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Myeloid Derived-Suppressor Cells as A Potential Target of Immunotherapy in Notch Dependent T-Cell Acute Lymphoblastic Leukemia

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ABSTRACT

Notch receptors play crucial roles in T-cell development, and their dysregulation leads to the development of T-cell Acute Lymphoblastic Leukemia (T-ALL), a condition that, as of now, lacks a definitive cure. Notch3 transgenic mice (*N3-tg*) represent a well-established model for T-ALL, where the constitutive activation of the receptor in immature thymocytes initiates an aggressive disease characterized by the expansion of tumoral T cells in the periphery. These tumor cells can trigger a Notch/IL-6-dependent accumulation of Myeloid-Derived Suppressor Cells (MDSCs), which, in turn, support tumor progression. MDSCs are an immature cell subset that inhibits immune responses, creating a conductive environment for tumor growth. Programmed cell death 1 (PD-1) is an inhibitory receptor of immune responses, recognized for its role as a suppressor of T-cell activation and proliferation when interacting with the PD-L1 ligand. MDSCs contribute to tumor progression through various mechanisms, including the expression of PD-L1, resulting in the inhibition of PD-1 expressing cells within the tumor microenvironment, such as T- and NK-cells.

The main aim of my thesis was to identify MDSC targets and mechanisms of action in the tumor microenvironment of our Notch-dependent murine model of T-ALL, with regard to their potential role in inhibiting NK activity, possibly through the PD-1/PD-L1 axis. Thus, this research ultimately aims to develop an innovative combined therapy for T-ALL, targeting tumor T cells, enhancing NK activity, and modulating MDSC function.

My data demonstrated that in *N3-tg* mice, number and function of NK cells decline significantly, while the percentage of them expressing PD-1 increases, during disease progression. This coincided with an expansion of functional MDSCs and in particular of the PD-L1^{+} fraction. This inverse correlation suggests us that NK impairment could be driven by MDSCs. Indeed, through *in vitro* cytotoxicity assay based on co-culture of NK cells with MDSCs, both from spleen of *N3-tg* mice, we confirmed that MDSCs can significantly hinder NK cell function.

Finally, treating *N3-tg* mice with anti-PD-L1 blocking antibodies markedly inhibits T-ALL progression, by significantly reductions in splenomegaly and absolute count of tumor cells. Moreover, the treatment led to a substantial decrease in overall MDSC numbers, particularly within the PD-L1-expressing subset. Concurrently, there was a

noticeable expansion of PD-1^{+} NK cells, exhibiting a significantly heightened cytotoxic activity compared to the control group.

In conclusion, my results suggest that in Notch-dependent T-ALL, MDSCs may hinder the anti-tumor activity of NK cells via the PD-1/PD-L1 axis, thus favoring disease progression. Then, molecules and cells of this network could potentially serve as prognostic markers and/or targets for innovative therapies.

INTRODUCTION

1. Myeloid-Derived Suppressor Cells (MDSCs)

The tumor microenvironment inhibits immune cell function through various mechanisms such a production of soluble modulators, low nutrient levels, hypoxic conditions, expressing immune-checkpoint receptors, high levels of myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs) (Melaiu O, et al, 2020). Understanding and targeting these factors is a crucial focus in cancer research, consequently impacting therapeutic effectiveness. Within this intricate context, Myeloid-Derived Suppressor Cells (MDSCs) assume a prominent role by inhibiting the host's anti-tumoral immune responses. The Myeloid-Derived Suppressor Cells (MDSCs) are a heterogeneous population of progenitors and immature cells of myeloid origins cells described for the first time around 20 years ago in oncological patients (Young MR, et al., 1987; Seung LP, et al., 1995). They are characterized by the ability to suppress innate and adaptive immune responses and expand during pathological settings such as cancer, infections, and inflammatory diseases. MDSCs consist of both myeloid progenitor cells and immature myeloid cells. In healthy individuals, myeloid precursors differentiate into mature cells, including granulocytes, macrophages, or dendritic cells (Sica A and Bronte V, 2007). In pathological conditions, the myeloid pathway expands to increase the production of myeloid leukocytes in the bone marrow, which are phenotypically and functionally different from normal myeloid cells, responding to threats such as infections, tissue damage, chronic inflammation, and cancer (Cassetta L, et al., 2019). Moreover, pathological activation results in the formation of myeloid cells with reduced phagocytic abilities, producing high levels of reactive oxygen species (ROS), nitric oxide (NO), and mainly anti-inflammatory cytokines. Consequently, MDSCs are unable to effectively perform the normal functions of myeloid cells and acquire a potent immunosuppressive potential (Condamine T, et al., 2015). These cells mainly accumulate in the blood and lymphoid organs during tumor progression but can also be recruited to the tumor site (Sica A and Bronte V, 2007).

The processes above, in turn, promote tumor growth through various mechanisms, including inhibition of NK cells and T lymphocytes immune-responses, alteration of the tumor microenvironment to facilitate tumor proliferation, creation of a favorable

environment for metastasis, stimulation of epithelial-mesenchymal transition (EMT), and promotion of angiogenesis. Consequently, MDSCs actively contribute to an immune-tolerant tumor microenvironment and hinder the effectiveness of cancer immunotherapies. In fact, the abundant presence of MDSCs in solid tumor patients has been associated with unfavorable prognosis and reduced overall survival (Law AMK, et al., 2020).

In murine models, two subtypes of MDSCs have been identified based on the expression of specific markers: monocytic M-MDSCs, morphologically and phenotypically resembling monocytes, and granulocytic/polymorphonuclear PMN-MDSCs, phenotypically and morphologically similar to granulocytes. Both subtypes in mice are characterized by simultaneous expression of two markers: CD11b and Gr-1. The two main subsets of MDSCs can be distinguished based on the variable expression of the marker Gr-1 (Gr-1hi cells are predominantly PMN-MDSCs, while Gr-1lo cells are mainly M-MDSCs). The Gr-1 marker is composed of two cell membrane molecules, Ly6C and Ly6G, each displaying distinctive expression patterns in the primary murine MDSC subtypes. Specifically, PMN-MDSCs exhibit the $CD11b^{+}LY6G^{+}LY6C^{low}$ phenotype, whereas M-MDSCs are characterized by $CD11b^{+}Ly6G^{-}Ly6C^{high}$ (Bronte V, et al., 2016).

In humans, characterizing MDSCs is more complex due to the absence of specific markers. Human PMN-MDSCs are defined as $CD11b^{+}CD15^{+}CD14^{-}$, where CD15 serves as a useful marker to discriminate the granulocytic component as an activation marker for human granulocytes. Conversely, M-MDSCs are defined as $CD11b^{+}CD14^{+}HLA\text{-}DR^{low/-}CD15^{-}$. CD14 is a typical surface marker for monocytes, indicating similarity to that cell type. Moreover, low, or absent expression of HLA-DR helps distinguish M-MDSCs from mature monocytes. Finally, the lack of CD15 expression differentiates M-MDSCs from G-MDSCs (Bronte V, et al., 2016). However, various distinct subsets of human MDSCs have been reported depending on the tumor context, including $CD11b^{+}CD33^{+}CD14^{+}HLA\text{-}DR^{low/-}$ cells (Garg A and Spector SA, 2014; Liu YF, et al., 2017; Bronte V, et al., 2016).

1.1 The Function of MDSCs

MDSCs drive the "immune escape" of tumors through the inhibition of both innate and adaptive anti-tumor immune responses. Furthermore, they actively promote tumor progression by stimulating neo-angiogenesis (Rivera LB and Bergers G, 2015), enhancing tumor invasion, and fostering the development of a pre-metastatic microenvironment. Studies have demonstrated that the immunosuppressive activity of MDSCs occurs both through the release of soluble factors and via cell-cell contact mediated by the binding between ligands and receptors on the cell surface. The primary factors responsible for suppressing T cells involve the expression of arginase (from the ARG1 gene) and inducible nitric oxide synthase (iNOS); the production and release of TGF-β, IL-10, and COX2, as well as reactive oxygen species (ROS); and other mechanisms such as cysteine sequestration, reduced expression of L-selectin by T lymphocytes, and induction of suppressive T cells or Tregs (Gabrilovich DI, et al., 2012). While MDSCs impact on T-cells is the most extensively studied, they also influence B-cells and Natural Killer (NK) cells (Jaufmann J, et al., 2020; Goh CC, et al., 2016; Stiff A et al., 2018). MDSCs are phenotypically characterized by a set of markers, none of which are unique to them, but they can be identified by their remarkable immunosuppressive and tumorigenic activities (Kumar V, et al., 2016; Parker KH et al., 2015; Marvel D and Gabrilovich DI, 2015). MDSCs utilize several mechanisms to modulate the immune system effectively. These include metabolite depletion critical for T-cell function, the secretion of immunosuppressive factors, the production of reactive oxygen (ROS) and nitrogen species (NO), the expression of immune checkpoint inhibitors, and regulation of lymphocyte trafficking. For example, MDSCs upregulate xc-transporters, allowing them to sequester cystine without releasing cysteine, which is essential for T-cell proliferation (Srivastava MK, et al., 2010). MDSCs exhibit immunosuppressive activity primarily through the expression of enzymes iNOS and ARG1 (Gabrilovich DI, et al., 2012). Specifically, Arginase-1 leads to a decrease in extracellular L-Arginine, resulting in reduced expression of the ζ chain of the CD3 receptor (Rodriguez PC, et al., 2002). This inhibition interferes with the expression of cell cycle regulators like cyclin D3 and cyclin-dependent kinase 4 (Cdk4) (Rodriguez PC, et al., 2007), leading to cell cycle arrest in the G1 phase. Nitric oxide produced by iNOS suppresses T cell functions by inhibiting JAK3 and STAT5 (Bingisser RM, et al., 1998), decreasing the expression of major histocompatibility complex class II molecules (MHC II), and triggering T cell

apoptosis (Rivoltini L, et al., 2002). Finally, the reaction between NO and superoxide generates peroxynitrite (PNT), which directly inhibits T cells by inducing TCR nitration, thereby diminishing their response to antigen-MHC complexes (Nagaraj S, et al., 2012). Moreover, nitration reduces the binding capacity of antigenic peptides on tumor cells to MHC molecules (Lu T, et al., 2011), also the nitration of specific T cell chemokines interferes with their migration (Molon B, et al., 2011). Reactive oxygen species (ROS) are byproducts of aerobic metabolism and include superoxide anion (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH). Elevated ROS levels are often associated with oxidative stress, causing damage to lipids, proteins, and DNA (Schieber M, et al., 2014). Increased ROS production is a prominent characteristic observed in MDSCs in tumor-bearing mice and cancer patients: inhibiting ROS in these cases leads to the complete abolition of MDSCs suppressive effects in vitro (Szuster-Ciesielska A, et al., 2004). Additionally, various factors produced by tumor cells stimulate the production of ROS by MDSCs, including GM-CSF, IL-6, and TGF-β. The induction od other suppressive subsets is another mechanism of action of MDSCs that, indeed, can release cytokines implicated in Tregs and TAM cell subsets, such as IL-10 and TGF-β (Sica A, et al., 2012). Specifically, TGF-β mediates the expression of $CD39^+$ and $CD73^+$ receptors on MDSCs, thereby activating the immunosuppressive CD39/CD73-adenosine pathway through which MDSCs inhibit T and NK cells (Li L, et al., 2018; Li J, et al., 2017). Moreover, microenvironmental signals such as HIF-1α can amplify the immunosuppressive activity of MDSCs, leading to increased PD-L1 expression (Noman MZ, et al., 2014). Additionally, MDSCs impact T cell migration by restricting the entry of naïve T cells into lymph nodes through ADAM17-mediated reduction of CD62L on T cells, and they also hinder the exit of effector T cells by NO-mediated reduction of adhesion molecules CD162 and CD44 (Consonni FM, et al., 2019). Initially, MDSCs, generated in response to inflammatory stimuli, contribute to tumor expansion by protect them from immune system attacks and recruiting pro-inflammatory $CD4^+$ T cells. Additionally, MDSCs play a role in remodeling the tumor microenvironment by promoting the invasiveness and angiogenesis of neoplastic cells. It has been demonstrated that they produce crucial mediators for neo-angiogenesis, such as those belonging to the vascular endothelial growth factor (VEGF) subfamily (Tartour E, et al., 2011). Through co-culture experiments of MDSCs with tumor cells, it has been shown that in metastatic tumors, MDSCs can support the epithelial-mesenchymal transition

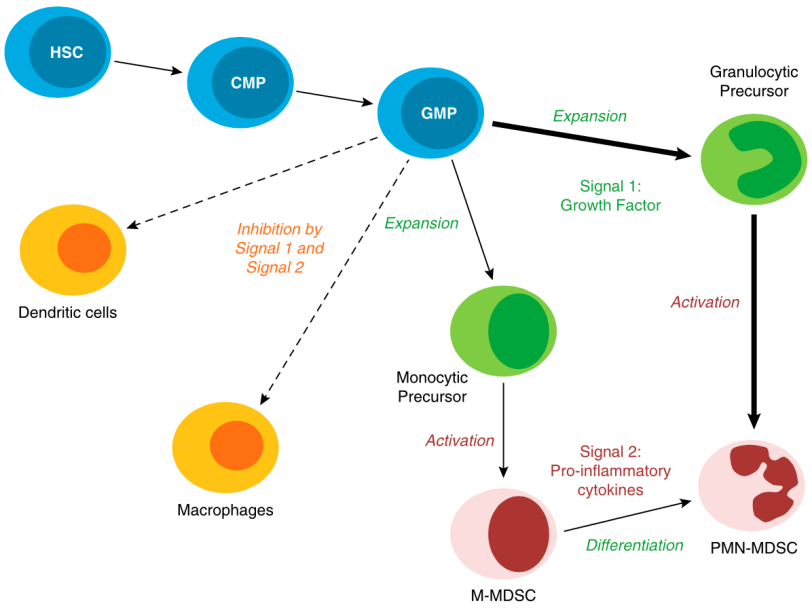
(EMT), prompting tumor cells to acquire a more invasive phenotype and enhancing their *in vivo* metastatic potential (Cui TX, et al., 2013).

In conclusion, MDSCs utilize a variety of intricate mechanisms to effectively dampen the anti-tumor activities of T and NK cells. They facilitate the accumulation and activation of immunosuppressive cells, such as Tregs and M2 macrophages, thereby playing a substantial role in promoting tumor progression.

1.2 The Generation of MDSCs: Differentiation and Activation

Regarding the induction and development of MDSCs, the most accredited theory refers to the "two-signal model" (Condamine T, et al., 2015). This model suggests that not one, but two different types of signals are necessary to drive the generation of functional MDSCs: one part aims to expand the immature myeloid cell population, while the other one activates these cells, transforming them into functional MDSCs. The first signal involves tumor-derived growth factors supporting the expansion of immature myeloid cells, notably including GM-CSF (granulocyte/macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), M-CSF (macrophage colony-stimulating factor), and other growth factors produced by tumor cells and tumor stroma (Condamine T, et al., 2015) and involves factor like STAT3, IRF8, C/EBPβ, Notch, adenosine A2b receptors, and NLRP3. The second signal drive the differentiation of immature cells into activated MDSCs. These factors primarily include pro-inflammatory cytokines originating from tumor stroma, and this phase involves the NF-κB, STAT1, STAT6, prostaglandin E2 (PGE2), and cyclooxygenase 2 (COX2) pathway (Gabrilovich DI, 2017) **(Figure 1).** It is crucial to note the role of the Signal Transducer and Activator of Transcription (STAT) family members (STAT3, STAT5, STAT6) in the expansion and activity of MDSCs (Gabrilovich DI and Nagaraj S, 2009). Specifically, the immature phenotype of MDSCs is linked to the constitutive activation of the transcription factor STAT3, which impedes the completion of cell functional maturation. STAT3 not only contributes to MDSC expansion but also mediates signals from growth factors and various cytokines, including IL-6. IL-6, an essential pro-inflammatory cytokine regulating multiple aspects of both innate and adaptive immune systems, is often found

at elevated concentrations in cancer patients and is frequently associated with an unfavorable prognosis. Studies have demonstrated IL-6 to be a significant factor in both expansion and activation of MDSCs (Wu CT, et al., 2012, Condamine T, et al., 2015). Finally, the combination of IL-6 and GM-CSF has proven as the best effective combination in inducing the accumulation of functional MDSCs in vitro from healthy mice's BM progenitor cells (Marigo I, et al., 2010). IL-6 plays a crucial role in making MDSCs more effective at suppressing the immune system. It does this by increasing the expression of PD-L1 and boosting the production of ROS and NO. Notably, MDSCs generated in the presence of IL-6 demonstrate heightened efficacy in inhibiting $CD8^+$ T cells, characterized by increased expression of Arg-1 and elevated ROS production when compared to cells generated without IL-6 (Weber R, et al., 2020). Lastly, the use of IL-6 promotes a more immature phenotype of myeloid cells by inhibiting their antigen presentation function (Beyranvand Neyad E, et al., 2021)



Condamine T, Mastio J, Gabrilovich DI. J Leukoc Biol. 2015 Dec;98(6):913-22.

Figure 1. MDSC Accumulation Is Regulated by Two Different Types of Signals*. The presence of tumor-derived growth factors* ***(Signal 1)*** *drives the expansion of monocytic and granulocytic precursors (GMP). These precursors require an activation signal* ***(Signal 2)*** *to acquire a suppressive phenotype to give rise to PMN-MDSC and M-MDSC. These 2 types of signals also inhibit myeloid cell differentiation into terminally differentiated cells, such as Dendritic cells and Macrophages. HSC, Hematopoietic stem cell.*

1.3 MDSC Role in Cancer Microenvironment

As noted above, MDSCs play a critical role in cancer by suppressing the immune responses against tumors, primarily by impeding the proliferation of T cells, cytokine secretion, recruiting regulatory T cells, and thus promoting the growth of the tumor. These cells are generated and accumulate during tumor progression, infiltrating the tumor mass and interacting with other MDSCs, tumor cells, and stromal cells, thus contributing to the creation of an immunosuppressive microenvironment (Umansky V, et al., 2019). Extensive research has focused on their involvement in various solid tumors, including melanoma, brain tumors, head and neck carcinoma, lung carcinoma, breast cancer, hepatocellular carcinoma, gastrointestinal tumors, pancreatic carcinoma, renal cell carcinoma, bladder carcinoma, ovarian carcinoma, and prostate carcinoma (Solito S, et al., 2014). The increase in peripheral blood MDSCs is associated with a poorer clinical outcome in patients with various types of cancer (Filipazzi P, et al., 2012; Zhang B, et al., 2013; Solito S, et al., 2011; Pico de Coaña Y, et al., 2013; Weide B, et al., 2014). Furthermore, several studies have suggested that a reduced immunosuppressive capacity of MDSCs correlates with therapeutic benefits in cancer patients treated with immune checkpoint inhibitors, particularly anti-CTLA-4 (Sade-Feldman M, et al., 2016; Gebhardt C, et al., 2015; Pico de Coaña Y, et al., 2013). Cancer cells actively contribute to MDSC differentiation. In particular, chronic inflammation in TME is characterized by pro-inflammatory cytokines such as PGE2, GM-CSF, G-CSF, M-CSF, SCF, S100 proteins, VEGF, TGFβ, and TNFα. These cytokines can alter the differentiation of myeloid cells, promoting the formation of MDSCs: modified monocytopoiesis of common myeloid progenitors (CMP) can give rise to suppressive cells derived from monocytic myeloid cells (M-MDSC) and tumor-associated macrophages (TAM), while altered granulopoiesis can lead to the generation of suppressive cells derived from polymorphonuclear myeloid cells (PMN-MDSC) and tumor-associated neutrophils (TAN) (Law AMK, et al., 2020). Additionally, MDSCs infiltrating the tumor can differentiate directly into potent immunosuppressive TAMs (Kumar V, et al., 2016). MDSCs, in turn, contribute to reshaping the tumor's surrounding environment by producing factors like VEGF and FGF. These factors stimulate the formation of new blood vessels in the tumor, as well as the mobility and invasion of tumor cells (Poschke I and Kiessling R, 2012). Interestingly, it has been reported that MDSCs can undergo a transformation into endothelial cells, thus contributing to tumor angiogenesis (Yang L.

et al., 2004). Furthermore, it has been discovered that TGF-β, HGF, and EGF produced by MDSCs infiltrating the tumor contribute to cancer-associated epithelial-mesenchymal transition (Toh B, et al., 2011). MDSCs have also been implicated in the formation of pre-metastatic niches for cancer spread (Law AMK, et al., 2020).

1.4 MDSCs in Hematological Malignancies

Extensive research has focused into MDSCs role in several solid tumors, yet their mechanisms in hematologic malignancies remain less explored (Lv M, et al., 2019). Most research in this area has been conducted in lymphomas and multiple myeloma. In a murine model of B-cell lymphoma, studies demonstrated an expansion of MDSCs that shared similar functional properties to those observed in solid tumors (Serafini P, et al., 2008). Furthermore, an analysis of the peripheral blood of non-Hodgkin lymphoma patients revealed a correlation between MDSC-mediated T cell suppression and an increase in their expression of Arginase-1, programmed death-ligand 1 (PD-L1), and S100A12 (a member of the S100 protein family involved in T cell suppression through increased PD-L1 expression on MDSCs) (Lin Y, et al., 2011). MDSCs have been observed in Multiple Myeloma (MM), where the primary site of tumor cells' emergence, the bone marrow, also serves as the site for MDSC generation. Direct contact between tumor cells and myeloid progenitor cells might elucidate their conversion and early accumulation in MM (Ramachandran IR, et al., 2013). In the context of multiple myeloma (MM), an increased abundance of MDSCs is evident in both peripheral blood (PB) and bone marrow (BM) among patients. This elevated presence is believed to contribute significantly to the onset and progression of the disease (Görgün GT, et al., 2013). Notably, MDSCs isolated from the PB of MM patients demonstrate a suppressive impact on T cells. This suppressive effect can be effectively neutralized by inhibiting the activities of Arginase-1 and iNOS (Romano A, et al., 2018). Unlike lymphoma and multiple myeloma, studies on MDSCs in leukemia, particularly acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL), are more limited. At diagnosis, peripheral blood in AML patients shows high heterogeneity in MDSC percentages with a $CD14^{-}HLA\text{-}DR^{-}CD33^{-}CD11b^{+}$ phenotype, and then their high presence and suppressive function in PB and BM correlates with tumor progression (Lv M, et al., 2019; Pyzer AR et al., 2017). Literature data reveal that the "V-

domain Ig Suppressor of T cell activation" (VISTA), a negative regulator mediating immune evasion in tumors, is highly expressed on MDSCs in AML patients. Silencing VISTA through siRNA highlights inhibition of MDSCs ability to suppress T cell activity (Wang L, et al., 2018). In Chronic Myeloid Leukemia (CML) patients, studies note a higher percentage of MDSCs with the $CD11b^{+}CD14^{-}CD33^{+}$ phenotype compared to healthy individuals, along with increased Arg-1 production known to inhibit T cells. Additionally, the study indicates heightened PD-L1 overexpression on myeloid cells, which, upon binding to its receptor PD-1 expressed on T cells, contributes to their suppression (Christiansson L, et al., 2013). For what concern acute leukemia, the observations are even more reduced. MDSCs increase in PB and BM of pediatric patients with B-cell acute lymphoblastic leukemia (B-ALL) compared to healthy controls (Liu YF et al., 2017; Hohtari H, et al., 2019). Further, in patients with chronic lymphocytic leukemia (CLL), an increase in M-MDSCs ($CD14^{+}HLA\text{-}DR^{low}$) has been observed compared to healthy controls. These cells inhibit T cell activation *in vitro* while promoting suppressive regulatory T cells. This phenomenon is linked to higher activity of the enzyme indoleamine 2,3-dioxygenase (IDO). It is interesting to note that tumor cells stimulate the production of IDO, suggesting a potential crosstalk between CLL cells, MDSCs, and regulatory T cells (Jitschin R, et al., 2014).

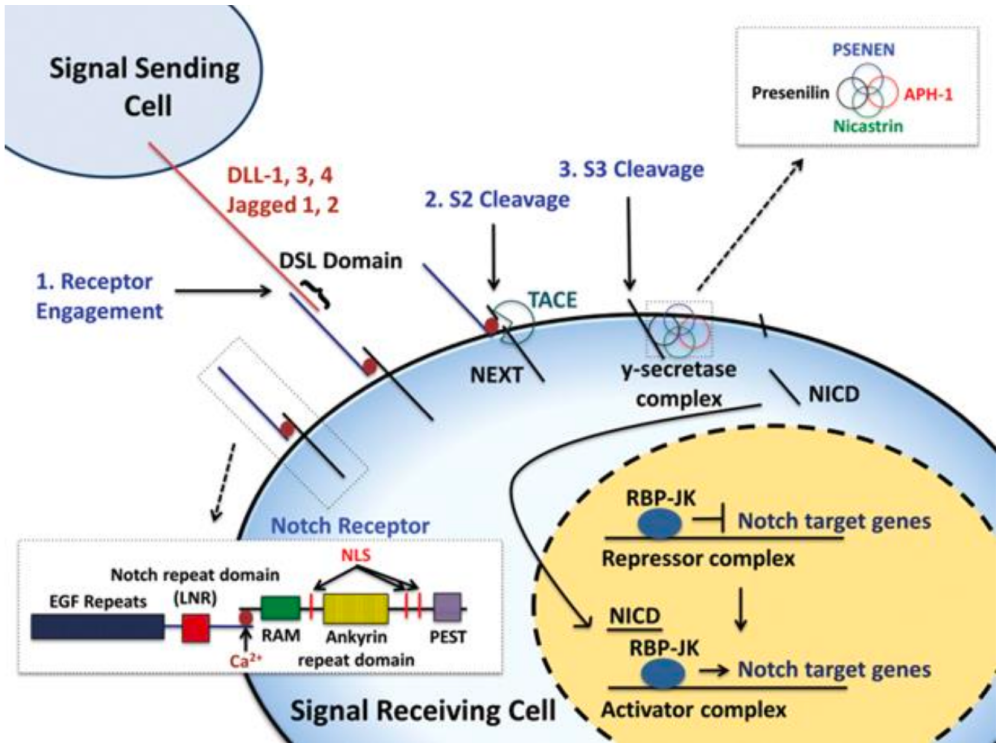
Finally, regarding the role of MDSCs in T-cell acute lymphoblastic leukemias (T-ALL), current literature lacks sufficient data (Lv M, et al., 2019). However, preliminary observations published by our group exist, serving as a vital premise for the purpose of this thesis, and will be further discussed.

2. The Notch Signaling Pathway

Notch is an evolutionarily conserved receptor found in various multicellular organisms, associated with development and differentiation. Abnormalities in signal transduction can lead to developmental anomalies, tumors, and other human diseases. Therefore, a comprehensive understanding of Notch signaling is essential for grasping the foundations of both development and cancer (McIntyre et al., 2020). Notch proteins, present on cell surfaces, are essential receptors guiding cell destiny by impacting proliferation, survival, apoptosis, and differentiation of different cell types. These receptors, found in mammals as Notch-1, -2, -3, and -4, interact with specific ligands—Delta-like (Delta-1, -3, -4) and Jagged (Jagged-1, -2). The architecture of Notch receptors consists of an extracellular domain, responsible for ligand interactions, and an intracellular domain, facilitating signal transduction. Within the extracellular domain, there are 29 to 36 tandem repeats of "EGF-like" (Epidermal Growth Factor) sequences that facilitate ligand interactions. Following these repeats are three LIN12/Notch repeats rich in cysteine ("LNR") and a heterodimerization domain (HD) (Radtke F, et al., 2010). The combined LNR and HD domains form the Negative Regulatory Region (NRR) of the receptor, playing a critical role in preventing Notch activation in the absence of the appropriate signal (Sanchez-Irizarry C, et al., 2004; Kopan R and Ilagan MX, 2009). The intracellular domain is responsible for signal transduction and contains a RAM domain (RBPJ-associated molecule) followed by six ankyrin-like repeats involved in binding with the CSL (CBF1, Suppressor of Hairless, Lag-1) transcription factor, a Transcription Activation Domain (TAD), and a PEST motif (rich in proline, glutamic acid, serine, and threonine), which regulates protein stability.

The Notch signaling pathway initiates upon ligand-receptor binding, causing two sequential proteolytic cleavages that result in the release of the Notch Intracellular Domain (NIC) from the transmembrane portion into the nucleus (Kopan R and Ilagan MX, 2009). Initially, the disintegrin and metalloprotease ADAM family, specifically ADAM 10 or ADAM 17, performs the first cleavage at the S2 site located in the extracellular domain of the Notch receptor (Kopan R and Ilagan MX, 2009). Following this, the γ-secretase complex, a multiprotein assembly comprising presenilin, nicastrin, APH-1, and PEN-2 proteins, executes the second cleavage at the S3 site within the receptor's transmembrane domain (Fortini ME, 2002). The resulting Notch Intracellular

Domain (NIC) can translocate into the nucleus, where it forms a complex with the CSL transcription factor. CSL is known as CBF-1 in humans, LAG in *C. elegans*, and RPB-Jk in mice. This NIC-CSL-DNA complex recruits' co-activators, including Mastermind-like1 (MAML1) (Wu L, et al., 2000) and histone acetyltransferases (Kurooka H and Honjo T, 2000). This interaction leads to the transformation of CSL from a repressor to an activator of transcriptional activity by removing CSL co-repressors **(Figure 2)**. The NICD/CSL signals in mammals target a spectrum of genes, including those from the Hairy/Enhancer of Split (HES) family, HRT/HERP genes, cyclin D1, p21, NF-κB, and numerous others. These genes play roles in various cellular processes, spanning inflammation, immune response, growth, development, and survival (Osborne BA and Minter LM, 2007). The Notch signaling pathway, crucial to numerous facets of cellular biology, is subject to regulation through a myriad of mechanisms. For instance, the Fringe family of proteins, a group of Golgi-localized glycosyltransferases, inhibiting Notch signaling by obstructing the ligand-receptor interaction through glycosylation of the receptor (Koch U, et al., 2003). Another target is the co-activator Mastermind (MAML-1), necessary for converting CSL into a transcriptional activator. Additionally, Fbw7, a component of the SCF-E3 ubiquitin ligase complex, ubiquitinates the receptor, targeting it for degradation (O'Neil J, et al., 2007; Thompson BJ, et al., 2008). Experimentally and therapeutically, the Notch signaling pathway can be negatively regulated by employing γ-secretase inhibitors (GSI). These inhibitors prevent proteolytic cleavage at the S3 site, hindering the release of the intracellular portion of Notch, thereby impeding signal transduction (Kopan R and Ilagan MX, 2004).



Kathleen M. Capaccione et al., Carcinogenesis. 2013 Jul; 34(7): 1420–1430

Figure 2. The Notch Signaling Pathway. *The figure illustrates the key stages of the Notch signaling pathway. It begins with the receptors binding to Notch ligands, present on the membrane surface of an adjacent cell. This interaction triggers enzymatic cleavage of the Notch receptor, leading to the release of its Intracellular Domain (NICD).*

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2.1 Notch Function During Thymocyte Maturation and in Development of T-cell Acute Lymphoblastic Leukemia (T-ALL)

The Notch signaling pathway regulates cell function and differentiation processes within the hematopoietic system and plays a pivotal role in T lymphocyte development (South AP, et al., 2012). Precursors of T lymphocytes enter the thymus and, through a coordinated series of events involving proliferation and differentiation phases, acquire the ability to recognize and respond to antigenic stimuli. Their differentiation occurs from certain progenitors that migrate from the bone marrow to the thymus, the primary

lymphoid organ. Differentiation steps within the thymus are marked by the differential expression of the TCR co-receptors, CD4 and CD8. Thus, in mouse, there are $CD4^-CD8^-$ double-negative (DN) thymocytes, the most immature lymphocytes found in the pro-T stage, characterized by the absence of typical mature lymphocyte surface molecules. These thymocytes, concomitant with the onset of the pre-T stage, differentiate into two alternative lineages, giving rise to Tγδ and Tαβ lymphocytes based on the rearrangement of TCR chains. Specifically, the rearrangement of the β-chain genes occurs prior to that of the α-chain. In fact, in thymocytes that will develop into Tαβ lymphocytes, an initial formation of a "pre-TCR" takes place, which is composed of the newly synthesized β-chain and the invariant pTα chain. Later, this pTα chain will be replaced by the mature α-chain. At this stage of T lymphocyte development, a crucial event known as "beta selection" occurs. Only DN thymocytes that have successfully rearranged the β-chain express a functional pre-TCR on their membrane, through which they receive signals for survival, proliferation, and differentiation towards next stages. As T lymphocytes mature in the thymus, they progress from being $CD4^-CD8^-$ (Double Negative) to $CD4^+CD8^+$ (Double Positive) cells, then to $CD4^+CD8^-$ or $CD4^-CD8^+$ (Single Positive) cells. This transition involves crucial selection processes: positive selection retains cells recognizing antigen-MHC complexes, while negative selection eliminates those reactive to self-antigens, preventing autoimmunity (Bellavia D, et al., 2003). Notch receptors, a key regulatory family, influence T cell fate. Studies reveal different Notch receptors are active at distinct thymic stages (Bellavia D, et al., 2003). Regarding the maturation of more immature DN thymocytes, Notch1 mainly affects early stages of T-cell precursors, supporting T-cell development over B-cell fate (Hasserjian RP et al., 1996; Radtke F et al., 2010). Conversely, Notch3 seems to play a pivotal role in "beta selection" stages but decreases significantly in mature thymocytes (Felli MP, et al., 1999). The varied expression of the two receptors raises questions about specific roles in T cell differentiation. Notably, disturbances or mutations in the genes governing these specific processes can lead to lymphoproliferative conditions like T-cell Acute Lymphoblastic Leukemia (T-ALL), where normal T-cell development is disrupted, causing aberrant cell growth and function. However, the final outcome of Notch signaling pathway activation is extremely context-dependent, ranging from that of a classical oncogene, that blocks differentiation and stimulate uncontrolled proliferation of progenitor cells, as for T-cell compartment, to that of an onco-suppressor, as for

myeloid cell lineage, where instead, the inhibition of Notch signaling has been associated with pathological effects (Lobry C, et al., 2014).

"T-cell acute lymphoblastic leukemia" (T-ALL) is an aggressive hematological tumor arising from aberrant T cell progenitors and represents about the 15% of all pediatric cases and 25% of all adult cases of acute lymphoblastic leukemia. The prominent role of Notch in driving T-ALL has been firmly established since its initial association with aberrant translocation events in some cases of a rare T cell neoplasm (Ellisen LW, et al., 1991), and numerous studies conducted on murine models and human patients have further substantiated this theory. Data demonstrate that over 50% of human T-ALL cases are characterized by an activating mutation of Notch-1, making the activation of this oncogene the main feature of T-ALL. This mutation can affect two distinct regions of the gene: the first within the hydrophobic region of the heterodimerization domain (HD) in the extracellular portion of the receptor, leading to the destabilization of the domain itself and rendering the S2 site more susceptible to attack by metalloproteinases like ADAM (Malecki MJ, et al., 2006). The second class of mutations generates a shift in the reading sequence or results in aberrant stop codons that lead to alterations in the C-terminal PEST domain of the protein, causing an increase in the stability of the intracellular domain. Various experiments in mice have confirmed the oncogenic role of Notch-1. These experiments observed the emergence of immature phenotype T-cell leukemias in mice that were previously irradiated and subsequently transplanted with murine bone-marrow precursors overexpressing the intracellular domain of Notch-1 (Pear WS, et al., 1996; Pui JC, et al., 1999; Aster JC, et al., 2000). Subsequently, a transgenic murine model was created for the intracellular domain of the Notch-1 receptor (N1IC), controlled by the proximal promoter of the *lck* gene, which is specific to immature thymocytes, to constitutively activate Notch only in this cellular compartment. Surprisingly, in these lck-N1IC transgenic mice, thymomas were observed only sporadically and at advanced ages (Robey E, et al., 1996). Additionally, in leukemia human cell lines it was confirmed an essential role of this receptor in sustaining their growth (Weng AP, et al., 2003). However, activating mutations in Notch3 have also been observed to be relevant in T-ALL. First of all, a human acute T-cell leukemia cell line (TALL-1), is specifically characterized by a homozygous mutation in exon 26 of the Notch3 gene, without any mutations related to Notch1. This specific mutation (S1580L) is found in the negative regulatory region (NRR), which, due to its

proximity to the S2 site, appears to make the domain more susceptible to cleavage by ADAMs (Bernasconi-Elias P, et al., 2016). It is important to highlight that, despite Notch1 playing a central role in T-ALL development, its activation might not always be present. Dysregulation of Notch3 is also observed in T-ALL patients without Notch1 activation (Bernasconi-Elias P, et al., 2016), suggesting that Notch3 might define a particular subset of patients affected by T-ALL.

2.2 The *N3-tg* Mouse Model of T-ALL

Even before being confirmed in patients, the link between Notch and T-cell acute lymphoblastic leukemia (T-ALL) was strongly supported by a series of experimental studies conducted in murine models. Mice were engineered with overexpression of Notch1 intracellular domain in hematopoietic marrow precursors through retroviral vectro infections (Pear WS, et al., 1996; Campese et al., 2006), as well as conventional transgenic overexpression of Notch3 intracellular domain in immature thymocytes (*N3-tg* mice) (Bellavia D, et al., 2000). Both mouse models yielded similar results, confirming that members of the Notch receptor family act as oncogenes within immature T lymphocytes when deregulated. In particular, in *N3-tg* mice the constitutive expression of Notch3 intracellular domain (N3IC) was controlled by the murine proximal promoter of *lck*, specific to immature thymocyte cells at the later phase of DN stages (Bellavia D, et al., 2000). Consequently, ligand-independent activation of Notch3 was induced in the thymocyte compartment, leading to the development of a form of T-cell leukemia akin to the aggressive type found in human pediatric T-ALL. Unlike observed results for lck-driven ICN1 transgenic mice above, all lck-N3IC (*N3-tg*) mice developed T-ALL as early as 6 weeks of age (Bellavia D, et al., 2000), and most of these transgenic mice died around 14-18 weeks of life. The pathology observed shares similarities with a highly aggressive form of pediatric T-ALL, such as the presence of splenomegaly, generalized lymphadenopathy and peripheral accumulation of immature T cells, mainly with a $CD4^{-}CD8^{-}$ (DN)/$CD4^{+}CD8^{+}$ (DP) phenotype (Bellavia D, et al., 2002), rendering *N3-tg* mouse a valid study model. Constitutive expression of N3IC also induces alterations in the differentiation pattern of T cells, characterizing T-cell leukemia development. Specifically, *N3-tg* mice revealed an accumulation of immature DN and DP T cells in

thymic and peripheral tissues, with high expression of CD25 and pTα (the invariant chain of the pre-TCR complex), along with constitutive activation of the NF-κB transcription factor (Bellavia et al., 2000). These factors are typically involved in beta selection phase of thymocyte development, indicating clearly that the oncogenic role of Notch3 primarily manifests in this phase of immature thymocyte development. Notably, the accumulation in the periphery, particularly in the spleen and bone marrow, of DN and DP T cells, which normally reside exclusively inside the thymus, is a distinctive and pathognomonic sign of T-ALL progression. More recently, it has been observed that constitutive activation of the Notch3 signal in aberrant DP T cells of *N3-tg* mice is also responsible for promoting *in trans* an IL-6-dependent expansion of functional myeloid-derived suppressor cells (MDSCs) with a $CD11b^{+}Gr\text{-}1^{+}$ phenotype (Grazioli P, et al., 2022), suggesting a significant involvement of the immune TME in T-ALL progression.

The key characteristics of the conventional transgenic *N3-tg* murine model are illustrated in **Figure 3**.

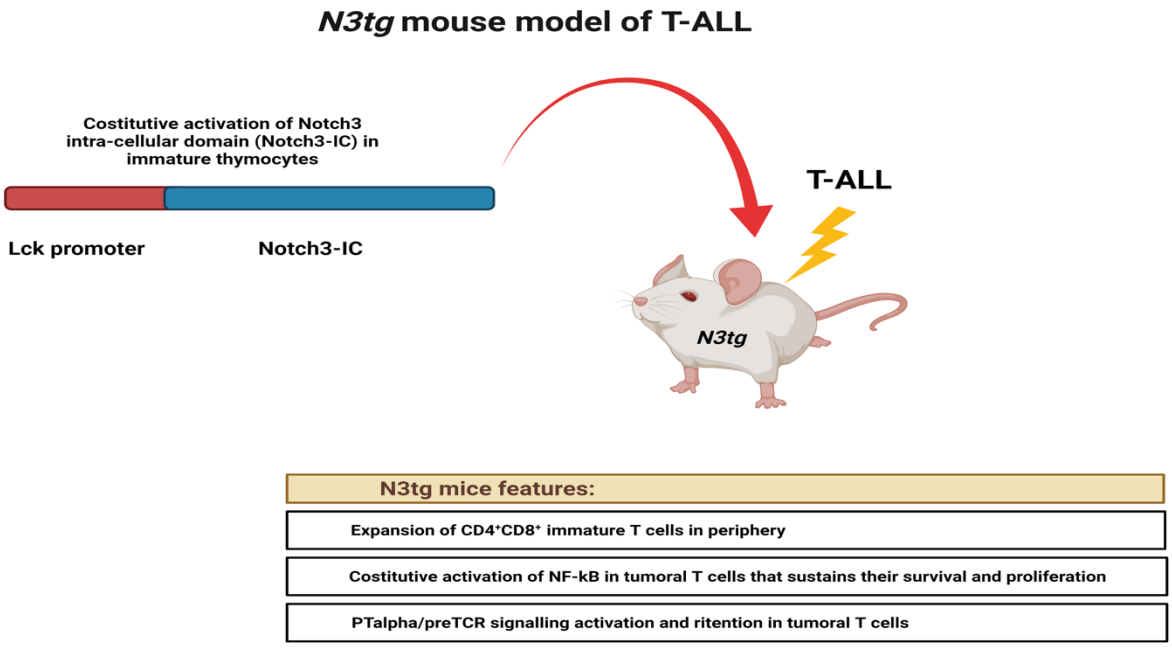

Figure 3. The N3-tg Murine Model of T-ALL. *The figure summarizes the main phenotypic characteristics of the N3-tg mouse model of T-ALL. Created with Biorender.com.*

2.3 Notch Signaling in MDSC Biology

The role of the Notch signaling pathway in myelopoiesis remains a subject of debate, but several pieces of evidence emphasize Notch's critical role in maintaining progenitor cells and impeding the terminal differentiation of myeloid cells, while others suggest its involvement in the completion of the differentiation process for mature myeloid cells (Cheng P, et al., 2014). Although the role of Notch signaling in myelopoiesis remains somewhat controversial, accumulating evidence has demonstrated its involvement in the development of Myeloid-Derived Suppressor Cells (MDSCs) (as reviewed in Grazioli P, et al., 2017).

Various data indicate that disruption of the Notch signal in bone marrow stromal cells or hematopoietic progenitor cells might trigger myeloid proliferation, potentially leading to the accumulation of $CD11b^{+}Gr\text{-}1^{+}$ cells with MDSC features (Amsen D, et al., 2015). In transgenic mice overexpressing ADAM10, thus altering proteolytic cleavage process of Notch transmembrane subunits and leading to an accumulation of intermediate molecules, a blockade in B cell development, a delay in T cell development, and a systemic increase in $CD11b^{+}Gr\text{-}1^{+}$ cells were observed (Gibb DR, et al., 2011). Another study demonstrated that inhibiting Notch signaling in tumor-bearing mice promotes the generation of less immunosuppressive PMN-MDSCs and disrupts M-MDCS formation. This suggests that modulating the Notch pathway could potentially alter the differentiation pattern and function of MDSC subpopulations (Wang SH, et al., 2016). This inhibition suppresses the STAT3 pathway in MDSCs, and when activated by IL-6, STAT3 reverses the effects of Notch signaling deficiency in MDSCs. This highlights a potential interconnected pathway involving Notch, IL-6, and STAT3 in MDSC development (Wang SH, et al., 2016). In a seminal paper from Gabrilovich's group, the authors demonstrated that an expansion of MDSCs at the expense of dendritic cells, is induced by an inhibition of Notch signals in hematopoietic progenitors of tumor-bearing mice, as well as in oncology patients (Cheng P et al., 2014). From a mechanistic point of view, the CKII kinase modifies Notch1 by adding phosphate groups to its intracellular domain (ICN1), disrupting its interaction with the CSL transcription factor. Treating tumor-bearing mice with CKII inhibitors restored normal Notch target gene expression and facilitated proper development of dendritic cells (Cheng P, et al., 2014). It is noteworthy that the increase in CKII is mediated by

factors released from the tumor, suggesting the involvement of non-cell-autonomous mechanisms (Cheng P, et al., 2014).

On the other hand, also the activation of Notch signaling pathway was associated with MDSC development. Peng and colleagues revealed in a breast cancer model that MDSCs play a role in promoting and sustaining the pool of tumor stem cells. This effect is mediated by both IL-6-dependent STAT3 phosphorylation and Notch activation, facilitated through nitric oxide release. As a result, the suggestion is made that targeting Notch and STAT3 signaling pathways could hold promise as a therapeutic approach for breast cancer (Peng D, et al., 2016). In addition, studies have highlighted that inhibiting Notch signaling through γ-secretase or anti-jagged antibodies restricts MDSC function in cancer contexts. This suggests a beneficial impact of Notch activation on this particular subset (Sierra RA, et al., 2017; Mao L, et al., 2018), in both solid and hematologic neoplasms.

In the context of leukemic pathologies, the role of Notch in potentially influencing MDSC onset and their crosstalk with leukemic T cells remains poor explored in the literature. Our lab team has recently gained intriguing new insights (Grazioli P, et al., 2022), demonstrated MDSC accumulation in the spleen, peripheral blood, and bone marrow of the Notch-3 dependent T-ALL transgenic mouse model (*N3-tg* mice). Indeed, these mice showed a significant increase in $CD11b^{+}Gr\text{-}1^{+}$ cells that are, indeed, functional MDSCs capable of exerting a dose-dependent suppression of *wt* T splenocyte proliferation (Grazioli P, et al., 2022). Mechanistically, the role of the pro-inflammatory cytokine IL-6 in this context was investigated. Studies confirm IL-6 as a significant factor in MDSC expansion and activation (Condamine T, et al., 2015). Our observations were conducted through co-culture experiments, using *wt* bone-marrow myeloid precursors and DP T cells obtained from *N3-tg* model. The study found that when neutralizing anti-IL-6 antibodies were introduced during co-culture, the resulting suppression test showed less immunosuppressive activity by MDSCs. These findings suggest that the dysregulation of Notch in tumor DP T cells of *N3-tg* mice may induce MDSCs *in vitro* through an IL-6-dependent mechanism. To validate this statement, experiments were conducted *in vivo* using NSG (NOD/LtSz-scid IL2rγ null) immunodeficient mice. These mice lack T, B, and NK cells but retain $CD11b^{+}Gr\text{-}1^{+}$ cells (Shultz LD, et al., 2005). Transplanting these models with DP T cells isolated from *N3-tg* transgenic mice showed an expansion of the myeloid compartment, accompanied by

the appearance of functional MDSCs. Strikingly, we demonstrated that eliminating MDSCs in *N3-tg* mice, by treating them with anti-IL-6 or anti-Gr-1 antibodies, significantly reduced the fitness of DP T tumor cells and ultimately, the T-ALL progression. Further, we also demonstrated that the human Notch3-dependent T-ALL cell line, TALL-1, induced MDSCs from PBMCs of healthy donors with the $CD11b^{+}CD33^{+}CD14^{+}HLADR^{low/neg}$ phenotype. This induction was again dependent on both Notch activation and IL-6 signaling mechanisms (Grazioli P, et al., 2022).

In conclusion, tumor T cells from Notch-3-induced T-ALL significantly impact on tumor microenvironment by inducing MDSCs. However, these preliminary studies require further exploration, as there is still limited understanding regarding the mechanisms of action and specific targets of Notch-dependent MDSCs in T-ALL.

3.The Mechanism of Action of NK Cells

Toward the end of the '80s, the mechanism by which NK cells eliminate tumor cells while sparing healthy ones was proposed. This concept emerged when researchers discovered that murine NK cells could destroy mutated lymphoma cells that didn't express MHC-I molecules on their surface. This suggested NK cells tend to preferentially target cells lacking MHC-I expression (Kärre K, et al., 1986). In parallel experiments on human cells, specific inhibitory receptors for MHC-I, known as Killer Ig-like receptors (KIR), were identified. Additionally, activating variants of KIR receptors were found. Molecular analyses revealed significant differences in the transmembrane and intracytoplasmic portions of these receptors (Montaldo E, et al., 2013). While inhibitory KIR receptors feature long intracytoplasmic tails with ITIM inhibition motifs that activate SHP1 and SHP2 phosphatases to reduce signaling molecule activation, thus dampening NK cells, activating KIR receptors show short intracytoplasmic tails and signaling polypeptides with ITAM motifs (DAP12), crucial for signal transduction (Parham P, 2005). Besides inhibitory KIR receptors binding to HLA-I molecules, another significant group consists of CD94/NKG2A, B heterodimeric receptors, recognizing HLA-E (Braud VM, et al., 1998). Additionally, various inhibitory NK receptors that are non-specific for MHC were identified, including cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and PD-1 (Bai R and Cui J, 2022). In the

absence of inhibitory signals, NK cell cytotoxicity requires the action of a series of activating receptors. In humans, spontaneous cytotoxic activity is mainly triggered by NKG2D, DNAM-1, and natural cytotoxicity receptors (NCR), while CD16 receptor on NK cells binds opsonized cells, facilitating antibody-dependent cellular cytotoxicity (ADCC) (Zamai L, et al., 2007). NKG2D and DNAM-1 recognize stress-induced ligands expressed by various tumor cell lines. On the other hand, natural cytotoxicity receptors (NCR) - NKp46, NKp30, and NKp44 - act through recognizing unknown ligands and mediate the destruction of numerous tumor cells.

Among the receptors activated on murine NK cells there are NKG2D, 2B4, Ly49 (especially Ly49D and H), and the CD94-NKG2C complex. Notably, $CD27^{hi}$ NK cells have shown cytotoxicity independently of NKG2D against Yac-1 target cells, suggesting these NK cells can eliminate target cells via a different activation pathway than NKG2D (Hayakawa Y, et al., 2006a). Although the set of activating receptors appears similar among various subsets of murine NK cells, inhibitory receptors present distinctions. $CD27^{lo}$ NK cells express a higher percentage of inhibitory isoforms like Ly49C, Ly49I, and KLRG1. NK cells lacking KLRG1 have demonstrated greater efficiency in carrying out their functions, suggesting KLRG1 expression acts as an inhibitory mechanism for NK cell proliferation and activity (Hayakawa Y, et al., 2006a). During the initial phase of innate immune responses, murine NK cells receive the first activation signals via inflammatory cytokines like IL-1, IL-12, IL-18, or Type I interferons (IFN) (Hayakawa Y, et al., 2006a). Upon stimulation, NK cells become activated killer cells, proliferate, increase cytokine production, and regulate the expression of effector molecules like perforin, granzymes, and Fas ligand (FasL) (Zamai L, et al., 2007). The elimination of target cells might occur through the targeted release of lytic granules containing granzymes and perforin after adhesion via integrins to form immunological synapses (Van den Broek MF, et al., 1995). Alternatively, NK cells might induce target cell death involving the so-called death receptors. Three different receptor/ligand systems can induce apoptosis during activation: TNF, binding to TNF-1 or TNF-2 receptors; FasL, binding to the CD95 receptor (APO-1/Fas) (Peter ME and Krammer PH, 2003); and TRAIL, which can bind to various TRAIL receptors (Medvedev AE, et al., 1997).

3.1 Alterations of NK Cell Function in Hematological Malignancies

The natural killer (NK) cells play a pivotal role in regulating tumor growth and fostering an anti-metastatic effect. Their significance has been affirmed through various experiments using murine tumor models. For instance, reducing NK cells before tumor transplantation has been shown to intensify tumor aggressiveness, leading to metastasis formation (Spiegel A, et al., 2016). Other research highlighted that an increased cytotoxic activity of circulating NK cells is associated with reduced cancer risk, emphasizing the crucial role of natural immune response against tumors (Imai K, et al., 2000). Another study in 2014 demonstrated that mice lacking the Tbx21 transcription factor (also known as T-bet), essential for NK differentiation, were more prone to metastasis formation after intravenous injection of melanoma cells (Malaisè M, et al., 2014). NK cells are recruited into inflamed tissues owing to various chemokine receptors on their cellular surface, including CXCR3, binding to chemokines released by tumor cells (Susek KH, et al., 2018). In particular, regarding hematologic malignancies, it has been hypothesized that the reduction in natural killer cells of NCR receptor expression represents a mechanism through which tumor cells become resistant to NK cell destruction. A study by Costello et al. (Costello RT, et al., 2002) examined the cytolytic capability of NK cells in patients with acute myeloid leukemia (AML). Most NK cells in leukemia patients exhibited low NCR density on their surface. Notably, a strong correlation exists between NCR density and NK cell cytolytic activity (Sivori P, et al., 1999), hence this profile was linked to reduced ability of NK cells to destroy leukemic cells. In contrast, in patients with multiple myeloma (MM), a hematologic malignancy involving the accumulation of plasma cells in the bone marrow, NCR receptor expression levels on NK cells were normal. However, the expression of the CD16 receptor was significantly reduced compared to healthy donors, potentially impacting the NK cells ability to mediate antibody-dependent cytotoxicity (Fionda C, et. al., 2018). The functional alterations of NK in hematological malignancies reported above, might stem from direct interaction between tumor cells and NK cells or the release of inhibitory substances from leukemic blasts and all of them can be exploited for therapy (as reviewed in Merino A, et al., 2023). The most frequently identified among inhibitory substances include cytokines like TGF-β, L-kynurenine, and prostaglandin E2. The release of such inhibitory substances appears to be a common process in hematologic diseases like AML and MM, as well as in various types of solid

tumors like melanoma, glioblastoma, and breast cancer. The inhibitory effect on NK cells by tumors can be further amplified by the presence of cells with immune-suppressive activity, such as tumor-induced regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs). In all these instances, the reduction of activating receptors on NK cells is associated with a functional impairment on NK cells infiltrating tumor tissues.

Finally, one of the few studies conducted on NK function in T-ALL, demonstrated *in vitro* and *in vivo* cytotoxic activity by NK-92 cells (representing activated human NK cells) against human leukemia cell lines. Approximately half of the samples from leukemia patients were sensitive to NK-92-mediated cytotoxicity *in vitro*, while normal human bone marrow cells were resistant (Yan Y, et al., 1998).

4. Structure and Function of the PD-1/PD-L1 Axis

Among all the immune checkpoints, the PD-1/PD-L1 axis holds particular significance as it is acknowledged as a therapeutic target in various types of malignancies. Programmed cell death 1 (PD-1) is an inhibitory receptor of immune-response, well-known for a critical role in suppressing T-cell activation and proliferation, upon interaction with the PD-L1 ligand. This surface receptor belongs to the CD28 family and is expressed on activated T cells, Natural Killer T cells, B cells and myeloid cells. The PD-1 molecule comprises an extracellular IgV-like domain, a transmembrane region, and an intracellular domain (Ishida Y, et al., 1992; Shinohara T, et al., 1994). This last domain contains two phosphorylation sites: the immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM), that are essential for inhibitory function of PD-1 (Patsoukis N, et al., 2020). PD-1 interacts with two B7 family ligands, PD-L1 and PD-L2. PD-L1 is widely expressed on antigen presenting cells (APCs) and hematopoietic cells, whereas PD- L2 is only expressed on macrophages and dendritic cells. (Mathieu M, et al., 2013). PD-1, found on activated CD4 and CD8 T cells, B cells, Natural Killer (NK) cells, macrophages, dendritic cells (DCs), and monocytes, plays a critical role in immune regulation (Dong Y, et al., 2017). In a healthy physiological state, the PD-1/PD-L1 pathway functions

positively, helping to regulate tissue inflammation and prevent autoimmune diseases. However, within tumorigenic environments, the collaboration between PD-1 and PD-L1 hampers the host's ability to mount an effective anti-tumor immune response, allowing tumors to evade immune detection. Tumor cells, including leukemic cells, often exhibit heightened PD-L1 expression, serving as an evasion tactic adopted by cancers to evade immune surveillance (Iwai Y, et al., 2002; Rezaeeyan H, et al., 2017). The initial indication pointing towards the role of the PD-1/PD-L1 pathway in suppressing anti-tumor immunity emerged from observations showing that the heightened expression of PD-L1 on P815 mastocytoma cells hinders the lytic activity of $CD8^+$ T cells through interaction with PD-1 in laboratory settings. Furthermore, this elevated expression enhances their *in vivo* capability to generate tumors and infiltrate tissues and organs (Iwai Y, et al., 2002). Additionally, within the tumor microenvironment (TME), cells like TAMs, MDSCs, and DCs frequently exhibit overexpression of PD-L1. Over the last decade, emerging studies have identified the presence of $PD\text{-}L1^+$ MDSCs in various tumor models (Fuse H, et al., 2016; Lu C, et al., 2016; Fujimura T, et al., 2012) and in cancer patients (Azzaoui I, et al., 2016). Several studies have shown that tumor-infiltrating MDSCs express a higher level of PD-L1 than their peripheral counterparts, suggesting microenvironmental regulation of PD-L1 expression (Noman MZ, et al., 2014; Fuse H, et al., 2016; Lu C, et al., 2016; Fujimura T, et al., 2012). Upon binding PD-L1, PD-1 triggers intracellular signaling pathways that deactivate immune cells, leading to their exhaustion. Consequently, therapeutic antibodies that block the PD-1/PD-L1 axis have emerged as successful strategies in boosting anti-tumor immune responses (Balar AV and Weber JS, 2017). The investigation of PD-1/PD-L1 signaling predominantly focused on activated T cells. Remarkably, PD-1 expression characterizes T cell exhaustion, a well-recognized state in chronic viral infections and cancer (Pauken KE and Whery EJ, 2015). The initiation of anti-tumor immune responses involves T cell activation after the presentation of tumor antigens within the context of MHC molecules by APCs (antigen-presenting cells). Upon activation, effector T cells migrate into the tumor site and start releasing several cytokines and cytolytic molecules, among them the IFNγ promotes PD-L1 upregulation on tumor cells. Tumor immune escape is due to the interaction of the upregulated PD-L1 receptor on tumor cells with inhibitory receptor PD-1 expressed on activated T cells. PD-1-PD-L1 binding induce T cell exhaustion impairing T cell functionality, proliferation, and cytotoxic activity (Annibali O, et al., 2018). However, PD-L1/PD-1 inhibition may not be sufficient in all patients and

not all those experience tumor shrinkage, durable responses, or prolonged survival, especially those with minimal activation of cancer-specific T cells, lack of strong cancer antigens or epitopes recognized by cells, poor infiltration of T cells into tumors (Kim JM and Chen DS, 2016). Even though, many studies are focused on the interaction of tumor cells expressing PD-L1 with PD-1^{+} T cells, the impact of PD-1/PD-L1 pathway on NK activation and function should not be underestimated. In many cancers, the crucial anti-tumor activity of Natural Killer (NK) cells is impaired by TME components, including MDSCs, and strategies aimed at rescuing/enhancing NK function have displayed great potential in therapy (Melaiu O, et al.,2020; Valipour B, et al., 2019).

4.1 The PD-1/PD-L1 In Hematological Malignancies and in T-ALL

The PD-1/PD-L1 axis stands as one of the primary pathways through which various cancer types, exhibiting increased PD-L1, evade the immune system. As previously mentioned, the interaction between PD-1/PD-L1 triggers various processes, primarily affecting T cells, such as exhaustion, anergy, and apoptosis (Zou W, et al., 2016). Thus, the balance between activation, cytotoxic function, and exhaustion is conditioned by the levels of PD-L1 expression in the surrounding environment. Tumor cells escalate PD-L1 expression, reducing functional capacity, proliferation, and cytotoxic action in cells expressing PD-1 (Tumeh PC, et al., 2014). Considering the promising outcomes obtained from inhibiting the PD-1 pathway in solid tumors (Hamanishi J, et al., 2016), recent times have witnessed new clinical and pre-clinical studies conducted in hematological neoplasms (Annibali O, et al., 2018). Classical Hodgkin lymphoma (cHL) is characterized by the presence of Reed-Sternberg (RS) tumor cells (Küppers R, 2009). Current therapy involves chemotherapy or autologous stem cell transplantation, yielding a 5-year progression-free survival of 40-60% (Gordon LI, et al., 2013). For this reason, understanding the interaction with the microenvironment and immune evasion strategies could prove beneficial in identifying new therapeutic agents for treating this malignancy.

The increased PD-L1 in RS cells and PD-1 in infiltrating lymphocytes create a potent inhibitory signal contributing to maintaining the suppressive microenvironment typical of cHL. This enables tumor cells to evade immune system recognition (Yamamoto R, et

al., 2009). Some studies have shown that genetic alterations on chromosome 9, where the PD-L1 gene is located, lead to high expression of this ligand in RS cells. Moreover, the Janus Kinase 2 (JAK2) gene, also present on chromosome 9, when overexpressed, further increases PD-L1 expression (Armand P, 2015). These results underscore the importance of the PD-1/PD-L1 immune checkpoint in cHL genesis and prognosis, suggesting that using PD-1/PD-L1 blocking antibodies could be beneficial in treating this patient group (Jelinek T, et al., 2017). Excessive PD-1 expression can also be found in Chronic Lymphocytic Leukemia (CLL), proposed as a potential diagnostic marker. The increase in strongly PD-1-expressing $CD8^+$ T cells is significantly linked to CLL development (Qorraj M, et al., 2017). Similarly, in other lymphoma types, particularly in Diffuse Large B-cell Lymphoma (DLBCL) and Follicular Lymphoma (FL), PD-L1 overexpression has been associated with reduced overall survival (Kiyasu J, et al., 2015). PD-L1 expression has been observed on both tumor cells and non-malignant cells infiltrating the tumor, as well as on macrophages (Andorsky DJ, et al., 2011). These pieces of evidence highlight the crucial importance of the PD-1/PD-L1 immune checkpoint in tumor genesis and progression, suggesting that the use of monoclonal antibodies inhibiting PD-1/PD-L1, such as Nivolumab (human anti-PD-1 monoclonal antibody), may be a beneficial option for treating these patient categories (Jelinek T, et al., 2017). Elevated PD-L1 inhibits the response of anti-tumor T cells, thus favoring tumor progression. Regarding tumor involving T cells, T-cell lymphomas with increased PD-L1 expression in the tumor microenvironment have been effectively treated by targeting PD-1 (Wilcox RA, et al., 2009; Phillips T, et al., 2016). However, in patients with T-cell neoplasms, blocking the PD-1/PD-L1 pathway can have contradictory effects, both protecting against cancer progression or favoring it, as both immune effector cells and tumor cells are T cells capable of expressing PD-1. For what concerns T-ALL, Studies on spontaneous preclinical models of T-ALL with alterations in Notch-1 signaling induced by thymic transplantation in immunocompetent models have highlighted the expression of PD-1/PD-L1 on leukemic cells. Specifically, it has been confirmed that the expression of these molecules increases with the increase in the tumor mutational burden (TMB), and that antibody therapy is effective when the TMB is high but less effective when the TMB is low (Gao J, et al., 2019). Therefore, TMB is a determining factor in the effectiveness of anti-PD-1 treatment Nivolumab has been studied in a phase II clinical trial involving patients with T-cell acute lymphoblastic leukemia (T-ALL). Unfortunately, after the enrollment of the first three patients, it was

discovered that the drug led to disease progression. Given these discrepancies, it is undoubtedly necessary to further investigate the use of PD-1 and PD-L1 monoclonal antibodies in the therapy of patients affected by T-ALL (Patel J, et al., 2023).

An intriguing recent study has indicated that leukemic stem cells (LSC) expressing the PD-1 receptor initiate the disease, as these cells exhibit high NOTCH1-MYC activity. Moreover, the signal transmitted by PD-1 protects these cells from apoptosis induced by the TCR receptor signal, suggesting that the use of PD-1 inhibitors could represent a promising strategy in T-cell acute lymphoblastic leukemia (T-ALL) therapy (Xu X, et al., 2023). In fact, the elimination of PD-1-expressing cells, removal of PD-1 in T-ALL cells, or interference with PD-1 or its ligand PD-L1 significantly reduces LSC and arrests disease progression.

Combined therapy using interference with PD-1 and chemotherapy significantly prolongs the survival of immunocompromised mice that received a transplant of murine or human T-ALL cells. Mechanistically, PD-1-expressing LSC exhibit elevated NOTCH1-MYC activity early in the disease. Additionally, the PD-1 signal protects LSC from apoptosis caused by the TCR signal through a cell-autonomous mechanism triggered by interaction with PD-L1 expressed on the same cell (Xu X, et al., 2023).

4.2 The Role of PD-1/PD-L1 Molecules in Mediating MDSC-NK Interaction in The Tumor Microenvironment

As highlighted earlier, the PD-1/PD-L1 axis is known for its involvement in tumor-tissue interactions between tumor cells and activated T cells. Recent studies have indicated an expanding horizon for this signaling pathway, extending its role beyond this dynamic. There's emerging evidence of PD-1 and PD-L1 expression in other critical players within the tumor microenvironment, such as MDSCs and Natural Killer cells. This suggests a broader interplay among various immune cell subsets, potentially influencing the immune landscape within tumors. In this context, PD-L1 plays a significant role in facilitating immune evasion in MDSC-dependent tumors (Liechtenstein T, et al., 2014). Multiple scientific pieces of evidence in literature

demonstrate that MDSCs limit the ability of NK cells to counteract tumors (Elkabets M, et al., 2010), and they do so through previously mentioned mechanisms like TGF-β, IDO, and adenosine production. However, MDSCs might directly interact with NK cells by expressing the ligand PD-L1. This interaction with the PD-1 receptor on NK cells could potentially inhibit their function. Studies have not only identified the presence of PD-L1$^+$ MDSCs in various tumor models (Fuse H, et al., 2016), but have also confirmed that tumor-infiltrating MDSCs express higher levels of PD-L1 compared to their peripheral counterparts, suggesting a microenvironment-mediated regulation of PD-L1 expression (Fuse H, et al., 2016). Tumor-derived factors can influence MDSC. Interestingly, GM-CSF can promote immunosuppression by inducing PD-L1 expression on MDSCs through STAT3 activation (Thorn M, et al., 2016). The expression of the PD-1/PD-L1 axis on NK cells has also been investigated. An immunosuppressive tumor microenvironment might contribute to the presence of dysfunctional NK cells, incapable of combating tumor growth. One mechanism through which the TME limits NK cell activity is by overexpressing the inhibitory receptor PD-1 on these cells. The increased expression of PD-1 on NK cells is associated with reduced functionality. Specifically, their ability to produce cytokines, as well as their cytotoxic and proliferative activities, becomes compromised (Pesce S, et al., 2017). NK cell subset expressing PD-1 have been identified in cancers, including multiple myeloma, Kaposi's sarcoma, ovarian and gastrointestinal carcinomas, lung cancer, and Hodgkin's lymphoma (Cheng M, et al., 2013). Thus, it remains the possibility that PD-L1$^+$ MDSCs may exert their function on NKs through the the PD-1/PD-L1 pathway and then, blocking antibodies could be exploited not only to restore T cell response but also anti-tumor activity of NK cells.

In this context, the aim of this thesis was to explore the potential involvement of the PD-1/PD-L1 in influencing tumor microenvironment of T-ALL, by mediating cross-talk between T tumor cells, MDSCs, and NK cells.

EXPERIMENTAL PART

5. Aim of the Work

The tumor immune microenvironment (TIME) comprises a wide range of molecules, intercellular signals, cancer cells, as well as pro-tumoral (i.e., Tregs and MDSCs) and anti-tumoral (i.e., T-cells and NK cells) subsets. TIME has a pivotal role in tumor immune suppression, distant metastasis, local resistance, and the targeted therapy response. Understanding the function and significance of each cell and molecule within the TIME is essential for cancer treatment. This knowledge allows us to target critical cells and signaling pathways, enhancing the effectiveness of tumor therapy. Immunotherapy, with its expanding therapeutic dimensions, is increasingly vital for treating cancer patients. Factors influencing the immune response in the TIME include the cell surface expression of immune checkpoint molecules. In recent years, the role of immune checkpoints as effective therapeutic targets has emerged in various solid tumors (melanoma, non-small cell lung cancer, renal cell carcinoma, head, and neck carcinoma) and hematologic malignancies such as Hodgkin lymphoma and adult T-cell leukemia/lymphoma (ATLL). Indeed, tumor cells exploit these pathways to deactivate anti-tumoral immune responses, evading immune surveillance and promoting cancer progression. The inhibition of immune checkpoints, a source of immune escape in numerous cancers, constitutes a key dimension of immunotherapy.

The overarching aim of this thesis is to investigate MDSCs as a therapeutic target in the context of T-cell Acute Lymphoblastic Leukemia (T-ALL). T-ALL is characterized by the uncontrolled proliferation of immature T cells and understanding how the immune system can be harnessed to combat this malignancy is crucial. In this context, MDSCs could play a role in creating an immunosuppressive tumor microenvironment by inhibiting both adaptive and innate immune responses. While extensively studied in various cancers, research on MDSCs in T-ALL, particularly the Notch-dependent subtype, is limited.

In our lab, we have created a mouse model that mimics an aggressive form of this disease, which is similar to the type seen in pediatric humans. This model, known as

N3-tg mice, overexpresses the intracellular domain of the Notch3 receptor (N3IC). Our previous research showed that in this model, immature tumor T cells induce MDSCs through a mechanism dependent on both the Notch signaling pathway activation and the presence of the cytokine IL-6. Interestingly, when we removed these MDSCs, we observed a reduction in the growth and spread of leukemia cells. This highlights the important contribution of MDSCs to the development of T-ALL. However, for this pathology, the primary target of the immunosuppressive action of MDSCs and the mechanism through which MDSCs promote tumor progression remain unknown. T-cells are usually the main target of MDSC suppression in cancer microenvironment, but, in T-ALL tumor cells are represented exactly by T-cells. Thus, in this thesis one of the primary focuses was to explore the possibility that NKs could be an important target of MDSC suppression and thus, to test the feasibility to reactivate innate immune responses by specifically enhancing the cytotoxic activity of Natural Killer (NK) cells. NK cells play, indeed, a pivotal role in immune surveillance and can recognize and eliminate abnormal cells, including cancer cells. Recent evidence in other malignancies has demonstrated that beside activated $CD8^+$ T cells, NK cells can also serve as a crucial target for the immunosuppression exerted by MDSCs.

Regarding the mechanism of immunosuppression exerted by MDSCs in the Tumor Microenvironment (TME), a recently suggested mode involves inhibiting the immune response through the PD-1/PD-L1 axis. This research endeavors to shed light on whether, within the T-ALL microenvironment, MDSCs impede NK cell activity, possibly through PD-1/PD-L1 axis, thereby contributing to the immune evasion orchestrated by leukemia cells.

In conclusion, the final purpose of this thesis was to investigate the potential role of the PD-1/PD-L1 axis in mediating the crosstalk between NK cells and MDSCs in the TIME of Notch-dependent T-ALL. Prospectively, these studies aim to enhance innovative strategies, combining immune checkpoint inhibitors (targeting PD-1/PD-L1 molecules) with therapies addressing the immunosuppressive role of MDSCs, contributing to the development of more effective and targeted immunotherapeutic approaches for Notch-dependent T-ALL treatment.

6. Materials and Methods

6.1 Mice

The experiments were conducted on mice, all with the same genetic background: C57Bl/6. Transgenic *N3-tg* mice were used, generated, genotyped, and characterized as previously described (Bellavia D, et al., 2000), along with age-matched wild-type (*wt*) mice as controls. The procedures employed in the animal experimentation comply with national guidelines regarding the ethical use of animals for scientific purposes as established in Legislative Decree No. 26/2014.

6.2 Cell Cultures

The cells were cultured at 37°C with 5% CO_2 in complete medium, specifically RPMI-1640 medium (GIBCO), supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin and streptomycin, and 2 mM glutamine. The murine T-cell lymphoma cell line YAC-1 was maintained in culture, as previously described (Lin Q, et al., 2021). The utilized cell lines were regularly screened to confirm the absence of mycoplasma.

6.3 Preparation of Cell Suspensions from Mouse Organs

Organs of interest were obtained post-mortem from *N3-tg* mice and their corresponding *wt* controls. Cell suspensions from the spleen were obtained through mechanical dissociation in RPMI medium and filtration through a 70 μM filter to eliminate unwanted tissue components. Subsequently, cells were collected in the pellet through centrifugation for 10 minutes at 1250 RPM at 4°C. Erythrocytes were removed by resuspending and incubating for 1 minute at room temperature with 1x RBC Lysis Buffer containing ammonium chloride ACK (NH_4Cl 0.15 M, $KHCO_3$ 1mM, Na_2EDTA-$2H_2O$ 0.1 mM), followed by washing with the addition of a 10x volume of RPMI and centrifugation for 10 minutes at 1250 RPM at 4°C. Finally, cells were resuspended in an appropriate volume of RPMI, and their total number and concentration were assessed through counting using a Burker chamber.

6.4 Flow Cytometry

After counting cells obtained from mouse organs, a corresponding volume of 1 x 10^6 cells per sample was transferred to 5 ml polystyrene tubes for FACS analysis. Staining was carried out for 30 minutes at 4 °C in the dark. Following this, samples were washed again by adding 2 ml of cold 1x PBS to remove unbound antibodies. For phenotypic analysis of mice, staining was performed using a mix containing the following specific mouse antibodies (BD, Bioscience): anti-CD4-PerCP.Cy5.5 (RM4-5), anti-CD8-APC (53-6.7), anti-CD11b-FITC (M1/70), anti-Gr-1-PE (RB6-8C5), enabling identification of both the $CD4^+CD8^+$ DP T cell population and $CD11b^+Gr\text{-}1^+$ myeloid cells (containing potential MDSCs). To identify NK cells, a mix containing anti-CD3-APC (145-2C11) and anti-NK1.1-FITC (P4/36) antibodies, all from BD, Bioscience, was prepared. Additionally, in combination with antibodies specific to the previously described subpopulations, anti-PD1 (29F.1A12) and anti-PD-L1 (MIH5) antibodies conjugated with appropriate fluorochromes (BD, Bioscience) were used to identify populations expressing these receptors. Samples acquired on the FACScalibur were analyzed using the CellQuest program (BD, Bioscience). Fluorescence in different samples was compared to that of corresponding isotypic controls, stained with the same protocol.

6.5 FACS-assisted Cell Sorting

Cell suspensions from the spleens of *N3-tg* mice were stained with anti-CD4, anti-CD8, anti-CD11b, and anti-Gr-1 antibodies, labeled with appropriate fluorochromes, to isolate $CD11b^+Gr1^+$ and $CD4^+CD8^+$ cells. Anti-NK1.1 and anti-CD3 antibodies were used to isolate the $NK1.1^+CD3^-$ subset. All relevant subsets were isolated (purity level ≥98%) using a FACSAriaIII sorter (BD Bioscience) equipped with BD FACSDiva software (BD Bioscience).

6.6 MDSC Suppression Test and $CD4^+CD8^+$ DP T Proliferation Assay

For the suppression assay of MDSCs, T cells from *wt* spleen were isolated by negative selection with the Pan T cell isolation kit II (Miltenyi Biotec) and stained with 2,5 μM CFSE (Sigma Aldrich). CFSE-labelled T cells were activated with coated 3 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (BD Bioscience) and 3.0 $x10^5$ cells/well (responders) were co-cultured with $CD11b^+Gr1^+$ cells (suppressors), sorted from the spleen of *N3-tg* mice at 5-6 weeks or 10-12 weeks, at the MDSCs:*wt* T cells ratio of 1:2. Co-cultures were performed for 72 h in 96-well plates. Then, samples were harvested and appropriately stained, and the proliferation of CFSE-labeled cells was evaluated by FACS analysis on gated $CD4^-CD8^+$ T cell subset. In experiments assessing the proliferation of tumor cells, DP T cells sorted, as above, from the spleens of *N3-tg* mice at 5-6 or 10-12 weeks of age were stained with CFSE, as described above, and co-cultured alone for 72 hours in 96-well plates, without any activation treatment. Afterward, samples were harvested, appropriately stained, and the proliferation of CFSE-labeled cells was evaluated by FACS analysis.

6.7 In Vitro Cytotoxicity Assay of NK Cells

The effector NK cells were isolated from the spleens of both *N3-tg* and control *wt* mice, as previously described. These NK cells were incubated overnight in an incubator using complete RPMI medium supplemented with IL-15 (Peprotech) at a final concentration of 50 ng/ml. The following day, YAC-1 target cells were stained with CFSE (2.5 μM). Subsequently, 5-10 $x10^4$ YAC-1 cells were co-incubated with NK cells from mice of different genotypes at different target-to-effector (YAC-1: NK) ratios (ranging from 1:0 to 1:20, as indicated in the figure legends). The co-incubation was carried out in a FACS tube at 37°C in a humidified atmosphere with 5% CO_2. After 6 hours, the cells were washed and treated with a vital dye (7-AAD, BD Bioscience). The mortality percentages were assessed for $CFSE^+$ YAC-1 target cells through FACS analysis, identified in the $7\text{-}AAD^+$ cell fraction. In another set of experiments, *N3-tg* tumor derived $CD4^+CD8^+$ (DP) T cells isolated from spleens of mice aged 10-12 weeks were labeled with CFSE (2.5 μM) and 10 x 10^4 of them were utilized as target cells. These cells were then co-cultured with

NK cells obtained from spleen of *N3-tg* mice aged 5-6 weeks, serving as effectors, following the previously described procedure. In experiments testing the potential role of MDSCs in suppressing NKs, $CD11b^{+}Gr\text{-}1^{+}$ MDSC cells were purified as previously described from the spleens of transgenic mice, and co-cultured with NK cells isolated from spleen of *N3-tg* animals for 18 hours at the NK:MDSCs ratios of 1:1 or 1:3, before performing the NK cytotoxicity assay on YAC-1 targets, as described above.

6.8 In Vivo Treatment of *N3-tg* Mice with Blocking anti-PD-L1 Antibodies

10-12-week-old *N3-tg* mice were treated with the rat anti-mouse anti-PD-L1 antibody (clone 10F-9G2) at a dose of 100 μg/mouse (from Bioxcell), resuspended in 200 μl of PBS 1x and administered via bi-weekly intraperitoneal injections for a total of two consecutive weeks. Control mice were treated with equivalent amounts of isotype control antibodies (Rat IgG2b Kappa, clone LTF-2, Bioxcell). At the end of the treatment period, the animals were sacrificed, and appropriate spleen subsets were isolated and assessed, as previously described.

6.9 Statistical Analysis

The results are presented as mean values ± standard deviation (SD). Statistical significance was determined using unpaired two-tailed Student's T-test to analyze the differences between two groups (conducted using GraphPad software). A p-value of ≤ 0.05 was considered indicative of a significant difference between two groups. *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$, not significant (ns)=$p > 0.05$. All data shown are representative of at least three independent experiments, each involving at least one mouse per group, unless expressly stated otherwise.

7. Results

7.1 Inverse Correlation of Tumor- and MDSC- *versus* NK-Cell Distributions and Functions During T-ALL Progression

In our prior publication (Grazioli P, et al., 2022), we identified that MDSCs might impact the outcome of T-ALL in *N3-tg* mice. However, the specific targets and underlying mechanisms of their actions are yet to be investigated. Given that Natural Killer (NK) cells play a critical anti-tumor role in various cancers but are often hindered by components of the Tumor Microenvironment (TME), including MDSCs, our current exploration aims to uncover potential numerical and functional changes within the TIME populations, including $CD4^+CD8^+$ (DP) T tumor cells, $CD11b^+Gr\text{-}1^+$ MDSCs and NK cells from *N3-tg* mice during T-ALL progression. To address this, we analyzed these subsets from the spleens of *N3-tg* mice at two age intervals, namely 5-6 weeks, and 10-12 weeks of age, representing an initial and an advanced stage of the disease, respectively. Using FACS analysis, we observed a significant increase of $CD4^+CD8^+$ DP T tumor cells in both percentages (43.1 ±6.2% *vs* 12.2 ±3.1%) and absolute numbers (134±10.5x10^6 *vs* 21.7 ±4.6x10^6) **(Figure 4A)**. Similarly, MDSCs **(Figure 4B)** displayed a substantial increase in both percentages (8.2 ±2.0% *vs* 4.9 ±1.4%) and absolute numbers (25.5 ±4.5x10^6 *vs* 8.9 ±0.9x10^6), in old vs young mice. Concurrently, we investigated the distributions of $NK1.1^+CD3^-$ **(Figure 4C)**, uncovering a dramatic reduction in both percentages (0.5 ±0.3% *vs* 3.0 ±0.8%) and absolute numbers (1.6 ±1.1x10^6 *vs* 5.4 ±1,4x10^6) of NK cells aged 10-12 weeks compared to those at 5-6 weeks.

In the previous experiments, we observed numerical alterations in the cells of the tumor microenvironment. Our next objective was to assess whether their functionality was impacted during the progression of the pathology. Regarding the functionality of tumor subset, flow cytometry analysis of sorted DP T cell, derived from the spleen of *N3-tg* mice and pre-labeled with CFSE, indicates a significant increase in proliferation during disease progression. In particular, there is a significant expansion in the proportion of DP T highly proliferating cells (as indicated by the M3 marker; 0.2 ±0.1% *vs* 14.7 ±2.9% at 5-6 *vs* 10-12 wks of age) **(Figure 5A)**. To assess the suppressive capacity of MDSCs in transgenic mice, we performed the "suppression test", *ex vivo*. We isolated MDSC cells

from the spleens of *N3-tg* mice and co-cultured them at a 1:2 ratio with *wt* T splenocytes used as responder cells.

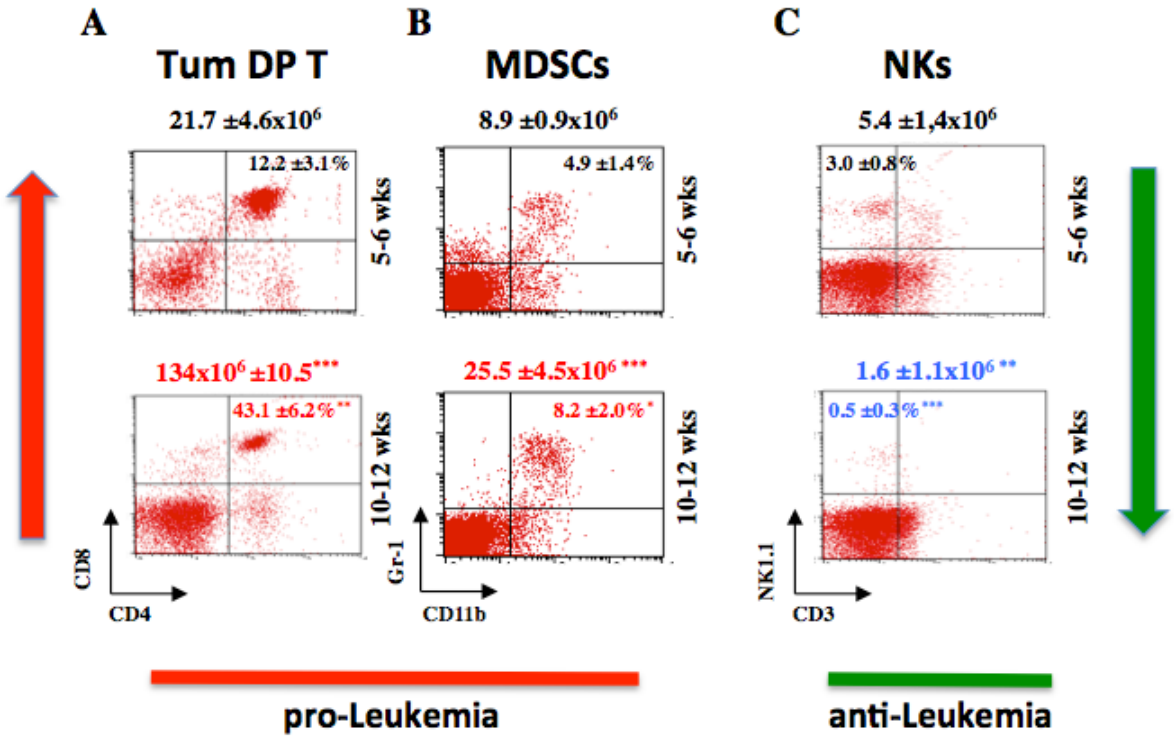

Figure 4. TIME Evolution During T-All Progression/1 (Subset Distributions)

Representative FACS analysis illustrates the percentages and absolute numbers of tumor DP T cells ***(A)****, MDSCs* ***(B)****, and NK cells* ***(C)*** *from the spleens of N3-tg mice during T-All progression at both the initial stage of the tumor (5-6 weeks of age) and the advanced stage at 11-12 weeks. FACS analysis was employed to calculate these values based on the distribution of* ***(A)*** *$CD4^+CD8^+$ subset for tumor cells,* ***(B)*** *$CD11b^+Gr\text{-}1^+$ subset for MDSCs, and* ***(C)*** *$NK1.1^+CD3^-$ subset for NK cells. Results are reported as mean values ± SD of three independent experiments (n = 4 mice at each age). *, P≤0.05; **, P≤0.01, and ***P ≤ 0.001 represent significant differences between values calculated at 10-12 wks and relative controls at 5-6 wks. Statistical significance was determined using unpaired two-tailed Student's T-test.*

These *wt* T lymphocytes were labeled with CFSE and activated with anti-CD3e and anti-CD28 antibodies. Following 72 hours of activation, we conducted a flow cytometry analysis to examine the CFSE profile of gated $CD4^-CD8^+$ proliferating cells. The analysis

clearly indicates that MDSCs from *N3-tg* mice increase their suppressive function with disease progression. **Figure 5B** illustrates that, indeed, co-culturing transgenic MDSCs from 5–6-week-old mice with *wt* T cells does not significantly hinder their proliferation rate of 85.7 ±8.9%, with respect to what observed with *wt* T cells cultured alone (proliferation rate of *wt* $CD4^{-}CD8^{+}$ T cells: 91.2 ±6.2%, not shown).

Instead, it is noteworthy that MDSCs derived from *N3-tg* mice aged 10-12 wks of age do exhibit a significant suppressive activity with respect to what exerted by MDSCs from *N3-tg* at 5-6 wks of age, by significantly reducing the proportion of proliferating cells (proliferation rate of *wt* $CD4^{-}CD8^{+}$ T cells: 31.3 ± 4.2% *vs* 85.7 ±8.9%).

In summary, over time, there is a gradual enhancement in the functionality of MDSCs. These cells are induced during leukemia, and notably, at the initial stage of pathology (5-6 weeks), they do not exhibit suppressive characteristics. However, their suppressive activity increases concomitantly with the progression of T-ALL pathology.

Finally, to evaluate NK cell function, we isolated NK cells from the spleens of *N3-tg* mice to perform a cytotoxicity assay against YAC-1 cells, a murine T-cell lymphoma cell line commonly used as target. YAC-1 cells were pre-labeled with CFSE, co-incubated with NK cells for 6 hours at the YAC-1: NKs ratio of 1:10, washed, and then treated with the viability dye 7-AAD. We used FACS analysis to evaluate the percentage of $7\text{-}AAD^{+}CFSE^{+}$ YAC-1 dead cells. The results showed that NK cells collected from *N3-tg* mice at the early stages of the pathology (5-6 weeks of age) are more functional than those from mice in the later stages of the disease (10-12 weeks of age), as evident from a target cell mortality rate of 35.7 ±7.2% *vs.* 4.1±0.9% **(Figure 5C)**. This suggests that, in the presence of a tumor, NK cells from *N3-tg* mice are actively performing their anti-tumor function in the initial stages of the pathology. However, in the later stage of leukemia they exhibit a drastic and statistically significant reduction in their cytotoxic function. Collectively, our findings demonstrate that, regarding both numbers and functions, pro-tumoral MDSCs increase and, conversely, anti-tumor NK cells decrease in the TIME of *N3-tg* mice, during T-ALL progression.

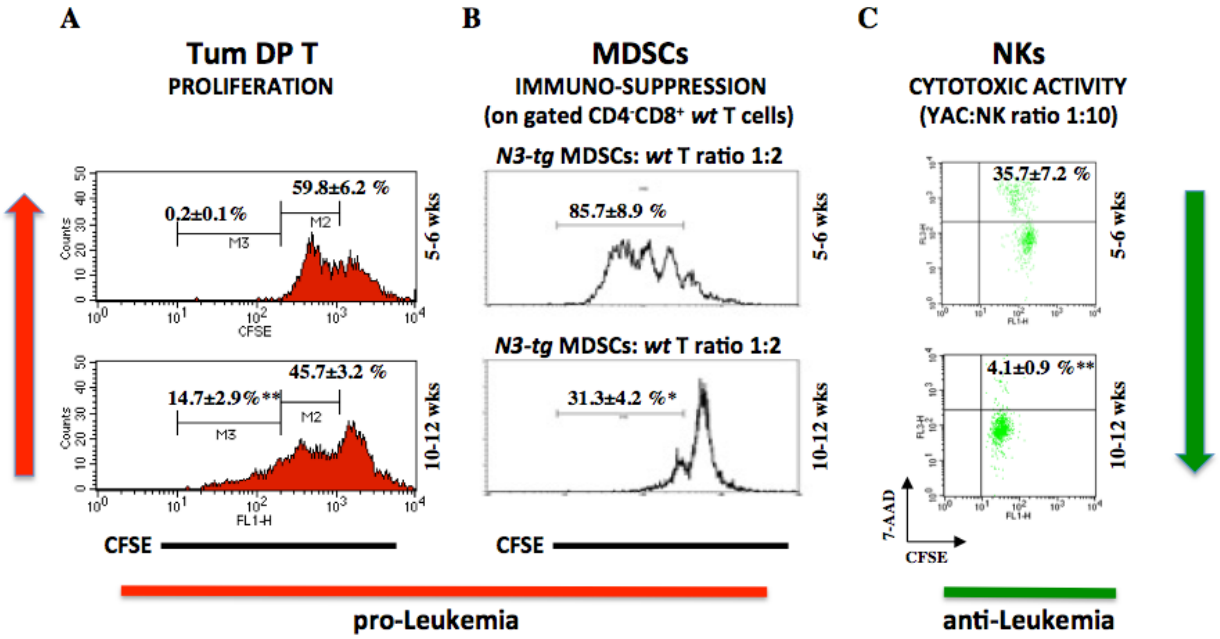

Figure 5. TIME Evolution During T-All Progression/2 (Subset Functions)

Representative FACS analysis of the functionality of tumor DP T cells, MDSCs and NK cells sorted from the spleen of N3-tg mice during T-ALL progression, at the indicated ages. ***(A)*** *In vitro proliferation assay with CFSE-labeled DP T cells cultured alone. The numbers within cytograms indicate the percentages of proliferating $CD4^+CD8^+$ cells, assessed through CFSE dilution via FACS analysis after 72 hours of culturing. In particular, M3 marker delimits highly proliferating DP T cells.* ***(B)*** *Representative FACS analysis of in vitro suppression assay with CFSE-labelled and activated wt T splenocytes (responders), cultured with $CD11b^+Gr\text{-}1^+$ putative MDSCs (suppressors), sorted from spleens of transgenic mice at the indicated ages, at the MDSCs: wt T cells ratio of 1:2. Numbers inside cytograms represent the percentages of proliferating $CD4^-CD8^+$ responder cells, assessed as CFSE dilution by FACS analysis at 72 hrs of culturing; the same test was conducted in parallel with $CD11b^+Gr\text{-}1^+$ from spleen of wt littermates, as a negative control (data not shown).* ***(C)*** *Cytotoxic activity of NK cells from spleen of N3-tg mice, at the indicated weeks of age, calculated by FACS analysis, as proportion of YAC-1 target cells (previously CFSE-labeled), killed in co-culture experiments, as evidenced by staining with 7-AAD. The numbers within the cytograms indicate the percentages of $CFSE^+$7-AAD^+ dead YAC-1 target cells. The results are related to co-culture experiments for 6 hours at the YAC-1: NKs ratio of 1:10.*

*Results are presented as mean ± SD of three independent experiments (n = 3 mice at each age), each sample performed in triplicate. *, P≤0.05, and **, P≤0.01, signify significant differences between values calculated at 10-12 wks of age and relative controls at 5-6 wks of age.*

Statistical significance was determined using unpaired two-tailed Student's T-test.

7.2 NKs From *N3-tg* Mice Kill Efficiently $CD4^+CD8^+$ (DP T) Tumor Cells

To confirm that NK cells from *N3-tg* mice effectively exhibit their activity on leukemia cells during the initial phase of the disease, we conducted the cytotoxic activity assay by co-culturing them directly with tumor *N3-tg* DP T cells. We sorted NK cells from the spleen of *N3-tg* mice aged 5-6 weeks. We also isolated by sorting tumor DP T cells from the spleen of *N3-tg* mice aged 10-12 weeks. DP T cells, serving as target cells, were labeled with CFSE and co-cultured either alone or in combination with graded numbers of NK cells as effector cells. **Figure 6** illustrates a notable dose-dependent NK cytotoxic activity with an increase that is evident across the DP T:NKs ratios of 1:5, 1:10, and 1:20, with corresponding DP T dead cell percentages of 18.2 ±1.1%, 27.6 ±1.5%, and 38.8 ±2.0%, respectively. In summary, NK cells from *N3-tg* mice during the initial phase of the disease (5-6 weeks of age) demonstrate cytotoxic activity on DP T tumor cells.

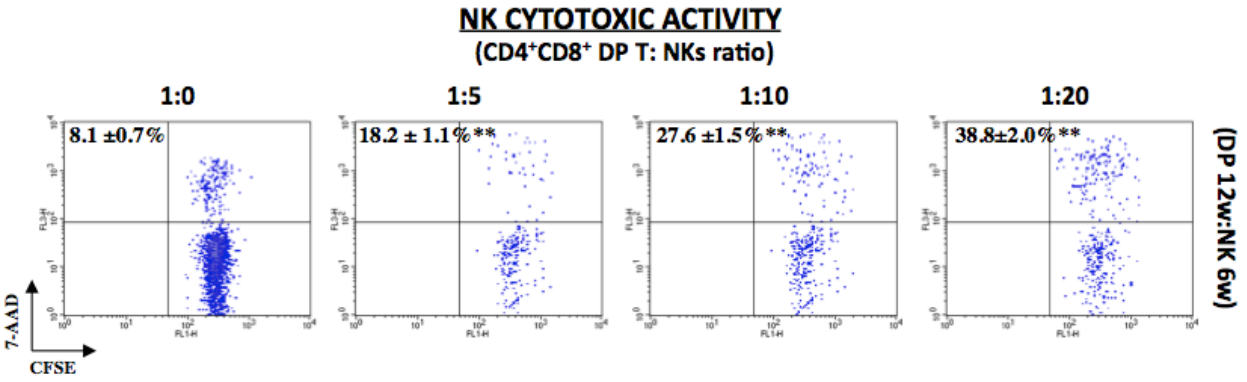

Figure 6. NK Cells Interact with Tumor DP T Cells Inside TIME of Notch3-Dependent T-ALL

*Representative FACS analysis of cytotoxic assay performed with splenic NKs from early-stage N3-tg mice (at 5-6 wks of age) on tumoral $CD4^+CD8^+$ DP T cells, isolated from spleen of N3-tg mice aged 10-12 weeks. The numbers within cytograms indicate the percentages of $CFSE^+$7-AAD^+ dead YAC-1 target cells. The results are related to co-culture experiments with a DP T:NKs ratios of 1:5, 1:10, or 1:20, respectively. The left panel displays results for target cells cultured alone as negative control (at DP T:NKs ratio of 1:0). Reported results are mean values ± SD from three independent experiments (n = 3 mice at each age), each conducted in triplicates. **, P≤0.01, represent significant differences with respect to the value reported for negative control (1:0 ratio). Statistical significance was determined using unpaired two-tailed Student's T-test.*

7.3 MDSCs From *N3-tg* Mice Inhibit Cytotoxic Activity of NK Cells

The natural and main target of MDSC suppression in the tumor microenvironment is represented by T lymphocytes. However, in the context of a murine model of aggressive T-cell leukemia, such as the one represented by the *N3-tg* mouse, where the tumor is predominantly composed of immature T cells (Bellavia D, et al., 2000), it seems improbable that they could be simultaneously the primary target of MDSCs induced by tumor cells themselves (Grazioli P, et al., 2022), which aims to facilitate the disease progression.

Thus, in an attempt to identify NK cells as potential specific targets for the suppressive activity of MDSCs in the tumor microenvironment (TME) of *N3-tg* mice, we repeated our cytotoxic activity assay of functional NK cells, as isolated from the spleens of transgenic mice at 5-6 weeks of age, corresponding to early stages of T-ALL (see Figure 5C, upper panel), in the presence of graded proportions of functional MDSCs, as isolated from the spleens of 10-12-week-old *N3-tg* mice, considering their lack of functionality at 5-6 weeks of age-(see Figure 5B, upper panel).

Results, as shown in **Figure 7A**, indicate a low percentage of dead cells in the negative control (2.1 ±0.2%), consisting of YAC-1 target cells alone, and a moderate rate of activity in the presence of NKs from *wt* mice (8.4 ±1.8% of dead target cells). Significantly, in co-culture experiments, cytotoxic activity of NK cells derived from the transgenic mouse is notably restrained by MDSCs in a dose-dependent manner **(Figure 7B)**, as also illustrated in the representative experiments of **Figure 7C**.

Overall, these experiments highlight the compromised cytotoxic activity of NK cells in *N3-tg* mice in the advanced stage of the disease, suggesting that this inhibition may be exerted by functional MDSCs present in the TME of *N3-tg* mice at the same time.

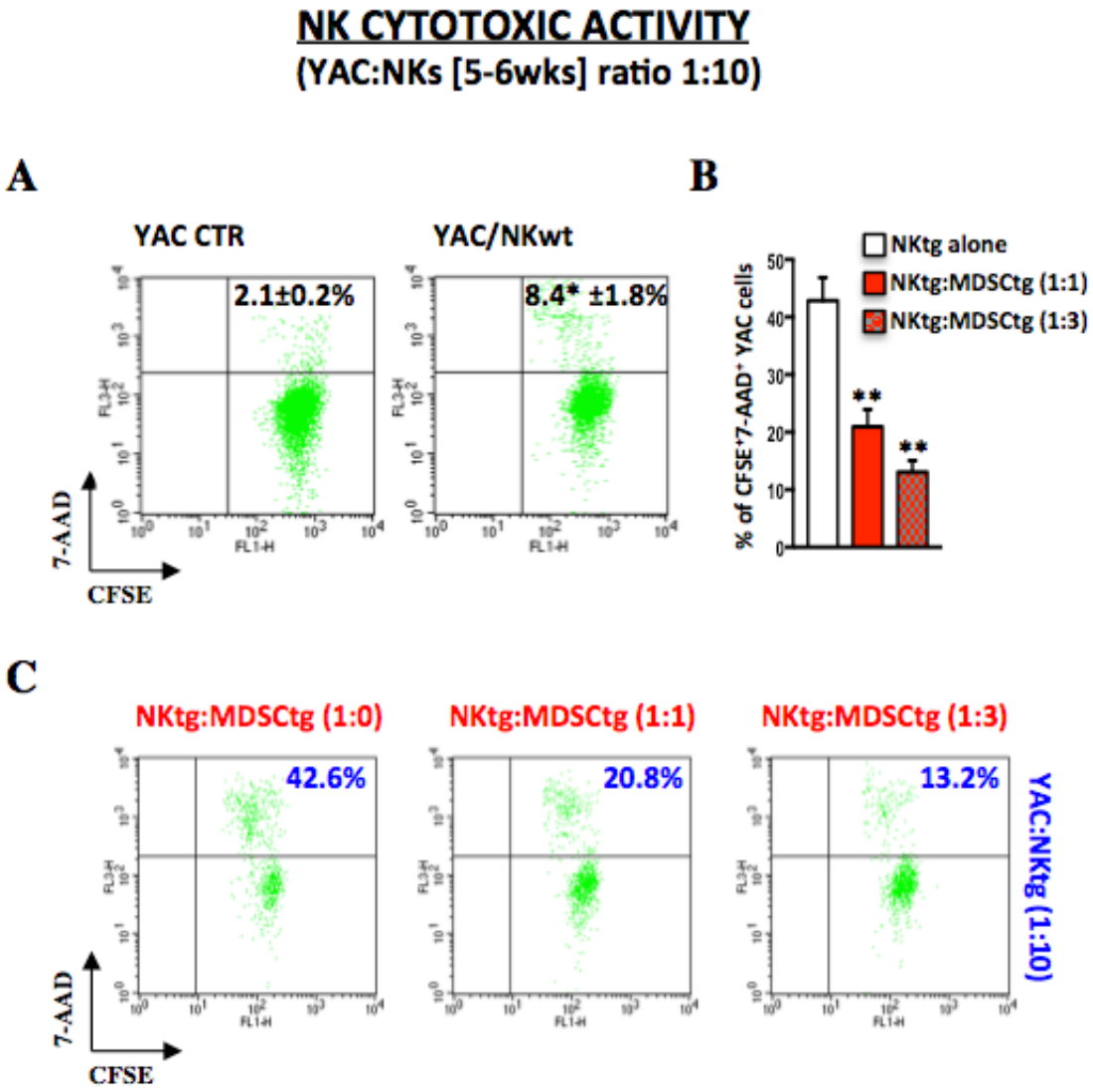

Figure 7. MDSCs Interact with NK Cells Inside the TIME of Notch3-Dependent T-ALL

Facs analysis of the cytotoxic activity of NK cells isolated from the spleens of control wt mice (NKwt) or N3-tg mice (NKtg) at 5-6 wks of age. Panel ***(A)*** *serves as controls, depicting the percentages ±SD of CFSE+7-AAD+ YAC-1 target cells cultured alone (left panel), or in combination with NKwt (right panel).* ***(B)*** *Mean values of the percentages ±SD of CFSE+7-AAD+ YAC-1 target cells induced by NK cells from N3-tg mice (NKtg), either alone or upon co-culturing with MDSCs from spleen of 10-12-wks-old transgenic mice (MDSCtg) at different NKtg:MDSCtg ratios (1:1 and 1:3). The panel* ***(C)*** *illustrate a representative experiment, as described in (B). Results in (A) and (B) are reported as mean ± SD from three independent experiments (n= 3 mice per group), each performed in triplicate. *, p≤0.05 and **, p≤0.01 indicate significant differences compared to the value recorded in the corresponding control. the YAC-1: NK ratio used in the illustrated experiments is always 1:10. Statistical significance was determined using unpaired two-tailed Student's T-test.*

7.4 The Proportions of NK/PD-1$^+$- and MDCS/PD-L1$^+$-Cells Increase During T-ALL Progression

After noticing the inhibitory effect of MDSCs on NK cytotoxic activity, it was essential to investigate the potential mechanism linking these two components. MDSCs exert immunosuppressive activity through various mechanisms, including the expression of PD-L1 and consequent inhibition of PD-1$^+$ cells (Yang T, et al., 2019). Then, our studies focus preliminarily on the dynamic changes in PD-1/PD-L1 expression over time, specifically within key cellular subsets of T-ALL TIME, such as NK cells, MDSCs and tumor DP T cells. By elucidating the nuanced behavior of PD-1/PD-L1 in these distinct cell populations, our goal was to unveil the regulatory mechanisms that govern the interplay between the immune microenvironment and the evolution of T-ALL. In our study, we initially directed our attention towards exploring the expression of PD-L1 on T-ALL tumor cells in our murine model, considering it is established overexpression in diverse tumor cell types. Using FACS analysis, we examined the distribution of specific markers (CD4, CD8, and PD-L1) in cell suspensions from the spleens of *N3-tg* mice at 5-6 and 10-12 weeks of age. The results interestingly reveal that the population of DP T tumor cells shows very low levels of expression of PD-L1 and no significant changes during T-ALL progression **(Figure 8A)**. Following this, we aimed to test the hypothesis that MDSCs could exert suppressive effects on NK cells through the PD-1/PD-L1 axis, thereby supporting tumor progression. We conducted flow cytometry experiments to specifically investigate (CD11b$^+$Gr-1$^+$) MDSCs expressing PD-L1, with inhibitory potential, along with (NK1.1$^+$CD3$^-$) NK cells expressing PD-1, with potential susceptibility to inhibition. Through staining experiments on cellular samples obtained from the spleens of transgenic mice at different stages of T-ALL progression (5-6 and 10-12 weeks of age), we observed a gradual and noteworthy increase in the proportion of these identified cell populations (NK/PD-1$^+$, MDSC/PD-L1$^+$) within the tumor microenvironment (TME) of *N3-tg* mice over time. The accumulation of these two subsets became particularly pronounced at 10-12 weeks of age, as illustrated in **Figure 8B and C**, respectively, a phase in which CD11b$^+$Gr-1$^+$ cells reached their full immunosuppressive function as MDSCs (Grazioli P, et al., 2022), that coincided with a substantial compromise in the cytotoxic activity of NK cells, as already shown in Figure 5B ans 5C, respectively. Thus, our data confirms the possibility that the PD-1/PD-L1 pathway can be involved in mediating MDSC/NK interactions.

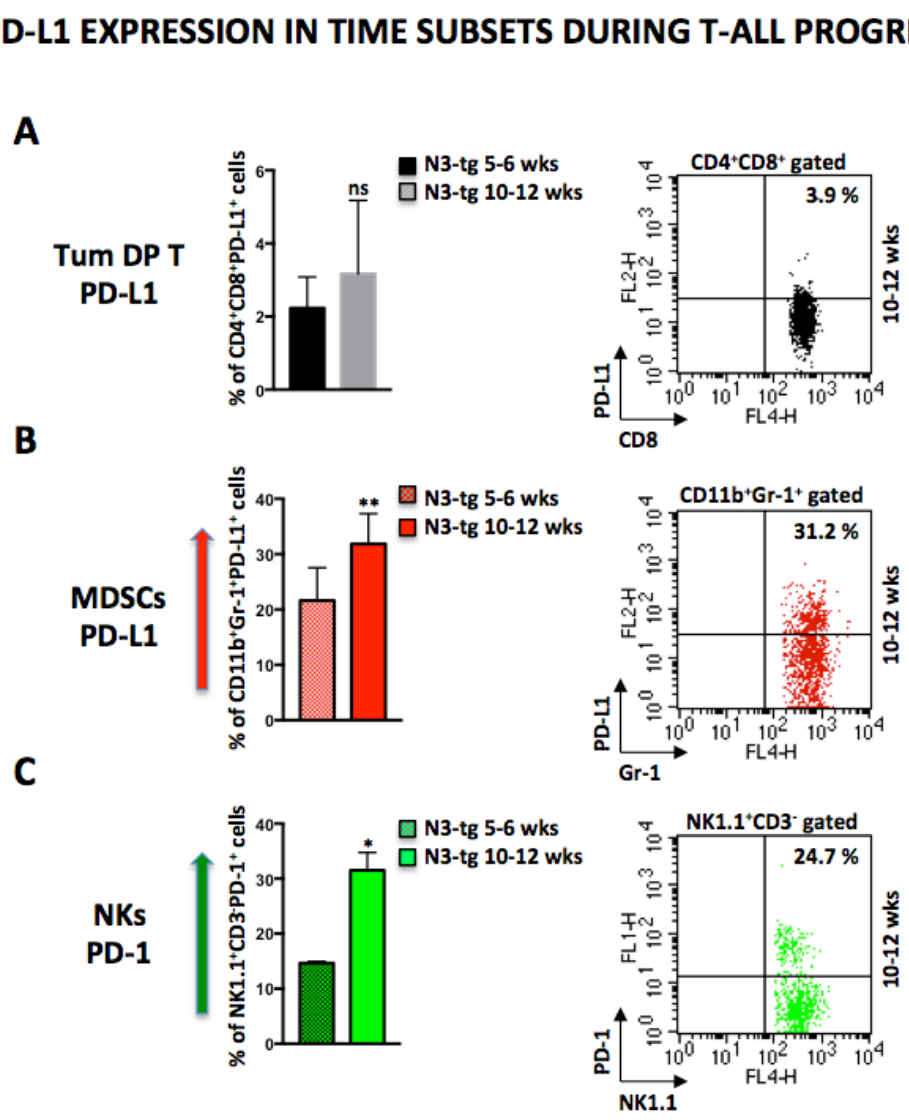

Figure 8. PD-1/PD-L1 Expression in TIME Subsets During T-ALL Progression

*The figure shows the results of FACS analysis of PD-L1 and PD-1 expression on relevant subsets of T-ALL microenvironment in the spleen of N3-tg mice during disease progression. Left panels report mean values of the percentages of DP T cells ($CD4^+CD8^+$) and MDSCs ($CD11b^+Gr\text{-}1^+$) expressing PD-L1 (**Figure 8A** and 8**B**, respectively) and of NK cells ($NK1.1^+CD3^-$) expressing PD-1 **(Figure 8C)** in the spleen of N3-tg mice, comparing observations at 5-6 weeks to those at 10-12 weeks of age. Right panels show representative cytograms from the corresponding analysis showed on the left panels. Numbers inside cytograms represent the percentages of $CD4^+CD8^+PD\text{-}L1^+$, $CD11b^+Gr\text{-}1^+PD\text{-}L1^+$ and $NK1.1^+CD3^-PD\text{-}1^+$ cells, respectively, in the spleen of N3-tg mice at 10-12 weeks of age. On the left panels, results are reported as mean ± SD of three independent experiments (n = 4 mice per group). ns, not significant; *, P≤0.05 and **, P≤0.01 represent significant differences between values at 10-12 wks of age with respect to the relative values at 5-6 wks of age. Statistical significance was determined using unpaired two-tailed Student's T-test.*

7.5 Treatment of *N3-tg* Mice with Anti-PD-L1 Antibodies Inhibits T-ALL Progression by Interfering with MDSC Expansion and NK Activity

The observations collected so far represent a crucial foundation for delving deeper into analyzing in an *in vivo* setting the PD-1/PD-L1 axis role in facilitating the interaction between Myeloid-Derived Suppressor Cells (MDSC) and Natural Killer (NK) cells. This interaction, it seems, may have implications for the progression of T-cell Acute Lymphoblastic Leukemia (T-ALL), by hindering the anti-tumor functions of NK cells. To test this hypothesis, we decided to treat *N3-tg* mice with blocking anti-PD-L1 antibodies, designed to inhibit the interaction between PD-L1 and its receptor PD-1, thereby blocking the inhibitory signals of this pathway. For this purpose, 10-12 weeks-old *N3-tg* mice were i.p. treated with the rat anti-mouse anti-PD-L1 antibody (clone 10F-9G2) or relative control antibodies at a dose of 100 ◎g/mouse, administered bi-weekly for two weeks **(Figure 9A)**. The choice of 10-12 weeks of age was made as a curative measure, because at this age we already have an accumulation of functional MDSCs and impaired NKs, respectively, in the TME of *N3-tg* mice (see Figure 5), especially of those expressing the PD-L1 and PD-1 molecules, respectively (see Figure 8). In *N3-tg* mice, the advancement of T-cell Acute Lymphoblastic Leukemia (T-ALL) is directly linked with splenomegaly and absolute count of double-positive (DP) T splenocytes (Bellavia D, et al., 2000). Moreover, the accumulation of Myeloid-Derived Suppressor Cells (MDSCs) suggests an important role in the advancement of T-ALL (Grazioli P, et al., 2022). Consequently, at the end of the treatment, we conducted flow-cytometry analysis in the spleens of *N3-tg* treated mice (anti-PD-L1) and their respective controls (CTR), to measure not only total yield of spleens and total count of $CD4^+CD8^+$ DP T splenocytes (**Figure 9B**, left and right panel, respectively), but also numbers of $CD11b^+Gr\text{-}1^+$ MDSCs and their proportions of $PD\text{-}L1^+$ cells (**Figure 9C**, left and right panel, respectively). Results showed that there was a notable decrease in all the parameters considered in animals treated with anti-PD-L1 compared to the control group (CTR). These results strongly suggest that blocking the PD-1/PD-L1 axis may have therapeutic potential in slowing down the progression of T-ALL, and also in inhibiting MDSCs accumulation in the TME, possibly impairing, in turn, their positive effects on tumor cell fitness, as previously reported (Grazioli P, et al, 2022)

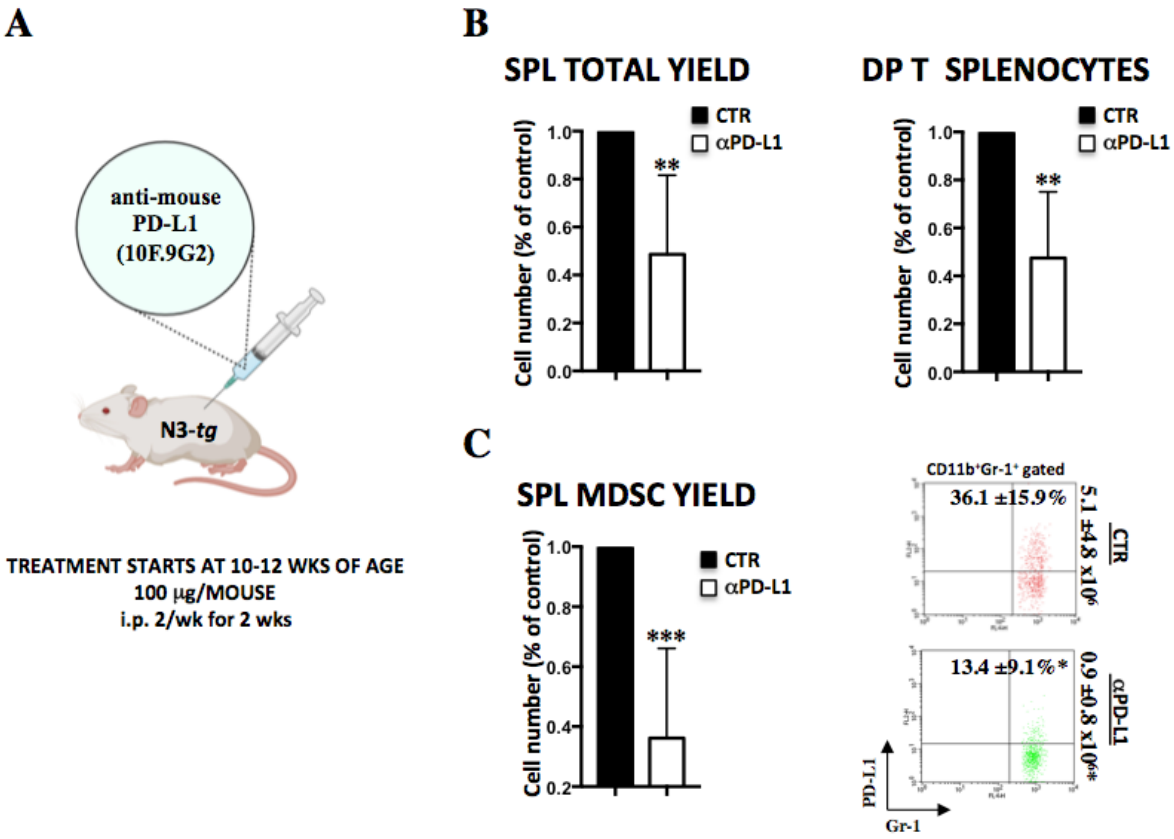

Figure 9. **Effects Of αPD-L1 Treatment On T-All Progression and MDSC Expansion**

*In **(A)**, a detailed schematic representation of the in vivo treatment protocol for N3-tg mice employing anti-PD-L1 blocking antibodies is elucidated. The graphs in **(B)** illustrate the total yield (left panel**)** and the count of $CD4^+CD8^+$ (DP) T cells (right panel) in the spleens of mice treated with anti-PD-L1, compared to controls treated with unrelated antibodies (CTR). In **(C)** the graphs report the total cell counts of $CD11b^+Gr\text{-}1^+$ MDSCs (left panel) and percentages and numbers of $CD11b^+Gr\text{-}1^+PD\text{-}L1^+$ MDSCs (right panel) in the spleens of mice treated with anti-PD-L1, compared to controls treated with unrelated antibodies (CTR), on the same mice as in (B). Numbers inside the cytograms represent the percentages $CD11b^+Gr\text{-}1^+PD\text{-}L1^+$ cells, and the numbers on the right part of the cytograms are the corresponding absolute numbers. In both the panels in (B) and in the left panel in (C), the results are calculated as the ratio between the values in treated mice and those in relative controls, expressed as % of control. The data in (B) and (C) are presented as the mean ± standard deviation (SD) from three independent experiments (n=6 mice per group). *, p≤0.05 **, P≤0.01, and ***P ≤ 0.001 represent a significant difference in values of treated mice compared to the value of relative controls. Statistical significance was determined using unpaired two-tailed Student's T-test.*

The next step of our investigation focused on assessing how blocking the interaction between PD-1/PD-L1 axis impacts on NK cells during disease progression. To examine the NK cell population, we employed cytometer staining assays with specific antibodies. We evaluated the NK cell populations in the spleens of *N3-tg* mice that were treated with anti-PD-L1 antibodies, or with isotype control antibodies, as previously outlined**.** The analysis of the NK cell population following treatment reveals a significant expansion in the percentage of PD-1^{+}NK cells in treated mice (αPD-L1) compared to their respective controls (CTR) (42.4 ±10.7% *vs* 18.8 ±11.6%) **(Figure 10A)**. This suggest that, by blocking the action of PD-L1^{+}MDSCs on PD-1^{+}NKs, these last subsets may start expanding again. More importantly, we assessed the cytotoxic activity of sorted NK cells on YAC-1 targets, after the treatment, revealing that NK cells isolated from *N3-tg* mice treated with anti-PD-L1 exhibited a notable enhancement in cytotoxic activity compared to the control group **(Figure 10B)**, and this increase is statistically significant (21.2± 6.7% *vs*. 3.9± 1.8%).

In summary, the application of anti-PD-L1 treatment led to a dual improvement in the TIME of *N3-tg* murine model of T-ALL: on one hand, it inhibits disease progression and MDSCs accumulation; on the other hand, it enhances the cytotoxic activity of NK cells. This dual enhancement holds significant promise in the context of immune-mediated therapies, particularly in the treatment of conditions were bolstering the immune system's ability to target and eliminate aberrant tumor cells is crucial, as for the T-ALL.

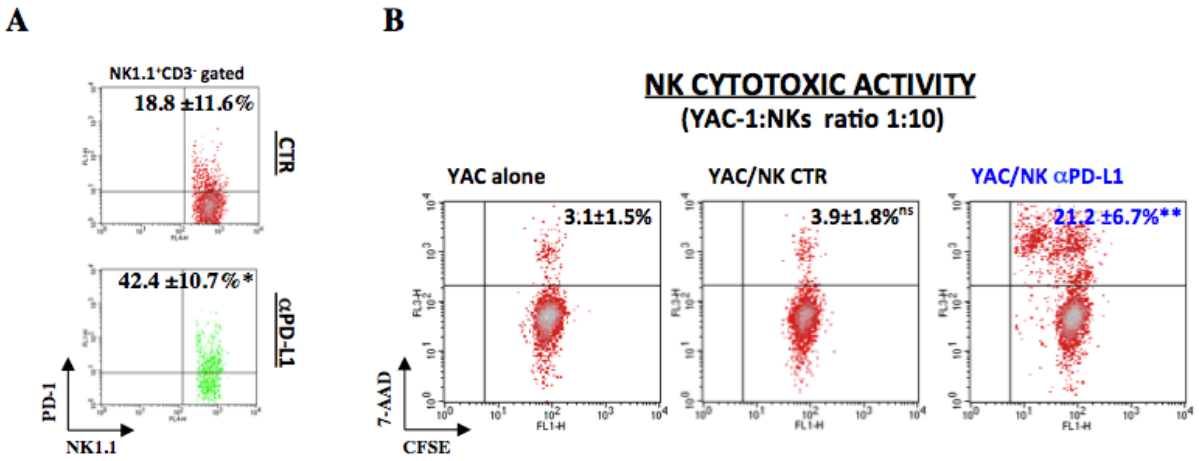

Figure 10. Effects Of αPD-L1 Treatment on NK Cell Activity

(A) *FACS analysis of the percentage of NK cells expressing the PD-1 receptor in the spleen of N3-tg mice at the end of the treatment with anti-PD-L1 antibodies, (αPD-L1), compared to their respective controls (CTR). Numbers inside the cytograms represent the percentages of NK1.1⁺CD3⁻PDL1⁺ cells. The results are reported as mean ± SD of three independent experiments (n = 6 mice per group), on the same mice, as in Figure 9. In* ***(B)*** *cytotoxic activity of NK cells from the spleen of N3-tg mice at the end of the treatment with anti-PD-L1 antibodies, (αPD-L1), compared to their respective controls (CTR), calculated by FACS assay, as proportion of YAC-1 target cells (previously CFSE-labeled), killed in co-culture experiments, as evidenced by staining with 7-AAD. The numbers within the cytograms indicate the percentages of CFSE⁺7-AAD⁺ dead YAC-1 target cells. The results are related to 6 hrs of co-culture experiments at the YAC-1: NK ratio of 1:10. The left panel represent negative control, consisting of YAC-1 target cells, cultured alone. The results are reported as mean ± SD of three independent experiments (n = 3 mice per group), on representative mice from those analyzed in (A), each performed in triplicate. ns= not significant; *, P≤0.05 and **, P≤0.01 represent significant differences with respects to the relative controls. Statistical significance was determined using unpaired two-tailed Student's T-test.*

8. Discussion

The tumor microenvironment (TME) transforms the immune system from a defensive force to a pro-tumoral entity, facilitating neoplastic progression by promoting immune response evasion. Within this context, myeloid-derived suppressor cells (MDSCs) play a crucial role in regulating the TME, exerting suppressive functions (Zhao Y, et al., 2023) MDSCs primarily act by suppressing the T anti-tumor response, serving as a key mechanism supporting neoplastic cells across various tumor types. However, alternative mechanisms may come into play. Specifically, MDSCs could directly support the survival of tumor cells (Lyu A, et al., 2020), they can provide indirect protection through the generation of Tregs, or target components of innate immunity, thus hindering their anti-tumor immune response (Gabrilovich DI and Nagaraj S, 2009). Notably, MDSCs may specifically attack Natural Killer (NK) cells (Bruno A, et al., 2019), leading to their suppression.

The experiments described in this thesis were conducted on a transgenic murine model for the intracellular domain of Notch3 (the *N3-tg* mouse), affected by a highly aggressive and early form of T-cell acute lymphoblastic leukemia (T-ALL). This model resembles the condition found in children for which effective therapeutic interventions are not completely established, especially after disease relapse.

In our previous publication (Grazioli P, et al., 2022), we demonstrated that $CD4^+CD8^+$ (DP) tumor T cells, that expand in the periphery and characterize T-ALL in *N3-tg* mice, induce the development of MDSCs *in trans*, through a Notch/IL-6-dependent mechanism. Moreover, MDSC depletion inhibit T-ALL progression in *N3-tg* mice (Grazioli P, et al., 2022).

Based on these premises, the main purpose of my thesis was to shed light on the potential targets and the associated mechanisms of action of $CD11b^+Gr\text{-}1^+$ MDSCs induced in the TIME of *N3-tg* T-ALL.

Regarding the MDSC target, our analysis focused on alternative cells compared to T lymphocytes, which typically represent their canonical target in most solid and hematological tumors where they are present. In our model of T-cell leukemia, indeed, it is unlikely that T cells, that are themselves malignant, would be the main targets of suppressive cells generated by the tumor to facilitate its own progression. So, we shifted our attention to NK cells, as recent descriptions emphasized their importance as targets of MDSCs in various types of tumors (Melaiu O, et al. ,2020; Stiff A, et al., 2018).

Hypothesizing a possible NK/MDSCs crosstalk, we preliminary examined the evolution of the tumor immune microenvironment (TIME) of *N3-tg* mice, focusing on characterizing $CD4^+CD8^+$ (DP) T tumor cells, MDSCs, and NK cells during the disease progression, both numerically and functionally. Our Flow-cytometry experiments revealed a significant decrease in both number and function of the NK cell population in 10-12-week-old mice, in a later stage of the disease, that corresponds to a notable increase of highly proliferating $CD4^+CD8^+$ (DP) tumor T cell proportion and to a significant expansion of functional MDSCs, with respect to mice at an initial stage of T-ALL, at 5-6 wks of age. In particular, we found that NK cells from *N3-tg* mice in the initial stages of the disease (5-6 wks of age), can efficiently eliminate tumor (DP T) cells; however, their cytotoxic activity decreases significantly as the tumor progresses. Conversely, functional MDSCs are virtually absent at 5-6 wks of age and they reach a peak of their suppressive activity at 10-12 wks of age. This inverse correlation suggests that MDSCs may be responsible for the changes observed in NK cell subset.

In a preliminary, but essential investigation, we established that MDSCs are indeed, capable of significantly inhibiting NK function, as measured through *in vitro* cytotoxic activity assays of NK cells from *N3-tg* mice, in the presence/absence of functional MDSCs from the same animals.

In this context, a likely mechanism by which MDSCs may inhibit NK cells may involve the PD-1/PD-L1 axis, an immune-checkpoint dysregulated in various tumors. This pathway has been extensively investigated in recent years, presenting significant therapeutic implications. It serves as a potential regulatory mechanism for inhibiting the anti-tumor response of cells expressing the inhibitory receptor PD-1, particularly cytotoxic $CD8^+$ T cells and NK cells. The inhibition is orchestrated by cells capable of expressing the PD-L1 ligand, primarily tumor cells, and also MDSCs (Yang T et al., 2019). Therefore, we evaluated the expression of these molecules in various populations of the tumor immune microenvironment (TIME) of *N3-tg* mice. Initially, we observed a significantly increased percentage of NK cells expressing the PD-1 receptor. This prompted further investigation. Despite PD-L1 being overexpressed on neoplastic cells in many tumors, when we assessed its expression on $CD4^+CD8^+$ (DP) tumor T cells from *N3-tg* mice, we discovered on them very low expression levels of PD-L1, that show no significant changes during T-ALL progression. This–makes it challenging to speculate on a predominant role of DP T cells in regulating the immune response through the PD-L1 pathway. Instead, we demonstrated a reproducible and significant expansion of the

MDSC population expressing PD-L1. This finding leaves open the possibility that this specific population may potentially exert suppression of NK cells through the PD-1/PD-L1 axis.

Our findings strongly justified testing the *in vivo* effects of administering anti-PD-L1 antibodies to *N3-tg* mice, aiming to analyze the consequences of inhibiting the interaction between the two cell populations above (PD-1^{+}NKs and PD-L1^{+}MDSCs) on leukemia progression. Our experiments, which involved treating *N3-tg* mice with anti-PD-L1 blocking antibodies, unequivocally demonstrated that disrupting the PD-L1/PD-1 interaction can effectively inhibit the progression of T-ALL. This inhibition was evidenced by a significant reduction in splenomegaly and absolute number of CD4^{+}CD8^{+} DP T cells, both widely recognized parameters of diagnostic significance to follow leukemia progression in murine models of Notch-dependent T-ALL (Bellavia D, et al., 2000). Additionally, it is important to note that this treatment significantly reduces the numbers of MDSCs, and particularly, of the PD-L1^{+}MDSC subset. In parallel, we also identified an increased expansion of PD-1^{+}NK cells, that exert a significantly heightened cytotoxic activity compared to the control group.

In summary, the experiments illustrated in this thesis strongly suggest that the PD-L1 ligand may play a key role in mediating an inhibitory effect of MDSCs on the PD-1^{+}NK cell population within the TIME of Notch-dependent T-ALL, and ultimately, on the disease progression (**Figure 11**).

Importantly, these findings provide a necessary premise for further investigation on the mechanisms driving NK/MDSC/T tumor cell interactions in T-ALL, by using more complex murine models, as well as human patients. Further, these studies offer valuable insights for the development of a novel combined therapeutic strategy for Notch-dependent T-ALL, focusing on inhibiting immune-checkpoint signals, limiting the immunosuppressive function of MDSCs, and restoring NK cell functionality.

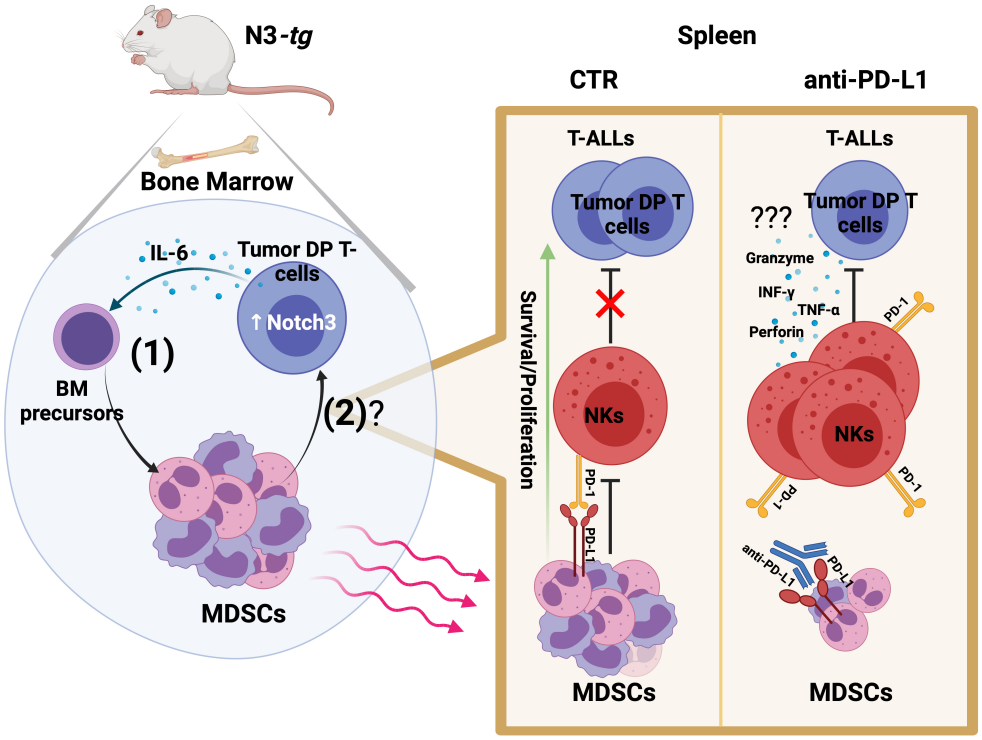

FIGURE 11: The Notch3-Dependent Induction of MDSCs And Their Possible Role on NK cell activity and T- ALL Progression. *On the left part, the figure summarizes the in vivo results on our Notch3-dependent model of T-ALL (N3-tg mice), as already reported (Grazioli P, et al., 2022). Inside the Bone-Marrow, aberrant T cells, mainly represented by $CD4^{+}CD8^{+}$ DP T cells (DP T-cells) induce functional MDSCs (MDSCs) from BM precursors, through a mechanism influenced by Notch-signaling deregulation inside DP T cells (↑Notch3) and the presence/release of IL-6 in the TME (1). In turn, MDSCs sustain DP T cell proliferation and/or survival (2), thus contributing to T-ALL progression through mechanisms that we hypothesized, as indicated by a question mark (?) and explained on the right part of the figure. These mechanisms are further delineated within the rectangle box. Based on the results of this thesis, MDSCs in the spleen of N3-tg mice may improve T-ALL cell (T-ALLs) survival/proliferation, either directly (green arrow) and, more likely, indirectly by blocking anti-tumor immune responses of NKs, possibly through the PD-1/PD-L1 axis. Indeed, the treatment of N3-tg mice with anti-PD-L1 antibodies can induce a dual improvement in controlling tumor progression, concurrently boosting the cytotoxic activity of NK cells (through mechanisms that needs to be defined, as indicated by **???**), while also reducing the accumulation of functional MDSCs, compared to the controls (CTR). Created with Biorender.com. Modified by Grazioli et al, 2022.*

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