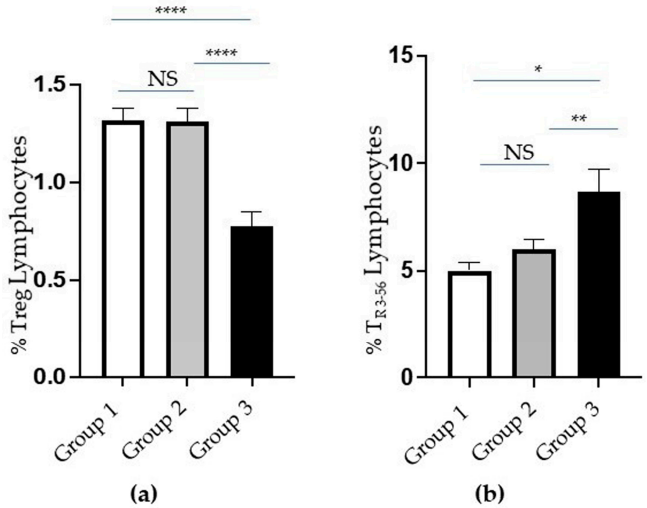

Figure 4. Analysis of T regulatory (Treg) and T_{R3-56} cells in groups of COVID-19 patients based on increasing severity. (**a**) The percentage of Treg and (**b**) T_{R3-56} lymphocytes in Groups 1 (white columns), 2 (grey columns), and 3 (black columns) of patients. Standard error (SE) bars are reported at the top of the columns. Statistical analysis (*Mann–Whitney test*) is reported: $p \leq 0.05$ (*); $p \leq 0.005$ (**); $p < 0.0001$ (****) and not significant (NS).

2.3. The Cytokines in the Patients Stratified on the Severity of COVID Disease

We analyzed the serum cytokine concentration in the three groups of patients (Table 1). Intriguingly, the TNF-α was significantly different between Group 1 and Group 2 ($p = 0.0011$) and between Group 1 and Group 3 ($p = 0.0075$). No statistic difference was observed between Group 2 and Group 3 (Table 1). In addition, no statistic differences were observed in IL-6, IL-17, and IL-10 concentrations between groups of patients (Table 1).

Table 1. Serum cytokine concentrations in Groups of COVID-19 patients based on increasing severity *.

	Group 1 Mean ± SE	Group 2 Mean ± SE	Group 3 Mean ± SE	Mann-Whitney (*p* Value)
TNF-α (pg/mL)	3.07 ± 0.04	9.45 ± 2.37	4.30 ± 0.61	**0.0011** group 1 vs. group 2 **0.0075** group 1 vs. group 3 NS group 2 vs. group 3
IL-6 (pg/mL)	49.61 ± 6.24	170 ± 50.71	52.77 ± 13.74	NS group 1 vs. group 2 NS group 1 vs. group 3 NS group 2 vs. group 3
IL-17 (pg/mL)	3.34 ± 0.27	4.86 ± 0.91	3.44 ± 0.61	NS group 1 vs. group 2 NS group 1 vs. group 3 NS group 2 vs. group 3
IL-10 (pg/mL)	7.31 ± 0.45	10.71 ± 1.93	11.65 ± 3.70	NS group 1 vs. group 2 NS group 1 vs. group 3 NS group 2 vs. group 3

* ELISA serum concentrations are reported. Significative values are reported in bold. Mean ± standard error (SE) and *p* value are reported.

2.4. The Patients Stratified on the Basis of a Cut-Off Calculated on the T_{R3-56} Cell Distribution

Given that the percentage of T_{R3-56} cells increased in patients with more severe clinical conditions, while the percentage of Treg cells appears reduced, we focused our attention on the T_{R3-56} regulatory level in our patient cohort.

In this regard, the percentage of T_{R3-56} cells in the enrolled patients ranged widely from 0.3 to 20.5. The mean percentage value was 6.3, with a standard deviation (SD) of 4.5 and a SE of 0.7. Therefore, it is a distribution with the percentage values spread over a very wide range. To identify the more relevant existing correlations between T_{R3-56} lymphocytes and the cells and molecules involved in the antiviral response, we arbitrarily focused on those patients whose percentage value was higher than the 75th percentile (8.2%) and above the mean $\pm$ 3 × SE (8.4%).

Adopting this criterion, we applied a cut-off of 8.4% to stratify the patients, resulting in a small group of patients (n = 24), with very high levels of T_{R3-56} cells (TR3-56^{High} Group).

Interestingly, this group is predominantly comprised of individuals from Group 2 (n = 8) and Group 3 (n = 14).

This finding supports the increase in CTLs observed in patients with more severe clinical conditions (Figure 2b) and the corresponding rise in T_{R3-56} cells in Group 3 (Figure 4b). Additionally, we observed a positive correlation between the percentage of T_{R3-56} cells and immune effector cells: CTLs and NK cells (Table 2). In addition, the percentage of T_{R3-56} negatively correlates to the monocytes in TR3-56^{High} Group (Table 2).

Table 2. T_{R3-56} cells positively correlate with Interleukin (IL)-17, Natural Killer (NK), and Cytotoxic T cells (CTLs) in the TR3-56^{High} Group of patients *.

	% T_{R3-56} Lymphocytes	
	Slope	***p* Value**
TNF-α (pg/mL)	0.1336	0.6730
IL-17 (pg/mL)	0.6786	0.0106
% Lymphocytes	−0.3719	0.0735
% Monocytes	−0.6431	0.0007
% Neutrophils	0.2351	0.2688
% T lymphocytes	−0.1228	0.5675
% B lymphocytes	−0.3902	0.0594
% NK lymphocytes	0.4456	0.0291
% CTLs	0.4507	0.0271

Table 2. *Cont.*

	% T_{R3-56} Lymphocytes	
	Slope	***p* Value**
% Th lymphocytes	−0.3846	0.0635
% Th1 lymphocytes	0.2662	0.2086
% Th17 lymphocytes	−0.1446	0.5001
% Treg lymphocytes	−0.2540	0.2311

* Spearman correlation is reported: r = slope; p = *p* value. The Spearman correlation coefficients range from −1 to +1. The sign of the coefficient (*r*) indicates whether it is a positive or negative monotonic relationship. A positive correlation means that as one variable increases, the other variable tends to increase as well. A negative correlation means that as one variable increases, the other tends to decrease. Values closer to −1 or +1 represent stronger relationships compared to values closer to zero. Significative values (*p*) are reported in bold.

Notably, the percentage of T_{R3-56} positively correlates to the IL-17A production (Table 2).

No correlations were observed between T_{R3-56} and the other cells (lymphocytes, neutrophils, T, Th, Th1, Th17, Treg, and B cells) and TNF-α (Table 2).

3. Discussion

The current study aimed to investigate the presence of T_{R3-56} cells [33] during the antiviral inflammatory response in COVID-19. By elucidating the role of these cells, research could expand on previous findings regarding CD3+CD56+ T cells in SARS-CoV-2 infections [62].

Our current analysis reveals that the overall lymphocyte population among WBCs was significantly lower in Groups 2 and 3 than in Group 1. Such evidence emphasizes that the trend was consistent across the entire cohort, regardless of treatment status.

A significant increment in Group 3 patients is exhibited by NK lymphocytes. This occurrence confirms that these effectors are involved in identifying and eliminating virus-infected cells in COVID-19 patients [9–13].

Additionally, Groups 2 and 3 exhibited reduced percentages of T cells.

Conversely, a significant increase in CTLs was revealed in Group 3, highlighting an active effort by the immune system to eliminate the SARS-CoV-2-infected cells. B lymphocytes progressively increased from Group 1 to Group 3, suggesting that an active humoral immune response against SARS-CoV-2 is aimed at neutralizing the virus [9–13,27]. Notably, the increase in both T cells and B cells correlated with disease severity in our cohort of patients.

Furthermore, Group 3 exhibited a reduced percentage of Th1 cells [9–13,18].

The percentage of Th17 cells [9–13,25,26] significantly increased in Groups 2 and 3 when compared to Group 1. This increase may signify the onset of chronic inflammatory conditions in patients at the most severe stage of COVID-19 [9–13,25,26].

Although T lymphocytes decreased with the severity of clinical conditions (from Group 1 to Group 3), they were accompanied by an increase in CTLs in Group 3. Therefore, we evaluated the activation status of T lymphocytes across the three groups. Interestingly, the percentage of activated T lymphocytes significantly increased in Group 2 compared to Group 1. Conversely, in Group 3, the percentage of activated T lymphocytes was lower than in Group 2 and similar to that in Group 1.

We investigated the presence of T lymphocytes involved in regulating immune responses, which could explain the observed reduction in lymphocyte activation status. In this context, it is noteworthy that the percentage of Treg cells [34–36] exhibits a significant decrease in Group 3.

The T_{R3-56} subset could provide novel insights, distinct from the cytotoxic role typically attributed to the CD3+ CD56+ T cell population in several diseases [37–56] and in SARS-CoV-2 infection [62,63].

Notably, the increased presence of $T_{R3\text{-}56}$ cells in Group 3 may suggest a compensatory response to heightened inflammation and immune activation driven by the observed increases in CTLs, B cells, and Th17 cells during severe phases in our patient cohort.

We analyzed serum cytokine concentration in the three groups of patients. Interestingly, TNF-α was significantly elevated in Groups 2 and 3. However, no statistical differences were observed in the levels of other cytokines between these patient groups. This finding suggests a potential role for TNF-α in influencing the severity of clinical conditions within these groups.

The concomitant increase in $T_{R3\text{-}56}$ cells along with the rise in CTLs, B cells, Th17 lymphocytes, and TNF-α suggests an overall activation of the immune system. $T_{R3\text{-}56}$ cells may play a regulatory role in balancing the immune response to prevent the excessive immune reactions, hyper-inflammation, and tissue damage observed in severe cases of COVID-19.

In patients not receiving therapy and belonging to the TR3-56^{High} Group, we observed that higher percentages of $T_{R3\text{-}56}$ correlate with elevated levels of CTLs and NK cells.

In addition, there is a positive correlation between the percentage of $T_{R3\text{-}56}$ cells and IL-17 levels in the TR3-56^{High} Group. IL-17 is produced by various immune cells, including Th17 cells, γδ T cells, natural killer T cells, and innate lymphoid cells [9–13,25,26]. No correlations were found between the other cell types and cytokines in the TR3-56^{High} Group.

The exacerbated immune response in COVID-19 necessarily involves an uncontrolled engagement of immune effectors and the release of pro-inflammatory molecules [1–7,9–13,17,19,25,26,32]. In this scenario, it is plausible to consider the observed increase in $T_{R3\text{-}56}$ cells in our cohort of patients as an attempt to mitigate the exacerbated immune response. Such occurrence points to the regulatory ability of $T_{R3\text{-}56}$ cells in COVID-19, as described in other pathological conditions [57,58,61].

Our findings highlight the need to further investigate the functional significance of $T_{R3\text{-}56}$ cells in the context of SARS-CoV-2 infection and other viral diseases. While our study provides preliminary evidence of a potential correlation between $T_{R3\text{-}56}$ cells and disease severity, additional research is necessary to fully understand the mechanisms behind this association and to explore the therapeutic implications.

4. Materials and Methods

4.1. Patients

All patients were clinically classified upon hospitalization according to the WHO classification [65]. Briefly, COVID infection was diagnosed using molecular analysis (RT-PCR) on nasopharyngeal swabs. Patients were classified according to the WHO ordinal scale, which categorizes their condition into Groups 1 to 7: (1) not hospitalized with normal activities; (2) not hospitalized but unable to resume normal activities; (3) hospitalized without supplemental oxygen; (4) hospitalized with supplemental oxygen; (5) hospitalized with high-flow oxygen, non-invasive ventilation, or both; (6) hospitalized with extracorporeal membrane oxygenation (ECMO), invasive ventilation, or both; and (7) death [65]. The study analyzed a cohort of 106 hospitalized COVID-19 patients with varying clinical severity, classified according to WHO severity categories 3 to 7. To ensure a robust retrospective analysis, we divided the patients into three groups: Group 1 (WHO Group 3), Group 2 (WHO Group 4), and Group 3 (WHO Groups 5, 6, and 7), following previously established methods [20,64]. This classification was based on the oxygen therapy requirements, reflecting the severity of the clinical condition of patients: Group 1 included patients not requiring supplemental oxygen (n = 22, 12 males and 10 females. Age range of 33-78, with a mean age of 56 years); Group 2 included those receiving supplemental oxygen (n = 60, 28 males and 32 females. Age range of 26–91, with a mean age of 62 years); Group 3 comprised patients needing high-flow oxygen, invasive ventilation, or ECMO (n = 24, 18 males and 6 females. Age range of 32–97, with a mean age of 69 years). In Group 3, 8 males died (age range of 63–95, with a mean age of 81 years). The number of recruited patients from the first COVID 19

wave was similar to that in the second wave (50 vs. 56), as is the distribution of patients from the first and second waves across Groups 1–3. To ensure that the patient population was not influenced by treatments, in the current retrospective evaluation we exclusively selected the data analysis of those hospitalized patients who did not previously undergo anti-inflammatory steroid therapy or azithromycin [66]. None of the patients received anti-SARS-CoV-2 vaccination prior to hospitalization.

Patients were admitted to the Section of Infectious Diseases of the University Federico II (Naples, Italy) during the first and second waves of the pandemic (2020–2021) [64]. Whole blood samples were collected at admission and after one week of hospitalization in tubes containing EDTA or free from anticoagulant and then immediately analyzed by flow cytometry. Serum samples were separated from blood cells after the collection.

Ethical approval for the study was obtained from the Ethical Committee of the University Federico II of Naples (protocol code 138/20, 14 April 2020). The study was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

4.2. Flow Cytometry

Immunophenotyping analysis was performed by multicolour flow cytometry, as described [20]. Briefly, CD45 was used to gate the viable lymphocyte cells. From this gate, CD3+ CD4+ cells were identified as Th, while CD3+ CD8+ as CTLs. Among Th, Th1 and Th17 were distinguished by specific surface markers, i.e., CXCR3 and CCR6, respectively. Moreover, human leukocyte antigen DR (HLA-DR) molecules were used as activation markers expressed on activated T lymphocytes. CD3 and CD45, and CD56 and CD19 were used to T (CD3+ CD45+), NK (CD45+ CD3- CD56+), and B (CD45+ CD19+) cell distribution for each patient. Treg cells were identified as CD3+ CD4+ $CD25^{High}$ $CD127^{low}$, and $T_{R3\text{-}56}$ as CD3+ CD56+, as described [57].

4.3. Serum Cytokine Analysis

Serum IL-17A, IL-6, IL-10 and TNF-α levels were analyzed using human-specific enzyme-linked immunosorbent assay (ELISA) Max™ Set Deluxe kits (BioLegend, Inc., San Diego, CA, USA), as described [20]. The concentration values (pg/mL) of each cytokine were obtained by interpolating the absorbance values on the respective calibration curve.

4.4. Statistics

The statistical analysis was performed using the *Mann–Whitney test* to compare the differences between Group 1 and Group 2, Group 1 and Group 3, and Group 2 and Group 3. The correlations between variables were evaluated by Spearman's rank-order correlation and Spearman's rank correlation coefficient (rs) was calculated. Statistical analysis and graphics were performed by Prism 9, GraphPad Inc. (San Diego, CA, USA). p values < 0.05 were considered as significant.

5. Conclusions

Our findings offer valuable insights into the complex immunological dynamics of SARS-CoV-2 infection and underscore the potential role of $T_{R3\text{-}56}$ cells as a remarkable component in the immune response against SARS-CoV-2.

Our findings might suggest the regulatory ability of $T_{R3\text{-}56}$ cells in COVID-19. However, these cells could also be involved in cytotoxic and antiviral secretory functions, potentially serving as effector cells in this context, as suggested by other studies on CD3+ CD56+ T cells [37–56]. It is reasonable to hypothesize that $T_{R3\text{-}56}$ cells might adapt their regulatory functions and exhibit additional effector roles in specific contexts, such as during infections or inflammations.

This intriguing hypothesis, which suggests the remarkable plasticity of the immune system [66,67], requires support from studies demonstrating this mechanism.

In this regard, studies on Tregs also suggested a versatile role: beyond their traditional function of suppressing immune responses to prevent autoimmune diseases and maintain immune balance, Tregs were found to exhibit dynamic functionalities [68,69]. Tregs also demonstrate cytotoxic activity against tumor cells through granzyme-dependent mechanisms [70]. This newly described ability enables them to directly target and eliminate tumor cells, which contrasts with their conventional role as immune suppressors. Moreover, Tregs can interact with non-immune cells and reside in non-lymphoid tissues, where they perform non-immunological functions primarily related to tissue repair and organ homeostasis [36].

Similarly, CD3+ CD56+ T cell subtypes, including the proposed $T_{R3\text{-}56}$ cells, may play a dynamic role in the plasticity of the immune response [44–50,52–56]. These cells could adapt to different peripheral tissue environments, where they may exhibit both significant suppressive effects on effector lymphocytes and engage in complementary effector functions that enhance immune responses. Therefore, considering both perspectives, our hypothesis is that $T_{R3\text{-}56}$ cells may play a dual role in the immune response to SARS-CoV-2 infection. On one hand, their regulatory function can help mitigate excessive inflammation and tissue damage, thereby contributing to immune homeostasis and facilitating tissue repair processes. On the other hand, the heightened presence of $T_{R3\text{-}56}$ cells in severe cases of COVID-19 may also reflect a broader immune response aimed at contrasting the viral infection.

Although this retrospective study may have limited clinical applicability due to the numerous mutations in SARS-CoV-2 and the evolving nature of COVID-19 since the first and second waves, its primary goal is to enhance our understanding of $T_{R3\text{-}56}$ cells. The study aims to elucidate their potential role in viral infections and propose their use as biomarkers. Moving forward, expanding our knowledge of $T_{R3\text{-}56}$ cells, as well as CD3+ CD56+ T cells and their interactions with the immune system, could facilitate the development of targeted therapies for managing infections and other diseases where these cells are critically involved.

Study Limitations

Being a retrospective analysis, our study did not evaluate the functional effects of $T_{R3\text{-}56}$ cells, which limits our ability to assess their potential regulatory capacity or effector functions. Additionally, the study did not compare the data with a control group of healthy and/or pre-pandemic subjects, as the focus was on comparing patients with varying degrees of disease severity. No correlation was analyzed based on gender and age of the subjects in Groups 1–3. No evaluation was conducted on the SARS-CoV-2 strains infecting the patients. Comparative analysis with other viral infections beyond SARS-CoV-2 was not performed. Finally, the study did not include a longitudinal assessment of the patients, as all of them, following hospitalization, underwent therapies capable of altering the immune response.

Author Contributions: Conceptualization, G.C., G.R. and G.T.; methodology, F.C., M.G., G.S. and V.R.; formal analysis, F.C., G.C., G.R. and G.T.; investigation, F.C., M.G., G.S., M.R. and V.R.; resources, G.C., G.R. and G.T.; data curation, F.C., G.C., G.R., G.T., M.G. and V.R.; writing—original draft preparation, F.C., G.C., G.R. and G.T.; writing—review and editing, G.C., M.C., G.R. and G.T.; supervision, G.C., G.R., G.T. and M.C.; clinical management, I.G., B.P.; project administration, G.C., G.R. and G.T.; funding acquisition, G.C., G.R., G.T. and G.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical approval for the study was obtained from the Ethical Committee of the University Federico II of Naples (protocol code 138/20, 14 April 2020). All experiments were performed in accordance with relevant guidelines and regulations. The study has been performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the privacy.

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T_{R3-56} and Treg Regulatory T Cell Subsets as Potential Indicators of Graft Tolerance Control in Kidney Transplant Recipients

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Abstract: Identification of early signatures of immune rejection represents a key challenge in the clinical management of kidney transplant. To address such an issue, we enrolled 53 kidney transplant recipients without signs of graft rejection, no infectious episodes and no change in the immunosuppressive regimen in the last 6 months. An extensive immune profile revealed increased activation of the T cells, a decreased amount and growth ability of the Treg and a higher level of the T_{R3-56} regulatory T cell subset, described by us as involved in the preferential control of cytotoxic T lymphocytes. In renal transplant recipients, the high level of the T_{R3-56} cells associates with a reduction in both the amount and the growth ability of the Treg. Moreover, when the transplanted subjects were categorised according to their stable or unstable disease status, as defined by changes in serum creatinine $\geq$0.2 mg/dL in two consecutive detections, a higher T_{R3-56} level and defective Treg growth ability were observed to characterise patients with unstable graft control. Further studies are required to substantiate the hypothesis that immune profiling, including T_{R3-56} evaluation, might represent a valuable diagnostic tool to identify patients at risk of developing significant anti-donor allo-immune responses.

Keywords: regulatory T cells; Treg; T_{R3-56}; kidney transplant recipients; immune profile

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1. Introduction

Immune-mediated processes have been largely observed to underlie kidney homeostasis maintenance, also playing a key role in the progression of chronic kidney disorders. Indeed, the derangement of the complex balance involving regulatory cell subsets and the activity of adaptive immune effectors have been considered critical for the pathogenesis of kidney diseases [1].

Kidney transplantation represents the main therapeutic option to control end-stage kidney disease [2]. In this context, recognition of allo-specificities by recipient immune effectors, despite the progress in immune-modulating approaches, remains a leading cause of allograft injury and loss [3]. Although the antibody-mediated (humoral) rejection is considered the leading cause of progressive graft dysfunction and loss [4,5], the involvement of T cell-dependent immunity in both early and late events contributing to graft rejection remains a critical issue [4]. Indeed, the key role of helper T cells in the activation of an effective humoral response has been largely established [6,7]. In this context, the availability of valuable criteria to identify in time early immune-mediated injuries in kidney transplant recipients represents a still unmet target.

Fine-tuning of the immune response is usually obtained by multiple regulatory processes, all belonging to the immune tolerance network [8–10] whose perturbation is expected to associate with immune-mediated tissue injury. In this context, the key role of regulatory populations in the prevention of immune-dependent damaging events has been largely shown [11].

Regulatory cells represent a heterogeneous group of differentiated T cell subsets including the $CD25^+CD4^+$ regulatory T (Treg) cells, constitutively expressing the forkhead box P3 (Foxp3) transcription factor [12]. This cell subset has been largely demonstrated to control the immune-effector response in terms of clonal expansion, differentiation, cytokine profile and tissue migration and is indispensable for the maintenance of immune self-tolerance [13]. Recently [14,15], we described how human T cells co-expressing CD3 and CD56 molecules represent a novel regulatory subset, the $T_{R3\text{-}56}$, able to preferentially modulate cytotoxic function and cytokine production by cytotoxic T cells (CTLs). The involvement of $T_{R3\text{-}56}$ in the pathogenesis of Type 1 diabetes [14] as well as in immune-mediated haematological disorders has also been described by us [16,17] and by others [18].

CTLs play a relevant role in mediating transplant damage [19–21]. In this context, the expression of activation molecules like CD25, CD69, CD154, CD95 on the surface of circulating CTLs from kidney recipients, the increased frequency of terminally differentiated memory CD8 T cells [20] and a deranged Treg/CTL ratio in the transplanted tissue [22,23] have already been proposed as relevant markers to identify early immune-mediated injuries in kidney transplant recipients. Since the $T_{R3\text{-}56}$ regulatory T cells have been observed to preferentially control CTL functions [14–17], the possibility that these cells may be involved in the control of immune-mediated damaging processes of kidney grafts needs to be explored.

Therefore, we analysed the immune profile of 53 kidney transplant recipients without signs of graft rejection, with no infectious episodes and no change in the immunosuppressive regimen in the last 6 months. Our analysis, in order to investigate the immune profile features, likely associated with early immune-mediated injuries of the transplanted kidney, focused on circulating immune regulatory T cell subsets, as represented by the Treg and the $T_{R3\text{-}56}$ populations, as well as the adaptive and innate immune effectors.

2. Results

2.1. Higher Amount of Circulating $T_{R3\text{-}56}$ Regulatory T Cells and Decreased Level of the Treg Lymphocytes Characterise Kidney Transplanted Subjects Showing No Signs of Graft Rejection

Figure 1 and Supplementary Figure S1 depict the comprehensive immune profile analysis performed in the cohort of 53 kidney transplant recipients showing no signs of graft rejection, no infectious episodes and no change in the immunosuppressive regimen in the last 6 months, in comparison with 20 age/sex matched healthy controls.

As shown in Figure 1A–C, comparative analysis with controls showed that $CD4^+$ T cells were significantly decreased in transplant recipients both as a percentage (67.04 $\pm$ 1.74 in controls vs. 52.17 $\pm$ 2 in the kidney recipients; $p < 0.0001$), and as an absolute number (977 $\pm$ 30.52 10^9/L in controls vs. 765 $\pm$ 59.11 10^9/L in the kidney recipients; $p < 0.0001$). Concurrently, there was an increase in the circulating CTL percentage (33.47 $\pm$ 0.88 vs. 47.83 $\pm$ 2 in the kidney recipients; $p < 0.0001$) and number (486.5 $\pm$ 18.19 10^9/L vs. 678.3 $\pm$ 60.96 10^9/L; $p < 0.0001$), and a decrease in the CD4/CD8 ratio (2.16 $\pm$ 0.15 in controls vs. 1.23 $\pm$ 0.09 in the kidney recipients; $p < 0.0001$).

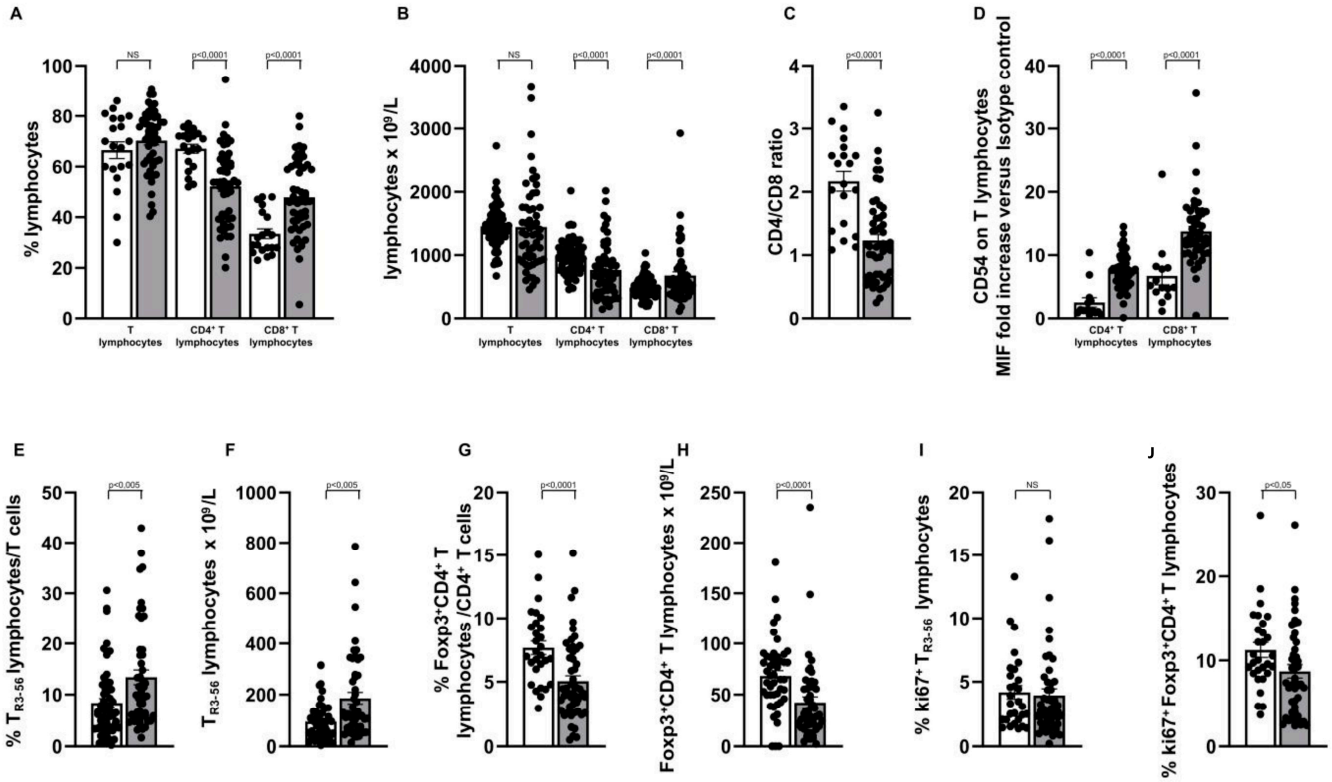

Figure 1. Increased amount of the circulating CTL and of the $T_{R3\text{-}56}$ regulatory T cells, higher expression of the CD54 activation molecule on T cell effectors and decreased amount and growth ability of the Treg subset characterise a cohort of allograft kidney recipients showing no rejection episodes, no infections and no changes in immuno-suppression therapy in the previous six months. White and grey columns indicate data obtained in healthy controls and kidney transplanted subjects, respectively. (**A**,**B**) Indicate percentage and number of circulating T, $CD4^+$ and $CD8^+$ T lymphocytes, as indicated; (**C**) Indicates CD4/CD8 ratio; (**D**) Refers CD54 expression level in $CD4^+$ and $CD8^+$ T lymphocytes, as indicated; as detailed in the Section 4, CD54 expression on the T cell effectors has been expressed as ratio of the mean intensity fluorescence (MIF) value for $CD4^+$ and $CD8^+$ T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb, as described [24]. (**E**,**F**) Indicate percentage and number of the circulating $T_{R3\text{-}56}$ regulatory T cells, respectively; (**G**,**H**) Refer to percentage and number of the circulating Treg population; (**I**,**J**) Indicate the growth ability of the circulating $T_{R3\text{-}56}$ and Treg populations, as represented by their intracellular expression of the ki67 molecule; Statistical evaluation of data was performed by means of the Mann–Whitney test. Statistical significance values are indicated.

Analysis of the surface expression of the CD54 molecule (Figure 1D), largely associated with antigen-dependent triggering of the T cells [25,26], revealed significant activation of both, $CD4^+$ (2.51 ± 0.75 in controls vs. 7.44 ± 0.37 in the kidney recipients; $p < 0.0001$) and CTL (6.72 ± 1.39 in controls vs. 13.77 ± 0.76 in the kidney recipients; $p < 0.0001$). We then focused on the regulatory subsets, as represented by the Treg as well as by the $T_{R3\text{-}56}$ T cell population. In comparison with healthy subjects (Figure 1E,F), the $T_{R3\text{-}56}$ regulatory T cell subset was significantly increased in the transplant recipients, both as a percentage (8.32 ± 0.89 in controls vs. 13.40 ± 1.43 in the kidney recipients; $p < 0.005$) and as a number ($94.05 \pm 10.61\ 10^9$/L in controls vs. $185.6 \pm 23.34\ 10^9$/L in the kidney recipients; $p < 0.005$) whereas Treg lymphocytes (Figure 1G,H) were reduced in transplanted subjects either as a percentage (7.66 ± 0.53 in controls vs. 5.05 ± 0.42 in the kidney recipients; $p < 0.0001$) or as a number ($68.37 \pm 5.24\ 10^9$/L in controls vs. $42.13 \pm 5.54\ 10^9$/L in the kidney recipients; $p < 0.0001$).

A key feature of the Treg subset is its high growth ability [27,28]. Thus, we evaluated this parameter in both circulating regulatory T cell subsets (Treg and T_{R3-56}), by detecting their ki67 intracellular expression. As shown, we observed (Figure 1J) a significant reduction in the ki67 intracellular expression in the circulating Treg lymphocytes of transplant recipients (11.23 ± 0.89 in controls vs. 8.73 ± 0.77 in the kidney recipients; $p < 0.05$). However, (Figure 1I) no significant difference (4.17 ± 0.54 in controls vs. 3.93 ± 0.51 in the kidney recipients) was revealed in the growth ability of the T_{R3-56} subset in comparison with healthy controls.

As shown in the Supplementary Figure S1, significantly decreased levels of the B lymphocytes (Figure S1A,B), a lower percentage of circulating iNKT cells (Figure S1C), no difference in the number of iNKT (Figure S1D), as well as in the percentage and amount of the circulating NK effectors (Figure S1E,F) were observed in the transplant recipients, in comparison with controls. Moreover, the comparative analysis of the growth ability of innate and adaptive lymphocytes between kidney recipients and controls revealed no significant difference in ki67 intracellular expression by $CD4^+$ and CTL T cells (Figure S1G,H), or in B lymphocytes (Figure S1I) or NK effectors (Figure S1J).

Thus, as compared with healthy controls, the immune profile of the cohort of kidney transplant recipients revealed the presence of activated adaptive effectors, of an increased amount of the T_{R3-56} lymphocytes and a decreased level and growth ability of the Treg subpopulation; in addition, increasing CTL, a reduced level of $CD4^+$ T cells, of B cells and of iNKT lymphocytes, was observed.

2.2. Decreased Growth Ability of the Treg Subset Characterises the Subgroup of Transplanted Subjects with a Higher Amount of the Circulating T_{R3-56} Regulatory T Cells

The cohort of kidney transplant recipients was characterised by increased activation of the CTL, higher levels of circulating T_{R3-56} regulatory T cells and a decreased amount and growth ability of the Treg population. Accordingly, we focused our investigation on the immune profile associated, in our cohort, with the highest levels of the circulating T_{R3-56} lymphocytes. In this context, kidney transplant recipients were categorised according to their level of circulating T_{R3-56} in two sub-groups characterised by a T_{R3-56}/T cell ratio higher or similar to the controls. The cut-off value (9.16% of the T lymphocytes) was arbitrarily established, as detailed in the Patients and Methods Section, by increasing by three SEM the median value observed in the healthy individuals enrolled in the study.

Light and dark grey columns show, in Figure 2, the results obtained in the group of transplant recipients characterised by T_{R3-56} lymphocyte levels similar to (≤9.16% of the T lymphocytes) or higher (>9.16% of the T lymphocytes) than the controls, respectively. Figure 2A,B show that kidney transplant recipients, regardless of the amount of circulating T_{R3-56} regulatory T cells, showed a decreased amount of the $CD4^+$ T cells, increasing levels of the CTL, a reduced CD4/CD8 ratio (Figure 2C) and a lower percentage and number of the Treg population (Figure 2E,F). Moreover, as depicted in Figure 2D, increasing activation of the T cell effectors, as evaluated by their CD54 expression, was found in the patients, as compared with the controls; however, a more consistent increase in CD54 expression was observed in the $CD4^+$ T lymphocytes of the transplant recipients with a higher T_{R3-56} level (6.39 ± 0.61 in the kidney recipients with a T_{R3-56} level ≤ 9.16% of the T lymphocytes vs. 8.13 ± 0.43 in the kidney recipients with T_{R3-56} > 9.16% of the T lymphocytes; $p < 0.05$).

As shown in Supplementary Figure S2A,B, the significant decrease in circulating B cells, found in the kidney transplant recipients versus controls, has been found to be more consistent in the subgroup of transplant recipients characterised by higher circulating T_{R3-56} regulatory T cells (6.70 ± 0.95 in the kidney recipients with a T_{R3-56} level ≤9.16% of the T lymphocytes vs. 4.30 ± 0.56 in the kidney recipients with the highest T_{R3-56} level; $p < 0.05$). Conversely, no difference was found in the level of iNKT (Supplementary Figure S2C,D) and of NK lymphocytes (Supplementary Figure S2E,F), in the patients, as compared with the controls, regardless of their level of circulating T_{R3-56} lymphocytes. Similarly, (Supplementary Figure S2G–J), no difference was revealed in the growth ability of $CD4^+$, CTL, iNKT and

B lymphocytes between controls and patients, independently of their level of circulating $T_{R3\text{-}56}$ lymphocytes.

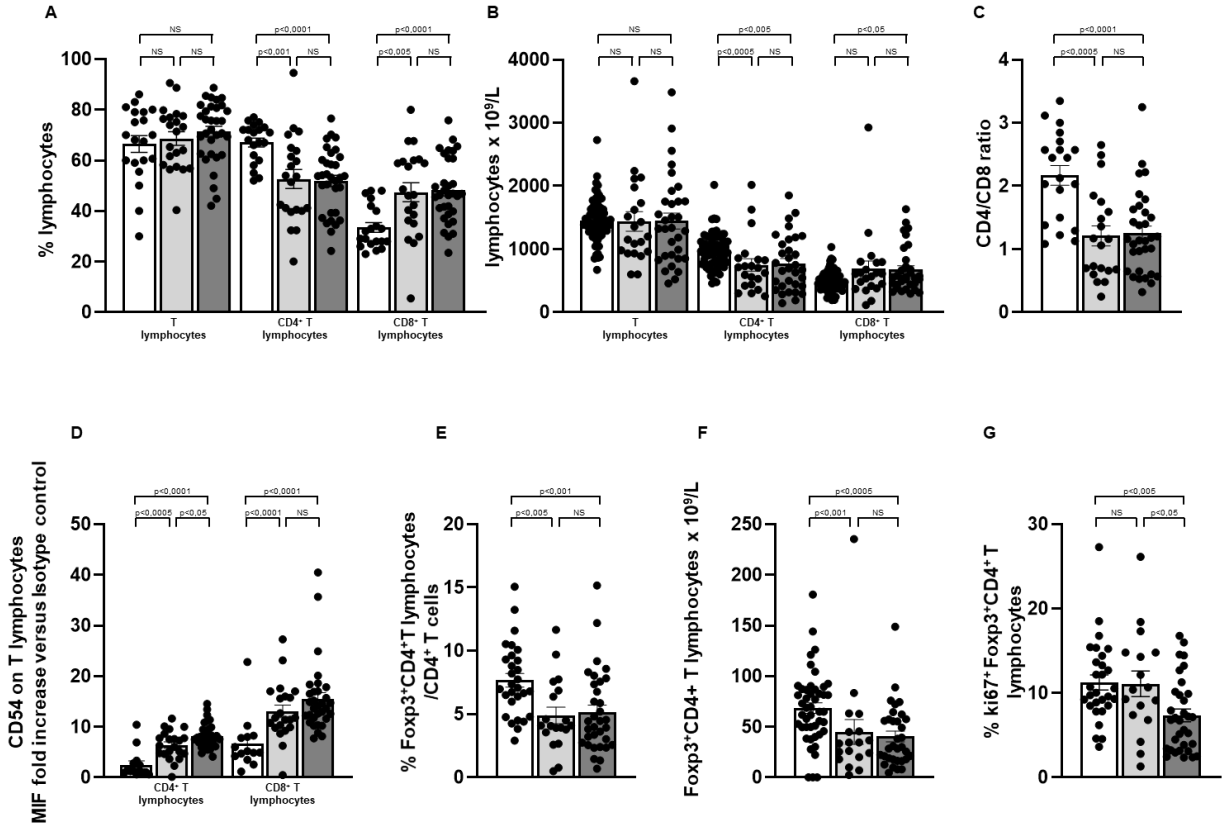

Figure 2. Allograft kidney recipients show association of highest level of circulating $T_{R3\text{-}56}$ regulatory T cells with significant decrease in the Treg growth ability and increasing CD54 expression by the $CD4^+$ T cell population. White columns indicate healthy controls; light and dark grey columns indicate transplanted subjects showing circulating $T_{R3\text{-}56}$ levels <9.16% or ≥9.16% of the T cell population, respectively; the 9.16 cut-off value was obtained by increasing by three standard errors the median value observed in healthy controls (see patient and method section for details). (**A**,**B**) Indicate percentage and number of circulating T, $CD4^+$ and $CD8^+$ T lymphocytes; (**C**) Shows CD4/CD8 ratio; (**D**) Refers to CD54 expression level in $CD4^+$ and $CD8^+$ T lymphocytes; as detailed in Section 4, CD54 expression on the T cell effectors was expressed as a ratio of the mean intensity fluorescence (MIF) value for $CD4^+$ and $CD8^+$ T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb. (**E**,**F**) Indicate the percentage and number of the circulating Treg in the different cohorts; (**G**) Indicates the growth ability of the circulating Treg population, as represented by their intracellular expression of the ki67 molecule; Statistical evaluation of the data was performed by means of the Mann–Whitney test. Statistical significance values are indicated.

Notably (Figure 2G), only the kidney transplant recipients showing a higher $T_{R3\text{-}56}$ level (>9.16% of the T lymphocytes) revealed a significant decreased growth ability of the Treg subset (11.08 ± 1.50 in the kidney recipients with a $T_{R3\text{-}56}$ level ≤ 9.16% of the T lymphocytes vs. 7.34 ± 0.75 in the kidney recipients with the highest $T_{R3\text{-}56}$ level; $p < 0.05$).

Thus, in renal transplant recipients, higher $T_{R3\text{-}56}$ levels are preferentially associated with increased activation of the $CD4^+$ T cells and decreased growth ability of the Treg subset.

2.3. The Presence of Higher $T_{R3\text{-}56}$ Levels in Kidney Transplanted Subjects Showing No Signs of Graft Rejection Associates with Early Signs of Unstable Graft Tolerance

Our immune profile analysis showed that, in our cohort of kidney transplant recipients, a higher amount of the circulating $T_{R3\text{-}56}$ regulatory T cells preferentially associates with a

decreased number and growth ability of the Treg, as well as with increased activation of the $CD4^+$ T lymphocytes. Thus, we investigated the possibility that the level of circulating $T_{R3\text{-}56}$ might represent a valuable criterion to identify kidney transplant recipients showing early signs of unstable control of the graft. With this aim, the kidney recipients were classified, according to their laboratory and clinical data, into a Stable and Unstable group, as detailed in the Patient and Method Section. Briefly, the Stable group was represented by subjects with a stable renal function and urinary parameters, while the Unstable group included patients showing changes in the serum creatinine level $\geq$0.2 mg/dL and/or proteinuria > 100 mg/day in 24-h urinary samples in two consecutive evaluations, despite no clinical predisposing conditions.

As shown in Figure 3, comparative analysis of the immune profile of the kidney recipients, grouped according to their Stable versus Unstable clinical conditions, revealed a significant difference between the two patient subgroups in the CTL number (546.89 $\pm$ 38.84 $\times$ 10^9/L in the kidney recipients with a Stable disease vs. 713.8 $\pm$ 80.43 $\times$ 10^9/L in the kidney recipients with an Unstable disease; $p < 0.05$), higher in the individuals belonging to the Unstable disease group, also showing (Figure 3B,C), a lower CD4/CD8 ratio (1.45 $\pm$ 0.14 in the kidney recipients with a Stable disease vs. 0.99 $\pm$ 0.09 in the counterparts with an Unstable disease; $p < 0.05$).

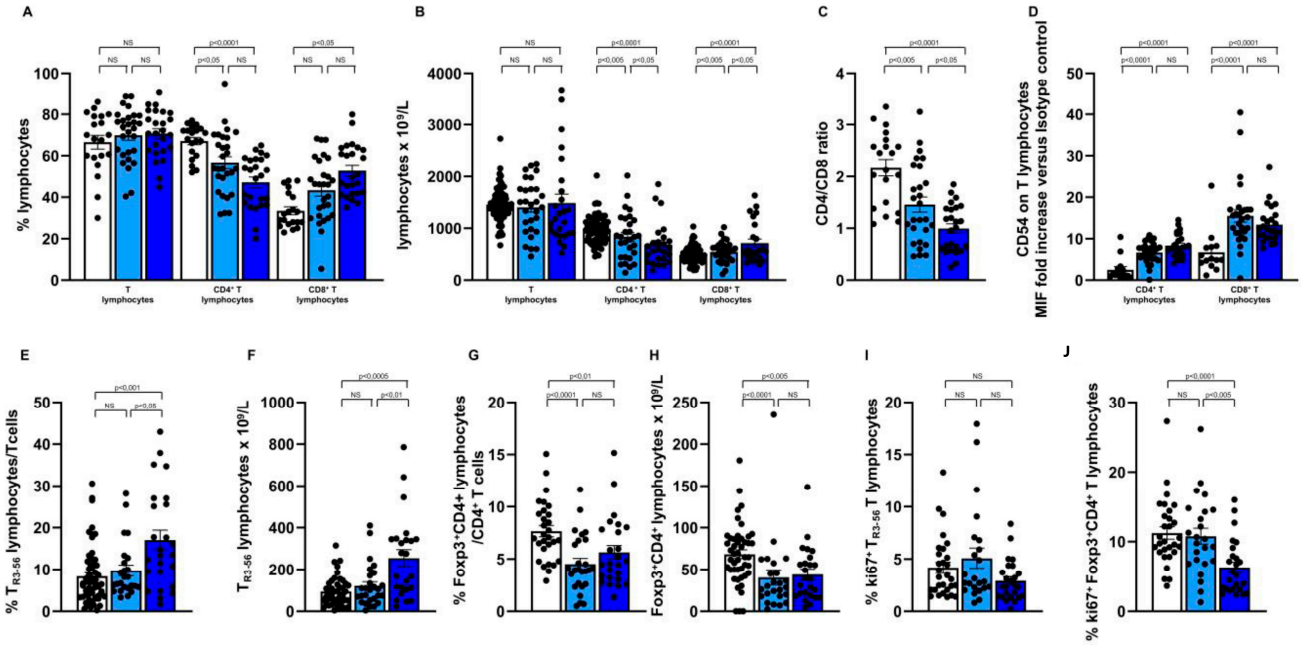

Figure 3. Increasing amount of circulating $T_{R3\text{-}56}$ lymphocytes and reduced growth ability of the Treg population characterise kidney transplant recipients showing unstable control of the graft. White columns indicate healthy controls; light and dark blue columns indicate transplanted subjects categorised, according to their clinical and laboratory profile, as belonging to the Stable or Unstable transplant recipient sub-groups, respectively. See Patient and Methods section for details. (**A**,**B**) Indicate percentage and number of circulating T, $CD4^+$ and $CD8^+$ T lymphocytes; (**C**) Indicates CD4/CD8 ratio; (**D**) Refers to CD54 expression level in $CD4^+$ and $CD8^+$ T lymphocytes; CD54 expression on the T cell effectors was expressed as a ratio of the mean intensity fluorescence (MIF) value for $CD4^+$ and $CD8^+$ T cells and the control MIF value obtained after staining the same cell populations with the isotype control mAb, as described [28]. (**E**,**F**) Show percentage and number of the circulating $T_{R3\text{-}56}$ lymphocytes; (**G**,**H**) Show percentage and number of circulating Treg; (**I**,**J**) Indicate the growth ability, as represented by their expression of the ki67 molecule, of the circulating $T_{R3\text{-}56}$ and Treg population, respectively; Statistical evaluation of data was performed by means of the Mann–Whitney test. Statistical significance values are indicated.

As shown (Figure 3E,F), a significant increase in the circulating $T_{R3\text{-}56}$ cells as a percentage (9.75 ± 1.31 in the kidney recipients with the Stable disease vs. 17.04 ± 2.36 in the counterparts with an Unstable disease; $p < 0.05$) as well as number (120.6 ± 19.27 10^9/L in the kidney recipients with Stable disease vs. 254.8 ± 40.32 10^9/L in the subgroup with Unstable disease; $p < 0.01$), associated with (Figure 3J), significant reduced Treg growth ability (10.77 ± 1.14 in the kidney recipients with Stable disease vs. 6.29 ± 0.78 in the kidney recipients with Unstable disease; $p < 0.005$), characterise the patient subgroup with Unstable disease. No difference has been observed in the amount of B cells, iNKT and NK lymphocytes (Supplementary Figure S3A–F) as well as in the growth ability of adaptive and innate immune effectors (Supplementary Figure S3A–F) comparing the subgroup of transplanted subjects with a Stable disease with the counterparts with an Unstable disease.

To investigate whether a high level of the circulating $T_{R3\text{-}56}$ cells, as defined by increasing by three SEM the median value obtained in healthy controls, might be significantly associated with an unstable graft control, we analysed the presence of circulating $T_{R3\text{-}56}$ cells at a percentage > 9.16% of T lymphocytes in kidney transplant recipients categorised in the Unstable versus the Stable disease subgroup, as defined in the Patients and Methods Section. As shown (Table 1), 19 out of the 25 subjects with an Unstable disease showed a $T_{R3\text{-}56}$ cell percentage > 9.16% of circulating T lymphocytes (defined higher $T_{R3\text{-}56}$ level), as compared with 10 out of the 25 subjects with a Stable renal disease ($p < 0.05$ by Fisher exact test; Odd Ratio 4.75; 95% CI 1.384 to 14.37).

Table 1. Higher levels of the circulating $T_{R3\text{-}56}$ cells significantly associate with Unstable disease [1] condition in a cohort of kidney transplanted subjects showing no infections, no rejection episodes and no changes in immuno-suppression therapy in the previous six months.

	N	Age Mean (Range)	Males/Females	$T_{R3\text{-}56}$ /T Cells <9.16 [2]	$T_{R3\text{-}56}$ /T Cells >9.16 [2]
	50	51.82 (35–68)	31/19	21	29
Stable Disease [1]	25	53.54 (38–68)	15/10	15 [3]	10 [4]
Unstable Disease [1]	25	50.32 (35–67)	16/9	6	19

[1] subgroup categorisation criteria have been detailed in the Patients and Method Section; [2] the number has been obtained increasing of three SEM the median value found in the healthy controls for $T_{R3\text{-}56}$ percentage/T lymphocytes (see Patient and Method Section for details); [3] significant different from the Unstable disease Group ($p < 0.05$ by Fisher exact test; Odd Ratio 0.21 (95% CI: 0.0695 to 0.722); [4] significant different from the Unstable disease Group ($p < 0.05$ by Fisher exact test; Odd Ratio 4.75 (95% CI 1.384 to 14.37).

Such data suggest that the level of circulating $T_{R3\text{-}56}$ cells might represent a valuable criterion to identify kidney transplant recipients with early signs of immune mediated damages to the graft, in the absence of any clinical and/or laboratory rejection treat.

3. Discussion

This study reveals that, in a cohort of 53 kidney transplant recipients not showing any clear clinical/laboratory sign of kidney rejection, higher levels of circulating $T_{R3\text{-}56}$ regulatory T cells are associated with unstable control of the transplanted kidney. This instability condition was unveiled by changes in the serum creatinine level ≥0.2 mg/dL and/or in proteinuria >100 mg/day in 24-h urinary samples in two consecutive bi-monthly evaluations, despite no clinical predisposing conditions. These findings propose that the evaluation of the circulating $T_{R3\text{-}56}$ T cell subset might serve as a potential indicator of early immune-mediated processes that could potentially impact graft tolerance.

Kidney transplant represents a major therapeutic option to effectively treat renal end-stage disease [1,2]. In this context, despite advancements in immune-modulating approaches, immune-mediated damaging processes continue to pose a significant challenge for allograft injury and loss. Moreover, valuable criteria to identify in time early immune-mediated injuries in kidney transplant recipients are still lacking.

The key role of T lymphocytes, able to orchestrate the whole immune response by controlling both humoral and cytotoxic activities, has been largely demonstrated [6,7].

T cell-dependent processes have been described as depending on multiple immune-regulatory networks involving different regulatory T cell subsets [10–13], only in part characterised by the expression of the Foxp3 transcription factor [15,29–33]. Moreover, the role of multiple inflammatory pathways, early after transplantation [34] and in long-term transplant recipients [35–38], has been largely referred to.

Co-expression of CD3 and CD56 molecules has been revealed by us to define the $T_{R3\text{-}56}$ regulatory T cell subset, preferentially involved in the control of cytotoxic T cell effectors in autoimmunity [14] as well as in haematologic disorders [16–18]. This study investigates the $T_{R3\text{-}56}$ subpopulation in a cohort of 53 kidney allograft recipients showing no signs of graft rejection, no infectious episodes and no change in their immunosuppressive regimen in the last 6 months.

Our extensive comparative immune profile of the kidney transplant recipients versus healthy controls revealed several peculiar features: i. increased expression of the CD54 molecule, largely associated with antigen-dependent T cell activation, by T cell effectors [25,26]; ii. increasing number and percentage of cytotoxic T cells; iii. decreased amount and growth ability of the Treg; iv. increasing percentage and number of the $T_{R3\text{-}56}$ regulatory T cell subset.

The constant availability of allo-antigens, as represented by the graft, has to be considered as a key feature underlying the immune scenario observed by us. Indeed, a low-grade pro-inflammatory microenvironment characterises the transplanted organ even in the presence of an effective immune-modulation therapy [1,2]. A significant association of $CD4^+$ and CTL activation with a reduction of the Treg subset has been largely found to underlie long-standing infection, transplantation and autoimmune diseases in human [34–39] and animal models [40,41]. In this context, the increasing $T_{R3\text{-}56}$ level in the transplanted subjects, when considering the immune-modulating role of such a T cell subset and the concomitant increase in the CTL population, might underlie an attempt to restore/maintain graft immune tolerance control in the presence of defective Treg-mediated immune-suppression.

Compelling evidence indicates growth ability as a major feature of the Treg population [38,39]. Such a treat has been largely associated with the need for a dynamic regulation of this T cell subset, specifically involved in the maintenance of a complex homeostatic balance. In this context, the decreased growth ability of the Treg subset might be likely related to an early derangement of the immune tolerance control of the graft in kidney recipients. Accordingly, the presence of an increasing level of activated helper T cells, the ones responsible for immune orchestration [6,7] in subjects showing highest amount of the $T_{R3\text{-}56}$ T cell regulatory population, might represent an attempt, in subjects showing early signs of graft functional recognition, to restore transplant tolerance control, thus avoiding clinical rejection episodes.

We found that categorising kidney transplant recipients based on their stable control of the graft, evaluated by changes in creatinine levels and 24 h proteinuria over two consecutive bi-monthly analyses, revealed a significant association of a higher $T_{R3\text{-}56}$ amount with an increasing CTL level and a decreased growth ability of the Treg population. Furthermore, our study revealed a significant association of the highest $T_{R3\text{-}56}$ levels with unstable graft control.

These observations, as a whole, propose a scenario in which early derangement of graft tolerance, in the presence of maintained kidney functional effectiveness, might be associated with increasing levels of the $T_{R3\text{-}56}$ regulatory T cell population and a low growth ability of the Treg subset. In this context, to minimise the pro-inflammatory effect related to dialysis pre-transplant treatment, a transplant vintage exceeding one year was considered in the enrolment criteria. Moreover, the absence of autoimmune diseases, as well as of viral infections in the enrolled cohort, was expected to focus the investigation on the peculiar

immune traits/cell subsets underlying the early events associated with deranged transplant tolerance. The mechanisms underlying such a complex scenario need further investigation.

The concomitant immune-modulating therapy that characterises the cohort of transplant recipients analysed by us is probably related to an inability to observe an inverse association of the T_{R3-56} amount with the activation level of their CTL target, as demonstrated in the haematological model [16,17].

The observation that in kidney transplant recipients, high levels of circulating T_{R3-56} regulatory T cells significantly associate with a decreased Treg growth ability, proposes T_{R3-56} evaluation as a potential marker of early defective graft tolerance control. Larger evaluations are needed in order to propose the potential employment of the analysis of the circulating T_{R3-56} T cell regulatory subset as a valuable indicator of graft stable control in kidney transplant recipients.

4. Materials and Methods

4.1. Patients

The study was carried out on 53 renal transplant recipients, all first transplanted from cadaver donors, in a regular follow-up at the Percorso Clinico Assistenziale in Nefrologia e Trapianto Renale, Azienda Ospedaliera Universitaria "Federico II", c/o the Dipartimento di Salute Pubblica of the Università Federico II (Napoli, Italy). Inclusion criteria were: age 18–65 years; transplant vintage: >1 year, with a bi-monthly clinical and laboratory control in the last 6 months; plasma creatinine < 3 mg/dL; haemoglobin value > 11 g/dL; white cell count > 4000/µL (neutrophils > 2000/µL); platelet count > 75.000/µL; absence of clinical signs of graft rejection, of infectious episodes and no change in the immunosuppressive regimen in the last 6 months. The subjects were randomly enrolled regardless of their dialysis vintage (usually between 2 and 4 years) as well as the pathogenic condition underlying the end-stage kidney failure requiring a kidney transplant. No anti-thymocyte serum was employed in the induction treatment. Exclusion criteria were: previous or combined transplantation; Panel Reactive Antibodies (PRAs) > 25% and/or presence of Donor Specific Antibodies (DSAs) at transplantation; presence of proteinuria exceeding 300 mg/day on 24-h samples; presence of hyperlipidaemia (baseline cholesterol and/or triglycerides values exceeding 220 and 200 mg/dL, respectively); evidence of autoimmune diseases or viral infections.

On the basis of these criteria, 53 consecutive patients, in a regular follow-up, were included in the study. These subjects were subsequently divided into two groups according to their laboratory data: patients with stable renal function and urinary parameters (Stable Group), and patients showing changes $\geq$ 0.2 mg/dL in serum creatinine level and/or > 100 mg/day in proteinuria 24-h urinary samples in two consecutive evaluations, despite no clinical predisposing condition (worsening hypertension, recurrence of the underlying renal disease, cardiovascular disease). These patients represented the Unstable Group. Plasma creatinine concentration was evaluated by an autoanalyser with a modified Jaffè method, urinary protein excretion by PCR ([urinary protein/urinary creatinine] $\times$ 1000, mg/mM) method; glomerular filtration rate was estimated with EPI-CKD formula. Immune-modulating treatments included Corticosteroids and Calcineurin inhibitors, as detailed in Table 2. Demographic and laboratory data of the enrolled subjects are shown in Table 2. The study, conducted in agreement with Good Clinical Practice guidelines, was approved by the Ethics Committee of the University Federico II of Naples (Protocol number: 66/11). All the procedures were in accordance with the Declaration of Helsinki, as revised in 2008. A total of 20 blood donors, age- and sex-matched with the patients, were enrolled into the study, as healthy controls; their baseline cholesterol and triglycerides values were always <200 mg/dL. All the patients and controls signed their informed consent to the study.

Table 2. Characteristics of the subjects enrolled in the study.

Kidney Recipient subjects (N = 53)	
SEX M/F (%)	30/23 (56/44)
AGE (Mean ± SD)	51.83 ± 14.04
TRASPLANT VINTAGE (years)	5.56 ± 4.2
White Blood Cell count ($\times 10^9$/L) Mean ± SD	8.278 ± 2.57
Neutrophil count ($\times 10^9$/L) Mean ± SD	5.308 ± 2.15
Lymphocyte count ($\times 10^9$/L) Mean ± SD	2.029 ± 0.83
Immunosuppressive drugs	
Tacrolimus Average dosage Mean ± SD	31/536.59 ± 2.63 mg
Cyclosporine Average dosage Mean ± SD	22/53168.1 ± 52.18 mg
Steroids Average dosage Mean ± SD	53/534.91 ± 2.13 mg
Healthy subjects (N = 20)	
SEX M/F; (%)	12/8 (60/40)
AGE (Mean ± SD)	45.75 ± 16.28
White Blood Cell count ($\times 10^9$/L) Mean ± SD	7.869 ± 1.862
Neutrophil count ($\times 10^9$/L) Mean ± SD	5.749 ± 1.378
Lymphocyte count ($\times 10^9$/L) Mean ± SD	2.120 ± 0.527

4.2. Cells, Immunofluorescence and Flow Cytometry Analysis

Blood samples were analysed by immunofluorescence by using FITC or Pe-Cy5 anti-human CD3 (BD Pharmingen, clone UCHT1), FITC anti-human CD4 (BD Pharmingen, clone RPA-T4), Pe-Cy7 anti-human CD8 (BD Pharmingen, clone RPA-T8), Pe-Cy5 or Pe-Cy7 anti-human CD56 (BD Biosciences, clone NCAM16.2), PE anti-human CD25 (BD, clone M-A251), PE anti-human CD54 (BD, clone HA58), FITC anti-human Ki-67 (BD, clone B56), all from Becton Dickinson Italia S.p.A., Milano, Italy. PE anti-human Vα24 (Beckman Coulter, clone C15), from Beckman Coulter S.p.a., Milano, Italy; FITC anti-human CD19 (eBioscience, clone HIB19), FoxP3-all (eBioscience, clone PCH101. from Thermo Fisher Scientific Inc., MA, USA. For intracellular detection of FoxP3-all and of Ki-67, a fixation and permeabilization FoxP3 buffer kit (eBioscence, from Thermo Fisher Scientific Inc., MA, USA), was employed according to the manufacturer's instructions. For the analysis of the CD54 expression level in T lymphocytes, fluorescence data were expressed as a ratio of the mean intensity fluorescence (MIF) value for the CD4 or CD8 T cell population and the control MIF value obtained after staining of the same cell subset with the isotype control mAb, as described [24]. All phenotypes referred to flow cytometry analysis of the lymphocyte population gated using forward (FSC) and side-scatter (SSC) parameters. Flow cytometry evaluation was performed by using an ATTUNE NxT acoustic focusing cytometer (Life Technologies; Thermo Fisher Scientific Inc., MA, USA). Data analysis was performed by using FlowJo Software (V10, LLC). PBMCs, for FoxP3 and ki67 detection, were isolated by centrifugation of the peripheral blood on a Ficoll-Paque cushion (GE Healthcare, Uppsala, Sweden) gradient. This evaluation strategy is expected to allow the direct analysis of the biological complexity of the immune profile in the peripheral blood. Accordingly, we focused on adaptive immune effectors, chiefly responsible for allo-antigen recognition/damaging, by evaluating their surface expression of the CD54 molecule, consistently associated with antigen-dependent T cell activation [25,26].

To evaluate possible oscillations in the results, two independent samples, obtained for each subject, were analysed at a one-week interval and produced substantially comparable results.

4.3. Statistical Analysis

Statistical evaluation of data, by using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA), was performed by a Mann–Whitney test and a Fisher's two-tailed exact test, as indicated. Two-sided p values of less than 0.05 were considered to indicate statistical significance.

5. Conclusions

Recognition of allo-specificities by recipient immune effectors has been largely recognised to underlie allograft injury and loss. Moreover, the availability of valuable criteria to identify in time early immune-mediated injuries in kidney transplant recipients, represents a still unmet target. Here, we show that increased activation of T cells, a decreased amount and growth ability of the Treg and higher level of the $T_{R3\text{-}56}$ regulatory T cell subset, consistently associated by us with the preferential control of cytotoxic T lymphocytes, characterise a transplant recipient cohort without signs of graft rejection, no infectious episodes and no change in the immunosuppressive regimen in the last 6 months. In addition, the highest level of the circulating $T_{R3\text{-}56}$ regulatory subset specifically associates with a reduction in the amount and growth ability of the Treg. Accordingly, unstable graft control, as defined by changes in serum creatinine $\geq$ 0.2 mg/dL in two consecutive bi-monthly detections, has been consistently associated with a higher $T_{R3\text{-}56}$ level and defective Treg growth ability. Further studies are required to validate the hypothesis that immune profiling, including $T_{R3\text{-}56}$ evaluation, might represent an early diagnostic tool to identify patients at risk of developing significant anti-donor allo-immune responses.

6. Limitations

One limitation of the study is represented by the lack of details about the underlying co-morbidities in the graft recipient cohort. Further studies are needed to deeply investigate such an issue.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms251910610/s1.

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